1-1-2011

MHC Class I-Dependent Acquisition and Maintenance of Natural Killer Cell Function

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by

Julie M. (Elliott) Chase

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

August 2011

Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

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Natural killer (NK) cells are hematopoietically derived immune cells of the lymphoid lineage. Their effector functions, including cytokine secretion and cytotoxicity, are controlled by the integration of signals received from both activating and inhibitory receptors. In a phenomenon known as "missing-self" recognition, NK cells eliminate self-tissues with aberrantly low or absent MHC class I surface expression, as is common in settings of viral infection and transformation, when these cells fail to engage the critical NK cell inhibitory receptors. Interestingly, NK cells that develop in an MHC class I-deficient environment are defective in natural killing and hyporesponsive to stimulation through their activation receptors, rather than hyperactive, as would be predicted by the missing-self hypothesis. Through contact between inhibitory receptors and their MHC class I ligands, NK cells are rendered both self-tolerant, as well as functionally competent. The process of MHC class I-dependent acquisition of NK cell function is termed "licensing."

Herein we devised an experimental system of adoptive transfer, where unlicensed, splenic NK cells from MHC class I-deficient donors were transferred to wild-type (WT), MHC class I-sufficient hosts. Following transfer, donor NK cells expressing an inhibitory receptor specific for a cognate host-MHC class I molecule gained function, consistent with licensing of these cells. Further work demonstrated that normal MHC class I expression is required on both hematopoietic as well as non-hematopoietic tissues in order to produce the licensed phenotype. These results indicate that NK-intrinsic expression of MHC class I is not required for normal function, leading to the conclusion that contacts with MHC class I in the *trans* orientation are sufficient for licensing. Maintenance of the licensed phenotype and the potential for its reversal were examined following the inverse adoptive transfer of licensed, splenic WT NK cells into MHC class I-deficient hosts. We found that maintenance of the licensed phenotype also requires MHC class I expression by both hematopoietic and non-hematopoietic cells, and that loss of either of these contacts results in the loss of NK cell function, suggesting that licensing is reversible. Additionally, these results demonstrate that contacts with MHC class I molecules in the *cis* orientation are insufficient to maintain the licensed phenotype.

Finally, in a second experimental model, parabiosis of MHC class I-deficient mice to WT partners, we explored the effect of bidirectional manipulation of MHC class I expression on NK cell function. Unexpectedly, we found a rapid and specific destruction of the spleen in the MHC class I-deficient partner, which appears to occur by a mechanism that is at least partially NK-dependent. These results suggest that factors not supplied in the context of adoptive transfer may contribute to the maintenance of function of WT NK

cells, and that the NK-mediated survey of missing-self seems to be restricted to tissues of hematopoietic origin.

In summary, these results demonstrate that NK cell licensing is both plastic and dynamic, fundamentally unique among the educational processes of immune lymphocytes. These results have important implications for the field NK immunotherapy, such as in the setting of peripheral NK cell adoptive transfer following bone marrow transplantation.

ACKNOWLEDGEMENTS

I would first like to thank my thesis advisor, Dr. Wayne Yokoyama, for generously providing funding and resources, and mentoring me through my thesis work. He has taught me much about the benefits of approaching science from a calm and even perspective. I would also like to extend my sincere thanks to the members of my thesis committee: Drs. Emil Unanue, Ken Murphy, Marco Colonna, Todd Fehniger and Thad Stappenbeck. While I am always happy to receive their approval, it is their criticism that I appreciate the most. Through their intellectual challenges, I believe that I have been made a better scientist.

Fellow members of the Yokoyama lab deserve thanks for fostering a pleasant work environment. In particular, I thank Beatrice Plougastel, whose patience and kindness were not lost on me. Our discussions always brought out the most excited part of my personality and motivated my science. Another former lab member, and extremely gifted scientist, Megan Cooper, deserves a great amount of thanks. During the early stages of this project, her rigorous questioning helped to give me direction and focus.

Other members of the Yokoyama lab that deserve particular thanks include Jennifer Laurent, for her excellent assistance and support. Also, I give many thanks to Liping Yang, who is a wonderful and sweet person, as well as a talented scientist. Without her help, the parabiosis experiments would not have been possible. I also thank Lorraine Schwartz and Tammy Derleth, whose friendship has been very special to me. I'd like to thank my fellow students in the lab, Claudia Vargas, Melissa Pak and Chris Affolter (a

fellow student and the best bay mate ever!). I will always have fond memories of afternoon espresso break and sweets shared with good conversation. A (recently) former member of the lab, Joe Wahle, deserves a great amount of appreciation, as my near-constant lab companion, as well as a true and dear friend. I'd like to thank all other current members of the lab: Darryl Higuchi, Takashi Ebihara, Maria Gainey, Jay Gao, Bijal Parikh, Josh Rivenbark, Jimmy Ma, Stephen Ferris, Diana Beckman and Samantha Taffner. A few former lab members who deserve special thanks include: Minji Byun, a great student mentor and a wonderful international traveling companion; Paul Klekotka, a fun lab mate and good role model; Helena Jonsson, a fellow student and a similarly passionate scientist; and Melissa Berrien, a continued friend.

Over the last five years, I have formed many new and fantastic friendships. I do thank all of the members of my graduate class (Holly (Smith) Akilesh, Sam Lam, Hyang Mi Lee, Sang-Hun Lee, Winnie Lee, Jim Mohan, Melissa Pak, and Aaron Rapaport). We have had many adventures: The Great Huzzah, 34 Club, and Potosi (catfish!) will not be forgotten. Lastly, and perhaps most importantly, I cannot begin to express the depths of my gratitude for the love and support of my family: Andrew Elliott, whose unwavering belief in me and my ability to succeed was often a sole and sustaining factor. My parents, who have provided encouragement at the times I needed it most, and finally, my brother, Steve. I will always be grateful that, while we attended separate universities and studied completely different subjects, we were graduate students at the same time and, therefore, in it together.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

AT	adoptive transfer
β_2m	beta-2 microglobulin
CFSE	carboxyfluorescein succinimidyl ester
d	day(s)
D8	H2-D^d transgene
FCS	fetal calf serum (=FBS, fetal bovine serum)
GM-CSF	granulocyte-monocyte colony stimulating factor
H2	histocompatibility locus (murine)
H2-D^b	'b' allele of the D gene, present in the H2 locus
H2-K^b	'b' allele of the K gene, present in the H2 locus
H-2^b	genotype consisting of 'b' alleles at the MHC I gene loci = K^b D^b L^{NULL}
	genotype of C57Bl/6 reference mice
HLA	human leukocyte antigen
hr	hour(s)
i.v.	intra venous
IFNγ	interferon gamma
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
KIR	killer Ig-like receptor
KODO	$K^{b-/-}$ $D^{b-/-}$
mAb	monoclonal antibody

MFI	mean fluorescence index
MHC I	major histocompatibility complex protein, class I
min	minutes
mL	milliliter
NCR	natural cytotoxicity receptor
NK	natural killer
NKC	natural killer gene complex
NKD	NK-deficient
PBS	phosphate buffered saline
PMA	phorbol myristate acetate
RBC	red blood cell
Rag1	recombinase activating gene 1
RT	room temperature
SCT	single-chain trimer ($H2K^b$-OVA)
SX	surgery
TKO	triple knockout
TNFα	tumor necrosis factor, alpha
Tg	transgene, transgenic
μg	microgram
w	week
WT	wild type
γ_C	common gamma chain (CD132)

CHAPTER 1:

Introduction

Natural killer cell ontogeny and function

Natural killer (NK) cells are hematopoietically derived immune cells of the lymphoid lineage. Unlike T and B lymphocytes, NK cells are classified as members of the innate immune system, along with cells of the myeloid linage, such as macrophages, dendritic cells and neutrophils [1]. NK cells are involved in the earliest phases of the immune response and have the capacity to execute effector functions without the need for prior stimulation, as was first revealed by their intrinsic tumor-killing capacity, earning them the name "natural killer" cells [2-4]. Intact NK cell effector functions are critical for a successful antiviral response [5-8], and these effector functions include both the ability to secrete immunomodulatory cytokines, such as interferon-gamma (IFNγ) and interleukin-12 (IL-12), as well as direct cytolytic function, via perforin and granzyme activity [9]. Importantly, natural killer cells do not express a single rearranged antigen-specific receptor, like T and B lymphocytes, but instead rely on the integration of signals from both activating and inhibitory invariant receptors for proper function.

Activating NK cell receptors and their ligands

Activating receptors on NK cells are structurally diverse and fall into two main categories: 1) the type II C-type lectin-like proteins, including the NKG2C:CD94 heterodimer and NKG2D homodimer expressed on both murine and human NK cells, as well as members of the Ly49 family of receptors in mice, notably Ly49D and Ly49H [10], and 2) the type I immunoglobulin super family (IgSF) proteins, including the natural cytotoxicity receptors (NCRs), such as NKp30 and NKp44 in humans and NKp46 in both humans and mice, as well as some members of the family of killer

immunoglobulin receptors (KIR) in humans. Although the ligand-binding domains of NK cell activating receptors are structurally varied, these receptors share other features in common, including a positively charged amino acid residue in the transmembrane region and a short cytoplasmic tail [11]. This truncated cytoplasmic domain renders activating receptors incapable of intrinsic signal transduction. However, the charged transmembrane amino acid allows for non-covalent association with other transmembrane adaptor proteins that do possess intrinsic signaling capacity, such as such as DAP12 (DNAX-associated protein of 12kDa, also known as KARAP), FcεRγI (commonly referred to as FcRγ), and CD3ζ [12, 13].

Ligands recognized by NK cell activating receptors are even more diverse than the receptors themselves (Table 1). They include self-derived proteins, for example the MHC Ib (major histocompatibility complex, class Ib) molecules Qa-1 and HLA-E, expressed in mice and humans respectively, which engage NKG2C:CD94 [10]. Additionally, the activating receptor NKG2D may be engaged via another class of ligands termed "induced-self" proteins, molecules not typically expressed on healthy cells but upregulated on unhealthy cells following viral infection or genetic transformation [14-16]. Examples include: H60, Mult-1 and the Rae-1 family members in mice, and MICA/MICB as well as the ULBP proteins in humans [17]. Some NK cell activating receptors are engaged by exogenous ligands, such as virally encoded proteins like viral hemagglutinin, directly recognized by NKp46 [18], and m157, recognized by Ly49H [6]. As can be seen, NK cells possess multiple activating receptors to sense pathogenic insult and initiate response.

Upon ligand binding by an activating NK cell receptor, the associated adaptor proteins, composed of two disulfide-linked signaling chains each containing a canonical ITAM (immunoreceptor tyrosine-based activation motif, D/ExxYxxL/Ix_{6-8}YxxL/I) in the cytoplasmic domain, initiate signal transduction. Both tyrosine residues in the ITAMs become phosphorylated by Src-family protein tyrosine kinases, allowing for the recruitment and binding of Syk-family protein tyrosine kinases, such as Syk and Zap70 [19], to the phospho-ITAM sequences. Binding of Syk-family members allows for phosphorylation of downstream effector molecules, such as PI(3)K (phosphoinositol-3 kinase), LAT (linker of activated T cells), and PLCγ (phospholipase C_γ), resulting in activation of the calcium signaling pathway and downstream effectors of the MAP kinase pathway, activating transcription factors such as NF-κB [13], and culminating in initiation of NK cell effector functions, such as cytokine production and degranulation.

Inhibitory NK cell receptors and their ligands

Inhibitory NK cell receptors, like activating NK cell receptors, can generally be classified into one of two structural groups: 1) the type II C-type lectin-like proteins, including the homodimeric murine Ly49 receptors, as well as the NKG2A:CD94 heterodimer, expressed in both mouse and human, and 2) type I IgSF members, such as the KIRs expressed in humans. Unlike activating NK receptors, the inhibitory NK receptors do not possess charged transmembrane residues for association with signaling adapter molecules. Instead, each inhibitory receptor is endowed with a long cytoplasmic tail, capable of intrinsic signal transduction [13].

Ligands for inhibitory NK cell receptors are less varied than the ligands for activating receptors, most being classical MHC I (major histocompatibility complex, class Ia) molecules (Table 1). The classical MHC I molecules are recognized by members of the Ly49 family in mice and the KIR2DL and KIR3DL proteins in humans. Qa-1 and HLA-E, β_2m-associated, non-classical MHC Ib molecules, are recognized by the CD94:NKG2A heterodimer in both species [20]. NK cells also express inhibitory receptors specific for other, non-MHC ligands [21], such as E-cadherin bound by KLRG1 [22, 23], although the significance of these interactions is less well understood.

NK cell inhibitory receptor signaling serves to counteract signals received through activating receptors. Each inhibitory receptor encodes a long cytoplasmic tail containing an intrinsic ITIM (immunoreceptor tyrosine-based inhibitory motif, I/L/V/SxYxxL/V) [13]. Ligation of inhibitory receptors leads to phosphorylation of the ITIM-tyrosine and subsequent recruitment and binding of phosphatase molecules, such as SHP-1 (SH2-containing protein tyrosine phosphatase-1), SHP-2 and SHIP (SH2-containing inositol phosphatase) [24, 25]. It is believed that these phosphatases then dephosphorylate activating signaling molecules, resulting in termination of the signaling cascade and inhibition of NK cell effector function. However, more recent theories of NK cell inactivation propose a role for these phosphatases in the prevention of actin cytoskeleton reorganization, thereby preventing clustering of activating receptors and initiation of downstream signaling events [26]. In any case, it is clear that engagement of inhibitory receptors recruits phosphatase molecules, which ‘counterbalance’ activating signaling, and serve to inhibit the initiation of NK cell effector function.

MHC I engagement and the theory of 'missing-self' recognition

The most familiar function of inhibitory ligand recognition by NK cells is that of 'missing-self recognition' (Figure 1) [27, 28]. NK cells provide defense against pathogenic insult and abnormal cell growth by assessing the surface of self-tissues for the full complement of MHC I expression. Virally infected or transformed tumorogenic cells display antigens through MHC I for presentation to CD8+ T cells. However, in an immune evasion strategy, many viruses have developed proteins to block efficient antigen processing, peptide loading and subsequent display of MHC I at the cell surface [29]. Similarly, tumors are often found to have accumulated mutations that interfere with display of antigens through the MHC I antigen-processing pathway [30]. Cells lacking surface MHC I fail to engage the critical NK cell inhibitory receptors, inciting anti-viral and anti-tumor NK cell function in the form of cytokine secretion and direct cytotoxic activity. It has been definitively demonstrated that engagement of an inhibitory receptor with its cognate MHC I ligand is the interaction responsible for the inhibition of NK cell lytic activity, providing a molecular basis for 'missing-self' recognition [31, 32].

Necessary role for MHC I in NK cell function

One might predict that NK cells isolated from mice deficient in the expression of MHC I molecules would be hyper-responsive, due to the absence of inhibitory receptor engagement. Using the strategy of targeted gene-deficiency (i.e. 'knockout') to delete genes encoding proteins necessary for MHC I expression, such as β_2-microglobulin (β_2m) and TAP (transporter of antigenic peptides), this hypothesis was tested. Unexpectedly, NK cells isolated from environments lacking MHC I were found to be defective in

natural cytotoxicity and hyporesponsive to activation through crosslinking of activating receptors [33-35]. Later studies confirmed that NK cells from humans with similar genetic deficiencies (defects in HLA [human leukocyte antigen] expression) demonstrate identical functional deficits [36].

For a number of years, the basis for poor NK cell function in MHC I-deficient hosts remained unclear. At the time these data were published, identities of NK cell activating and inhibitory receptors and their ligands were still being deciphered. One idea, known informally as the 'at least one' theory, proposed that NK cells in MHC I-deficient mice (and humans) remained self-tolerant, even in the apparent absence of interaction with an inhibitory MHC I molecule, through undetected interactions between (undiscovered) inhibitory receptors and ligands [37-39]. However, this theory was refuted when experiments revealed the presence of NK cells lacking inhibitory receptors specific for any of the expressed self-MHC alleles, in hosts with normal MHC I expression [40]. Importantly, not only were these NK cells self-tolerant, they were shown to have defects in functional capacity. Thus, it was determined that the presence of MHC I alone is insufficient for the generation of functional NK cells. Instead, normal NK cell function appeared to require the ability of an NK cell to specifically recognize self-MHC I.

NK cell licensing

More recently, the contribution of MHC I to the development and function of NK cells was examined in greater detail on a single cell level. Utilizing a cell-free stimulation assay, Kim *et al.* determined that the signaling defect in NK cells derived from MHC I-

deficient hosts is widespread, impacting activation through multiple receptors that associate with various signaling adaptor molecules, indicating a generalized deficiency in the reception of activating signals [41]. However, they show that cells remain responsive to chemical stimuli, demonstrating that downstream effectors of the activating signaling pathways remain intact.

The authors further demonstrate that transfection of primary cells with a self MHC-specific inhibitory receptor and subsequent exposure of these cells to the ligand improved their ability to produce cytokines in response to crosslinking of activating receptors. Furthermore, in order for the NK cells to gain function, it was demonstrated that the transfected inhibitory receptor must contain an intact ITIM domain. This result was particularly intriguing, as there is not great precedent for the transduction of a positive signal (i.e. gain of function) through a receptor that is believed to have a purely inhibitory function. The observed phenomenon was termed ‘licensing’ and is defined as the process whereby the interaction of an inhibitory receptor with a cognate self-MHC ligand confers capacity for full biological function in a mature NK cell (Figure 2).

Similar education effects have been observed for human NK cells via self-HLA recognition by killer immunoglobulin-like receptors (KIRs), receptors related by convergent evolution to murine Ly49 receptors [42-45]. Thus, self-MHC I engagement by NK cell inhibitory receptors appears to be a fundamental element in acquisition of NK cell effector function

Proposed mechanisms for licensing

The nature of the signal responsible for NK cell licensing remains unclear. There are two contrasting models to describe the mechanism of the gain of function induced by licensing: disarming and arming [46]. In the model of disarming, the signal delivered to the NK cell through the inhibitory receptor is of an inhibitory nature, consistent with the function of an ITIM-bearing Ly49 receptor in a mature cell. This signal serves to counteract some continuous signal received via engagement of an activating receptor with a self-ligand. The transmission of an inhibitory signal via recognition of self-MHC I by an inhibitory NK receptor is postulated to alleviate or balance the positive signal and rescue the NK cell from overstimulation. NK cells devoid of inhibitory receptors or unable to interact functionally with inhibitory ligands, as in the case of MHC I-deficient animals, receive only positive signals, resulting in the induction of anergy. The nature, identity and source of the endogenous activating ligands and receptors responsible for induction of positive signaling have not been identified.

In contrast to the theory of disarming is that of arming, in which recognition of self-MHC I by an inhibitory NK cell receptor results in transmission of a 'positive' signal, resulting in functional responsiveness. In the absence of this licensing signal, NK cells remain hyporesponsive. While this theory, transmission of a positive signal through a receptor traditionally classified as having only inhibitory function, may be difficult to conceptualize, it should be noted that receptors often possess the capacity to signal multiple functions, depending on context. For example, the major antigen receptor of the T cell (TCR), serves to activate T cell effector functions, but may also transmit signals

resulting in anergy or commitment to a regulatory lineage, rather than activation [47]. Regardless of which mechanism is correct, it is clear that the interaction of an inhibitory NK cell receptor with a self-MHC ligand is required for the generation of fully competent mature NK cells.

Licensing as a rheostat

Thus far, licensing has been presented as an all or none phenomenon. However, under the tuning or rheostat model, NK cells acquire function in a graded manner depending on the number of inhibitory receptor:self-MHC interactions [48]. When NK cells possess more inhibitory receptors capable of interacting with multiple alleles of MHC I, they show greater function following stimulation through their activating receptors. NK cells with fewer self-MHC I contacts are less responsive. In studies using mice with different numbers of MHC alleles [49], increased function of NK cells was observed in hosts expressing multiple MHC I alleles, indicating a positive correlation between expression of MHC I alleles and education or licensing of the NK cell population. Additionally, in studies using multicolor flow cytometry to identify NK cell populations expressing multiple, single or no inhibitory receptors for self-MHC I molecules, there was a similar effect: NK cells with more inhibitory receptors capable of binding host MHC I molecules displayed greater function [50, 51]. Therefore, it has been confirmed that there is a positive correlation between the number of inhibitory receptors contacting self-MHC and increased NK cell function.

Kinetics, maintenance and reversibility of licensing

While the importance of the interaction between inhibitory receptor and self-ligand is understood, many other details of NK cell licensing remain unclear. In particular, questions remain regarding the time required for licensing to occur and the location of its occurrence, the relationship of licensing to development, the nature of the intracellular signaling events transmitted via the inhibitory receptor, and whether licensing results in a permanent alteration of NK cell function, akin to lineage commitment, or whether it is mutable.

One interesting unexplored topic is the relationship of NK cell licensing to the development of NK cells. During development in the bone marrow, NK cells undergo a proliferative burst following Ly49 receptor expression [52]. One may hypothesize that the signal resulting in this proliferation is somehow related to the licensing of NK cells, as both processes seem dependent upon the interaction of Ly49 receptors with self MHC. Additional literature reports suggest a possible survival advantage for NK cells following the interaction of inhibitory Ly49 receptors with self MHC molecules [53], again implying a positive relationship between NK cell development and licensing. While these data suggest that licensing may be a developmental process, occurring in the bone marrow, the timing and localization of NK cell licensing have not been formally established.

Another unresolved issue pertaining to both licensing and development concerns the permanency and plasticity of licensing. In particular, it is unknown whether licensing of

NK cells results in a permanent alteration of their phenotype, akin to lineage commitment, or whether the phenotype is reversible, allowing a previously licensed cell to become unlicensed. Additionally, it is unknown whether the specificity of licensing may be altered, such that an NK cell could become 'relicensed' on a different set of MHC I alleles.

Contributions of this work

In this thesis work, we address some of these questions using an experimental system of adoptive transfer of mature MHC I-deficient NK cells, which results in the inducible licensing of transferred NK cells. Characteristics examined include responsiveness to crosslinking of activating receptors, ability to efficiently kill target cells, and surface phenotype. Later experiments address several central issues regarding licensing, including its kinetics, the factors required for its induction and maintenance, as well as the potential for reversal of the licensed phenotype. Implications of these results on the therapeutic use of human NK cells will be discussed.

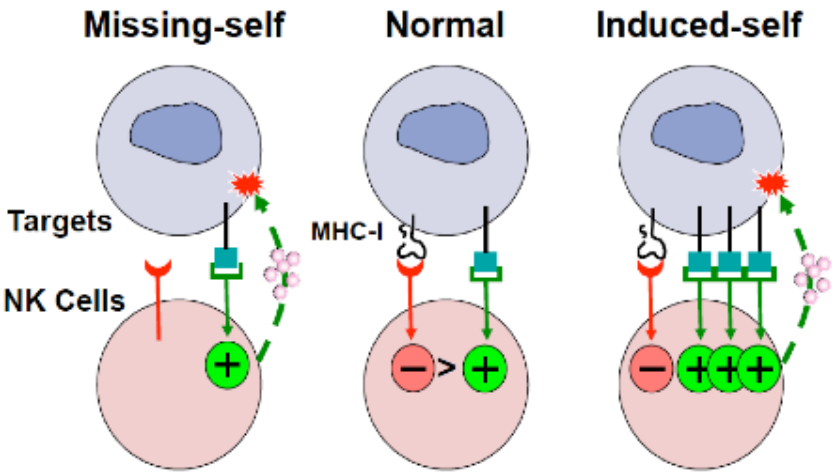
Table 1. Natural killer cell activating and inhibitory receptors and their ligands.

Natural killer cell function is dictated by an integration of signals through activating and inhibitory receptors. Listed are common activating and inhibitory NK cell receptors and their ligands. Receptors are expressed by both mouse and human, unless otherwise indicated.

		Receptor	Ligands		
			Murine	Human	Viral
Activating		NKG2C:CD94	Qa-1	HLA-E	
		NKG2D	Rae1 family H60, Mult1	MICA, MICB ULBP1-4	
		CD16	antibody Fc domain		
		NKp46			HA
	Murine	Ly49H			m157
	Human	KIR2DS1	-	HLA-C	
Inhibitory		NKG2A:CD94	Qa-1	HLA-E	
	Murine	Ly49C	$H2K^b$	-	
	Murine	Ly49A	$H2D^d$	-	
	Human	KIR2DL1,2,3	-	HLA-C	
	Human	KIR3DL1,2	-	HLA-A, HLA-B	

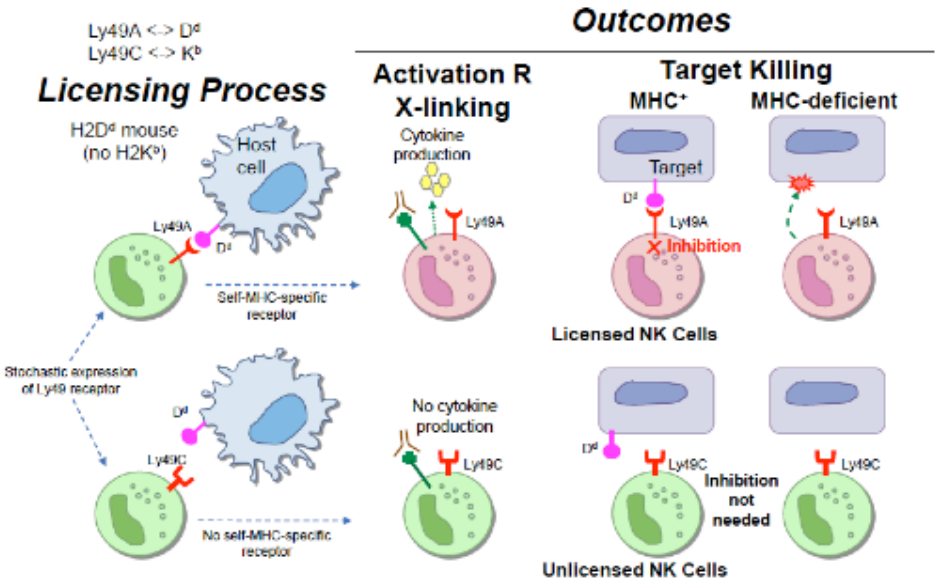
Figure 1. 'Missing-self' recognition results in NK cell activation.

NK cell function is dictated by an integration of signals through activating and inhibitory receptors. *Left:* Infected or transformed cell does not express surface MHC I ('missing-self'), and may or may not express activating ligands. NK cell effector functions of cytotoxicity and cytokine secretion are induced. *Center:* Healthy target cell expresses MHC I, inhibiting NK cell activation. *Right:* Infected or transformed cell expresses MHC I and viral or 'induced-self' proteins (e.g. H60, Mult-1). NK cell may become activated if a high number of activating receptors are also engaged.



Elliott and Yokoyama (2011) "Unifying concepts of MHC-dependent natural killer cell education." Trends in Immunology. In press.

Figure 2. NK cell licensing results in functional competence and self-tolerance. NK cells become licensed following interaction of an inhibitory NK receptor with a proper self-MHC I ligand. NK cells that are licensed are functionally competent to respond to stimulation via activating receptor crosslinking, and remain self-tolerant as a result of continued interaction between the inhibitory receptor and self-MHC I.



Elliott and Yokoyama (2011) "Unifying concepts of MHC-dependent natural killer cell education." Trends in Immunology. In press.

CHAPTER 2:

Materials and methods

Mice

C57BL/6 and C57BL/6 Ly5.1 mice were purchased from the National Cancer Institute (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME). β_2-microglobulin$^{-/-}$ mice were purchased from The Jackson Laboratory. $\beta_2m^{-/-}$ Ly5.1 mice were generated in house by crossing $\beta_2m^{-/-}$ mice with C57BL/6 Ly5.1 mice. $H2K^{b-/-}$ $H2D^{b-/-}$ $H2D^{d}$-expressing mice (KODO D8) were generated by crossing $H2K^{b-/-}$ $H2D^{b-/-}$ mice (purchased from Taconic Farms, Germantown, NY) with mice expressing a D^{d} transgene (D8), provided by D. Marguiles (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Triple knock-out (TKO) $K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ mice with transgenic expression of K^{b}-OVA MHC I as a single protein (TKO SCT-K^{b} Tg mice) were the generous gift of Dr. Ted Hansen (Washington University, Saint Louis, MO) [54]. Jinx mice, harboring a mutation in *Unc13d*, were kindly provided by Dr. Bruce Beutler (Scripps Research Institute, La Jolla, CA) and have been previously described [55]. Animals were housed in specific-pathogen free facilities at Washington University, and all mice were used between 6 – 16 weeks of age. Experiments were conducted in accordance with the animal protocol approved by the Animal Studies Committee at Washington University.

Antibodies, surface staining and flow cytometry

The following antibodies and reagents were purchased from BD Biosciences (San Jose, CA): anti-CD3 (145-2C11), anti-Ly5.1 (A20), anti-Ly5.2 (104), anti-NK1.1 (PK136), anti-Ly49A (JR9 and A1), anti-CD69 (H1.2F3), streptavadin APC (SA-APC), SA-PerCP-Cy5.5, SA-PE-Cy7. The following antibodies were purchased from eBioscience (San Diego, CA): anti-NKp46 (29A1.4), anti-IFNγ (XMG1.2), anti-K^{b}OVA (eBio25-

D1.16), anti-CD94 (18d3), anti-NKG2D (CX5), anti-CD11b (M1/70), anti-CD127 (A7R34), anti-CD49 (DX5), anti-CD107a (eBio1D4B). Anti-Ly49C (4LO33) and anti-Ly49H (3D10) were purified in our laboratory from hybridoma supernatants. (The 4LO33 hybridoma was provided by Suzanne Lemieux, Institut National de la Recherche Scientifique-Institut Armand-Frappier, Laval, Quebec, Canada.) All surface staining was performed on ice in staining buffer (3% FCS, 0.1% NaN_3 in PBS) containing anti-FcR antibodies (2.4G2). Samples were collected on a FACSCanto (Becton Dickinson, Franklin Lakes, NJ) using FACSDiva software (Becton Dickinson) and data were analyzed using FlowJo (TreeStar, Ashland, OR).

CFSE-label and adoptive transfer

For adoptive transfer experiments, donor splenocytes were purified by homogenization of spleens through a 70μM nylon filter. These cell preparations were red-blood cell lysed (10mM $KHCO_3$, 150mM NH_4Cl, 0.1mM EDTA pH 8.0) for 5min at RT and washed with media. Cells were resuspended in PBS, counted and washed, removing an aliquot to later determine NK percentage via antibody staining and flow cytometry. Cells were resuspended at 1×10^8 cells/mL in a 1μM CFSE (carboxyfluorescein diacetate succinimidyl ester, CellTrace™ Invitrogen) solution, and incubated at RT for 15min in the dark. Cells were washed twice in PBS and resuspended at 2.5×10^8 splenocytes/mL, and a small aliquot removed to verify the CFSE label via flow cytometry. Using a 28g insulin syringe, 200uL of the cell suspension was injected into the lateral tail vein of age-matched, sex-matched recipient mice.

In vitro stimulation and intracellular cytokine staining ('licensing assay')

Splenocytes were harvested from adoptive transfer recipients at day 7 post-transfer and stimulated with anti-NK1.1 (or anti-Ly49H) mAb as previously described [41]. Briefly, 1.0×10^7 splenocytes were plated into 6-well plates, that were either precoated with antibody (5μg/mL, 1.5 h at 37°C), contained media alone, or contained media with 0.5μg phorbol myristate acetate (Sigma, St. Louis, MO) and 4μg ionomycin (Calbiochem, La Jolla, CA). Cells were incubated for 1 h at 37°C in 5% CO_2. BrefeldinA (GolgiPlug, BD Biosciences) was added and cells were incubated for an additional 7h. Cells were then fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences), followed by antibody staining for intracellular IFNγ, as previously described [41].

Degranulation assay

Splenocytes were harvested from adoptive-transfer recipients at day 7 post-transfer, and NK cells were purified using magnetic separation columns (MACS NK isolation kit, Miltenyi Biotech, Germany). Separation purity was determined via flow cytometry (gating on $CD3^-$ $NK1.1^+$ cells), and NK cells were plated with target cells at a ratio of 1:1 (E:T) in 96-well V-bottom plates. Anti-CD107a antibody and monensin (eBioscience) were added to each well prior to incubation. Plates were incubated for 2 hours at 37°C, after which surface staining for flow cytometry was performed as described above.

Co-culture and kinetic analyses

Freshly isolated RBC-lysed splenocytes from C57BL/6 Ly5.1 and $\beta_2m^{-/-}$ donors were plated in 6-well plates at ratios indicated in the figures, at 1.0×10^7 cells/mL of R10 media

(RPMI, 10%FCS, L-glutamine, pen-strep)/well. For the kinetic analysis, a single donor population was prepared and injected into multiple recipients, which were analyzed on different days following transfer using the same set of reagents.

In vivo cytotoxicity assay

Splenocytes were harvested from WT and $\beta_2m^{-/-}$ donors by homogenization of spleens through 70μM nylon mesh. Cell preparations were RBC lysed and differentially labeled with 1μM or 2μM CFSE for 15min at RT, after which time cells were washed and resuspended at 1.5×10^8 cells/mL. A small aliquot was removed from each to verify the CFSE label by flow cytometry. 100μL of each cell preparation was taken, mixed and drawn into a 28-gage syringe, and injected into the lateral tail vein of age-matched, sex-matched recipients (resulting in a final injection of 1.5×10^7 WT cells and 1.5×10^7 $\beta_2m^{-/-}$ cells in a total volume of 200μL). 16hr post-transfer, recipient mice were sacrificed and spleens were homogenized and analyzed by flow cytometry for the presence of target cells remaining. The ratio of $\beta_2m^{-/-}$:WT targets recovered was determined.

Generation of bone marrow chimeras

The day of transfer, recipient mice were lethally irradiated (950rad). Using sterile technique, donor bone marrow was harvested by flushing femurs and tibias with a solution of 2% FCS in HBSS. Cells from multiple donors were pooled, washed and resuspended in RBC-lysis buffer for 5min at RT. Samples were washed with PBS, counted and resuspended at 1×10^7 cells/mL. Recipient mice were injected with 2×10^6 donor cells, via injection of the lateral tail vein. Mice were maintained on antibiotic

drinking water (sulfamethoxazole/trimethoprim, Hi-Tech Pharmacal Co, Inc, Amityville, NY) for 3w post-irradiation.

Generation of parabiotic mice

Mice were co-housed for 1w before SX, and opposing lateral sides were shaved 1d before the procedure. Mice were anesthetized with 5% isofluorane (1 – 1.5 L/min O_2), and maintained on isofluorane (2%) for the entire procedure. Using sterile tools and within a sterile field, skin was sterilized with betadine and an incision was made just distal to the patella. The incision was lengthened along the lateral aspect, continuing to the scapula and diverting along the brachial line, stopping just distal to the elbow. A margin of skin (~0.5cm) was separated away from the underlying connective tissue along the length of the incision, taking care to avoid puncture or compromise of the peritoneal cavity. Periodically through the procedure, the exposed mucosal layer was moistened with sterile saline solution. A single suture (chromic gut, Ethicon UG-203) was tied around the proximal head of the tibia, and another tied around the radius of the forearm. Mice were then placed in proximity and a single continuous suture was used to join the patellar ligament. The free ends of individual sutures from the tibia of each mouse were joined. This was repeated with the sutures of the radius. Skin was approximated and the incision was closed with 15-20 stainless steel wound clips (9mm, Beckton Dickinson, Autoclip®). Each mouse was injected with 0.1cc buprenorphine (0.02mg/mL) for pain management, and 1000cc 5% glucose in saline, for rehydration. Pairs were warmed under a heat lamp until mobile, then returned to standard cages, where a gel nutrition supplement, Clear H2O Hydrogel® (Portland, Maine), was placed on the bottom of the cage to facilitate

access. Pain medication was given for 3d post-SX, tapered from 0.1cc to 0.05cc. Antibiotics (trimethoprim/sulfamethoxazole) were administered via drinking water from the day of surgery through sacrifice.

Statistical analysis

Data are presented as mean ± SD. Statistics were generated using unpaired Student's *t* test within GraphPad Prism software (GraphPad, La Jolla, CA), and values $p < 0.05$ were considered significant.

CHAPTER 3:

Characterization of newly licensed NK cells

The goal of this thesis work is to determine factors that contribute to the MHC I-dependent gain of function, or licensing, of natural killer (NK) cells, as well as factors contributing to the maintenance of the licensed phenotype and the potential for its reversal. To more fully understand the processes surrounding licensing, as well as the potential for normal function in MHC I-deficient NK cells, we chose to utilize a system of adoptive transfer of MHC I-deficient NK cells to MHC I-sufficient hosts. Preliminary findings demonstrated that adoptive transfer of mature, peripheral, MHC I-deficient NK cells to wild type (WT) hosts led to increased capacity for cytokine production by previously hyporesponsive donor cells. We hypothesized that the gain of function is due to licensing of these cells, induced by exposure to host MHC I. We therefore sought to further investigate the gain of function, as we believed these findings would provide insight regarding the natural processes of NK cell licensing.

Introduction

A connection between MHC I expression and the generation of functional NK cells has been appreciated for nearly 20 years [33-35]. However, many of the details regarding the exact role for MHC I in NK cell education have remained poorly understood. Previous attempts to further characterize the contribution of MHC I to NK cell development and acquisition of function relied heavily on chimeric mice, in which the hematopoietic system of an irradiated host is reconstituted with the bone marrow from a genetically different donor [28, 33, 56, 57]. Results from these studies indicated that NK cells arising from MHC I-deficient bone marrow within a WT host (and vice versa) do not gain full functional capacity, as measured by a reduction in the *in vivo* rejection of MHC I-

deficient bone marrow or *ex vivo* killing of target cells. Interestingly, mixed bone marrow chimeras (WT+$\beta_2m^{-/-}$ marrow → WT hosts), yielded an intermediate killing phenotype. An important caveat to these experiments is that functional analysis (typically capacity for target rejection) was assessed for the bulk NK cell population. Therefore, the observed intermediate phenotype could not be definitively ascribed to full function of one subset and null function of the other, and/or equivalently intermediate function of some or all cells in the population. Thus, previous data utilizing bone marrow chimera systems to probe the influence of MHC I molecules on NK cell acquisition of function remained incomplete.

While experimental methods traditionally used to study NK cell biology had not yielded satisfying answers regarding the contribution of MHC I to NK cell development and function, important advances in experimental technique, including a cell-free stimulation assay (the 'licensing assay') [41] have allowed for progress in the field. In this particular assay, plate-bound monoclonal antibody with specificity for an activating NK cell receptor is used to crosslink the receptor, thereby stimulating cytokine production by NK cells during *ex vivo* culture. An inhibitor of the Golgi apparatus (BrefeldinA) [58] is added to the cultures, to allow for intracellular accumulation of cytokines, in particular IFNγ. The NK cells are then assayed for IFNγ production via intracellular staining and flow cytometry. Thus, cytokine production (i.e. functional capacity) can definitively be determined for each individual NK cell.

In our experiments, adoptive transfer of labeled cells followed by intracellular cytokine staining and analysis via flow cytometry permits direct comparison of IFNγ production between donor-derived NK cells and endogenous host NK cells, allowing for a quantitative assessment of the influence of host environment on donor cell function. Therefore, when paired with analyses of function at the single-cell level as in our experimental system, adoptive transfer becomes a useful tool with which to determine the contribution of MHC I to NK cell development and function.

NK cell adoptive transfer protocol

Briefly, the details of adoptive transfer are as follows: spleens are harvested from donor mice and splenocytes recovered from disrupted organs. Next, the splenocytes are treated with CFSE (carboxyfluorescein succinimidyl ester), a fluorescent dye that covalently labels cell membranes [59]. The cells are then washed, counted and aliquoted for i.v. injection into recipient mice. Typically, recipients were sacrificed 7 days post-injection, upon which time spleens were harvested, yielding a mixture of host- and donor-derived splenocytes, which were then experimentally manipulated.

Validation and optimization of adoptive transfer system

Initially, transfers of WT NK cells to WT hosts were performed, in order to optimize the experimental system, as well as examine the potential for artifact. Cells were injected into WT, immunoreplete hosts, which were not pre-conditioned (e.g. irradiation or NK-depletion) prior to injection. Upon harvest of host spleens 7d post-adoptive transfer (AT), the phenotype of host and donor splenocytes was examined by flow cytometry, paying

close attention to markers indicative of an activated NK cell phenotype. The phenotype of WT donor NK cells was equivalent to that of resting, WT host NK cells, indicating that donor cells are not activated by purification and adoptive transfer (Figure 3a). Additionally, WT NK cells did not proliferate in the WT hosts, (Figure 3b), as there was no dilution of CFSE, further demonstrating that adoptive transfer of NK cells did not initiate NK cell activation.

To maximize the number of donor NK cells recovered, we experimented with increasing the number of splenocytes transferred, from 5×10^7 up to ~8×10^7 (containing ~1.6×10^6 NK cells). However, increasing the number of transferred cells did not result in a greater recovery of donor NK cells from immunoreplete hosts. At 7d post-transfer, the average yield was consistently around 2×10^5 donor NK cells, comprising ~10% of the NK cell population in the recipient spleen. As we wished to recover the maximum number of donor cells possible, we next examined the potential for the use of immunodeficient mice as adoptive transfer recipients. We utilized doubly deficient $Rag2^{-/-}\gamma_C^{-/-}$ mice, which lack T, B and NK cells [60]. At 7d post-AT, WT donor NK cells recovered from $Rag2^{-/-}\gamma_C^{-/-}$ hosts had increased surface expression of activation markers, including: CD11b (Mac-1), CD11c and CD69 (Figure 4a). Additionally, the donor cells had proliferated to a great extent (Figure 4b). Taken together, these results strongly suggest that the WT donor NK cells had become activated in the immunodeficient hosts. These results were not particularly surprising, as other studies have noted the activating effects of homeostatic proliferation upon lymphocytes, in particular NK cells [61]. Therefore, while the use of immunoreplete hosts does limit the number of donor cells that may be recovered, in order

to avoid complications in data interpretation associated with activation of cells following homeostatic proliferation, we opted to use only immunoreplete hosts for the remainder of our studies.

WT NK-mediated rejection of MHC I-deficient splenocytes can be overwhelmed

In order to study the effect of MHC I on NK cell development and function, we wished to transfer MHC I-deficient splenocytes to WT hosts. It was considered that MHC I-deficient cells, such as those from $\beta_2m^{-/-}$ mice, would not successfully engraft in WT hosts, as it is known that MHC I-deficient grafts are eliminated following transfer to WT mice, in an NK-dependent manner [28, 33]. However, transfer of a sufficiently large number of cells can result in graft acceptance [62, 63], and we suspected we could produce a similar result. In order to verify this, we titrated down the number of MHC I-deficient splenocytes transferred to WT hosts, and found that grafts of fewer than 3×10^7 cells were efficiently eliminated. Additionally, we compared the survival of various MHC I-deficient immune cell subsets (e.g. T, B and NK) following their transfer to WT hosts, and found all populations engrafted/were eliminated equivalently. We therefore concluded that within the experimental time frame of 7d, transfer of a sufficient number of MHC I-deficient cells can, in fact, overwhelm the ability of endogenous WT NK cells to eliminate the MHC I-deficient graft, in a general mechanism that does not appear to favor retention of any hematopoietic lineage over another.

MHC I-deficient NK cells acquire the ability to make IFNγ following adoptive transfer to MHC I+ hosts

To examine the potential for gain of function by hyporesponsive, peripheral MHC class I-deficient NK cells, we harvested $\beta_2m^{-/-}$ splenocytes, CFSE-labeled them and injected them into MHC I-sufficient WT or control $\beta_2m^{-/-}$ hosts. At day 7, host spleens were harvested and bulk splenocytes were activated *in vitro* by anti-NK1.1 crosslinking, then analyzed for IFNγ production, via intracellular flow cytometry (i.e. the 'licensing assay'). Surprisingly, we found that following activating receptor crosslinking, donor-derived MHC I-deficient $\beta_2m^{-/-}$ NK cells recovered from WT hosts produced IFNγ at a level equivalent to that of host WT NK cells (Fig 5). In contrast, $\beta_2m^{-/-}$ donor cells recovered from $\beta_2m^{-/-}$ control hosts produced IFNγ at a frequency identical to host-derived $\beta_2m^{-/-}$ NK cells (Fig 5). This was equivalent to the hyporesponsive production of IFNγ by NK cells from unmanipulated $\beta_2m^{-/-}$ control animals (data not shown). Additionally, this greater capacity for cytokine production was also observed when NK cells were stimulated through another activating receptor, Ly49H, which uses a different intracellular signaling chain (DAP12, as opposed to $F_CR\gamma$, used by NK1.1, [64]) (Figure 6). Therefore, adoptive transfer of hyporesponsive, MHC I-deficient splenic NK cells appeared to result in the acquisition of global activation receptor-associated responses that correlated with host NK cell responsiveness.

Using intracellular staining and flow cytometry, we also examined the NK cell production of cytokines other than IFNγ, including TNFα and GM-CSF, following adoptive transfer. However, our stimulation to assess the licensed phenotype, cross-

linking of activating receptors, does not induce NK cells to produce large quantities of TNFα, making this a poor candidate for functional analyses. Additionally, although NK cells can be incited to produce large amounts of GM-CSF (confirmed by ELISA), this cytokine was not detectable by flow cytometry, under various conditions.

Gain of function is cell-intrinsic and not acquired during co-stimulation

Though our data indicated that MHC I-deficient NK cells had gained functional capacity after residence in WT hosts, it remained possible that the observed gain of function was not the result of a cell intrinsic change in the MHC I-deficient NK cells, but rather simply due to a positive influence of WT splenocytes on the MHC I-deficient cells during the *in vitro* stimulation. To test the requirement for residency in WT hosts, as well as identify the gain of function of MHC I-deficient NK cells as cell-intrinsic or cell-extrinsic, we performed an *in vitro* experiment in which freshly isolated $\beta_2m^{-/-}$ and WT splenocytes were mixed at various ratios and stimulated under the conditions utilized for the analyses of adoptively transferred cells. Regardless of the presence of WT cells in co-culture, $\beta_2m^{-/-}$ NK cells retained their hyporesponsive phenotype following activating receptor crosslinking (Figure 7). Additionally, transfer of supernatant from stimulated WT splenocyte cultures to wells containing $\beta_2m^{-/-}$ splenocytes did not result in increased functional capacity of $\beta_2m^{-/-}$ NK cells. Therefore, during the *in vitro* co-culture, WT splenocytes did not impart gain of function to MHC I-deficient NK cells by either contact-dependent or contact-independent mechanisms. These results indicate that the gain of function of the $\beta_2m^{-/-}$ NK cells following adoptive transfer to WT hosts is acquired during residence in the MHC class I-sufficient environment, not during co-

culture and stimulation, and that the gain of function is likely due to a cell-intrinsic change in the $\beta_2m^{-/-}$ NK cells.

Gain of function is not consistent with a 'memory' phenotype

It has been demonstrated that previously activated NK cells exhibit a memory-like property of enhanced cytokine production upon restimulation [65, 66]. Therefore, we sought to determine if our results could reflect prior donor NK cell activation. Prior studies showed that memory-like NK cells preferentially proliferate upon transfer [65, 66]. By contrast, donor $\beta_2m^{-/-}$ NK did not proliferate following residence in WT hosts, as evidenced by a lack of CFSE dilution (Figure 8a). Additionally, donor $\beta_2m^{-/-}$ NK did not spontaneously produce IFNγ or display markers of activation, such as CD69, at any time from days 1-7 post-transfer (Figure 8b and data not shown). Taken together, these data indicate that $\beta_2m^{-/-}$ NK cells were not activated following adoptive transfer to WT hosts, suggesting that acquisition of function is not due to the transition of $\beta_2m^{-/-}$ NK cells to a 'memory-like' state.

Gain of function correlates with licensing

We hypothesized that the gain in function of $\beta_2m^{-/-}$ NK cells following their transfer to WT hosts was due to licensing of these cells. Ly49C is an inhibitory NK cell receptor with a strong ligand in the H-2^b background, H2-K^b [67]. If transfer of hyporesponsive $\beta_2m^{-/-}$ NK cells into an MHC I-sufficient environment results in licensing of $\beta_2m^{-/-}$ NK cells, one should find a higher frequency of IFNγ+ cells among the Ly49C+ population than the Ly49C- population, as NK cells expressing a cognate inhibitory receptor for an

expressed MHC I molecule (in this case, Ly49C for K^b) would be capable of becoming licensed. Analyses were performed to examine the correlation between expression of Ly49C and production of IFNγ by donor NK cells, following adoptive transfer. When adoptively transferred $\beta_2m^{-/-}$ NK cells were analyzed for Ly49C expression in parallel with IFNγ production, it was found that $\beta_2m^{-/-}$ Ly49C+ NK cells do make IFNγ at a statistically significantly higher frequency than Ly49C- NK cells, following recovery from a WT host (Figure 9), suggesting that $\beta_2m^{-/-}$ NK cells become licensed as a result of their residence in MHC I-expressing, WT hosts.

Gain of function extends to cytotoxicity and degranulation

MHC I-deficient NK cells demonstrate defects in cytokine production, as well as cytotoxicity, as compared to WT NK cells [28, 33]. To examine the gain of cytotoxic function in $\beta_2m^{-/-}$ NK cells following adoptive transfer, we first performed a traditional ^{51}Cr release assay, using sorted donor $\beta_2m^{-/-}$ NK cells as effectors, purified from WT hosts 7d post-AT. However, recovery of a low number of transferred cells permitted very few E:T conditions, giving these results limited value.

Therefore, as an alternative to the direct cytotoxicity assay, we exposed transferred NK cells to sensitive targets and assessed the level of surface CD107a (LAMP-1), using antibody staining and flow cytometry. Surface expression of CD107a is used as a surrogate marker for NK cell cytotoxicity, as it is upregulated on the cell surface following degranulation, and killing is dependent upon degranulation [68]. Following transfer to MHC I-expressing hosts, $\beta_2m^{-/-}$ NK cells gained the ability to degranulate at a

level equivalent to that of host and control WT NK cells in response to YAC targets (Figure 10). By contrast, $\beta_2m^{-/-}$ NK cells adoptively transferred to $\beta_2m^{-/-}$ hosts remained defective in degranulation, demonstrating levels comparable to Jinx NK cells, which cannot degranulate due to a mutation in *Unc13d* [55], and therefore serve as a negative control. Furthermore, as with cytokine production, we found no increase in the ability of freshly isolated $\beta_2m^{-/-}$ NK cells to degranulate in response to target cells following co-incubation with WT NK cells *in vitro* (Figure 11). Taken together, these data indicate that the gain in capacity for degranulation, and presumably cytotoxicity, observed in MHC I-deficient cells is acquired following their residence in the WT host environment.

To complement flow-based degranulation data regarding gain of cytotoxic function following adoptive transfer, the utility of a flow-based *in vivo* target rejection assay was assessed. In pilot experiments, WT NK cells were transferred to MHC I-sufficient, NK-dysfunctional hosts (Jinx), to determine the maximum increase in cytotoxic capacity achievable following adoptive transfer of functional NK cells to an immunoreplete host. At day 7 post-AT, MHC I-deficient and MHC I-sufficient splenocyte targets were differentially labeled with CFSE and injected into these AT-recipient hosts. After 16-24hr, mice were sacrificed and remaining targets were assessed by flow cytometry of host spleens. We noted only a very modest increase (about 4.5%) in the total *in vivo* killing capacity of Jinx mice which had previously received an adoptive transfer of WT NK cells 7d before the target cell injection over untreated Jinx controls, even though the adoptively transferred WT NK cells were retained at a typical level (comprising ~10% of the host spleen at 7d post-AT). Therefore, the minimal change renders this *in vivo* target rejection

assay unrealistic for discriminating increase in cytotoxic capacity of MHC I-deficient cells following their transfer to lymphoreplete, MHC I-sufficient hosts (Figure 12).

Gain of function is acquired 48-72 hours post-transfer

Kinetic analyses were performed to more precisely determine the time required to establish the gain of function in MHC I-deficient NK cells, following their transfer to WT hosts. For these experiments, a large preparation of $\beta_2m^{-/-}$ donor splenocytes was injected into many experimental (WT) and control ($\beta_2m^{-/-}$) recipients on day 0. Mice having received injections of cells from the common preparation were sacrificed on subsequent days following transfer, so that in each experimental time course, all hosts possessed cells from an identical starting pool, facilitating chronological comparison. The results of these analyses revealed that the gain of function in MHC I-deficient NK cells occurs approximately 3 – 4 days post-transfer to WT hosts, and remains stable through 7d (Figure 13). Additional analyses indicate that the gain of function holds until at least 14 days post-AT. However, donor cell recovery is poor at these later time points, presumably due to continued elimination of MHC I-deficient grafts by WT host NK cells, hindering further analysis.

Phenotype and tissue distribution of cells following adoptive transfer

Previous studies indicate that splenic NK cells from β_2m-deficient animals are mature and appear indistinguishable from peripheral NK cells in WT mice [46, 52, 69, 70]. Regardless, one potential mechanism for the gain of function by transferred $\beta_2m^{-/-}$ NK cells could be differentiation of the cells following transfer. Phenotypic analyses at 7d

post-AT indicate that, regardless of the genotype of the host or donor, NK cells appeared indistinguishable in most aspects. For example, markers such as NK1.1, CD94, NKG2A, CD69, DX5, and 2B4 showed no difference in expression between $\beta_2m^{-/-}$ and WT NK cells, before or after adoptive transfer (Figure 14). Interestingly, other markers, in particular activation receptors such as NKp46, NKG2D and CD11b, were expressed at a slightly higher level on WT NK cells when compared with $\beta_2m^{-/-}$ NK cells, and their expression was slightly increased on $\beta_2m^{-/-}$ NK cells following transfer to WT hosts. The mechanism for the increase in activating receptor expression, as well as the significance of this increase, remains unknown. Overall, while there were small increases in the expression of some activating receptors, there were minimal changes in the expression of most surface molecules following adoptive transfer of $\beta_2m^{-/-}$ cells to WT hosts.

Tissue distribution of transferred and endogenous cells was compared following adoptive transfer of $\beta_2m^{-/-}$ splenocytes to WT hosts. Peripheral organs (spleen, liver, thymus, LN and BM) were homogenized and examined via flow cytometry. There were no obvious differences, defects or abnormalities in the distribution of donor NK cells within the host, as compared to distribution of WT NK cells.

Summary

To more fully understand the processes surrounding NK cell education and licensing, as well as the potential for normal function in MHC I-deficient NK cells, we chose to utilize a system of adoptive transfer of MHC I-deficient NK cells to MHC I-sufficient, WT hosts. Surprisingly, we discovered that, although hyporesponsive in an MHC I-deficient

environment, MHC I-deficient NK cells can acquire normal functionality in MHC I+ hosts. Our results are the first to demonstrate that MHC I-deficient NK cells retain the capacity for normal function and that cell-intrinsic MHC I expression is not required for NK cell function.

The gain of function observed for MHC I-deficient NK cells following their adoptive transfer to WT hosts applies to both NK-mediated cytokine production, as well as cytotoxicity, and does not appear to be related to either the maturation of these cells, nor the generation of a memory-like phenotype induced by activation. Additionally, this gain of function is cell-intrinsic, as it cannot be induced by co-stimulation with WT cells. Interestingly, we observed that NK cells expressing a cognate inhibitory receptor capable of interacting with the MHC I molecule are more likely to gain function than NK cells that do not express such a receptor. Therefore, consistent with all findings, we propose that this observed gain in function is due to licensing of the MHC I-deficient cells within the WT, MHC I-sufficient hosts, following exposure to MHC I molecules.

The length of time required for the process of NK cell education or licensing has never before been experimentally determined. Our kinetic analyses reveal that the gain of function following adoptive transfer of MHC I-deficient NK cells to WT hosts requires approximately 72 hours. Given that our data suggest the gain of function is due to licensing of the MHC I-deficient NK cells, it is interesting to consider the implications of this time frame on the process of licensing. If the mechanism of licensing were governed by differential phosphosignaling events at the plasma membrane, or physical

rearrangement of existing signaling components, one may predict the induction of function to occur quickly, on the order of minutes. Instead, we observe that it takes days for MHC I-deficient NK cells to become functional following adoptive transfer. Perhaps these data indicate that licensing requires modifications within the cell, such as new transcription, new translation of transcripts and protein synthesis, and/or chromatin rearrangement. While experiments examining the transcriptome of licensed and unlicensed NK cells have been fairly uninformative (unpublished observation, personal communication, Joe Wahle), experiments examining the newly synthesized proteome (via ribosomal IP) or changes in chromatin accessibility (via ChIP) may be revealing.

In conclusion, these data demonstrate that MHC I-deficient, hyporesponsive NK cells become functional following exposure to MHC I molecules expressed in WT hosts. Future experiments focus on the identification, as well as the necessary localization of molecules required to impart the gain of function, as well as for the maintenance of the functional phenotype and the potential for its reversal.

Figure 3. Transfer to an immunoreplete host does not activate WT NK cells.

Splenocytes were purified from WT (C57BL/6) donors, CFSE-labeled and i.v. injected into unconditioned WT hosts. At 7d post-transfer, host spleens were harvested and NK cell phenotype was assessed via antibody staining for surface antigens, followed by flow cytometry. Cells are gated as NK1.1+CD3-CD19- and were examined for **(a)** surface phenotype, using CFSE+/- to discriminate donor and host cells, and (**b)** proliferation, as measured by CFSE dilution. Results are representative of 2 independent experiments.

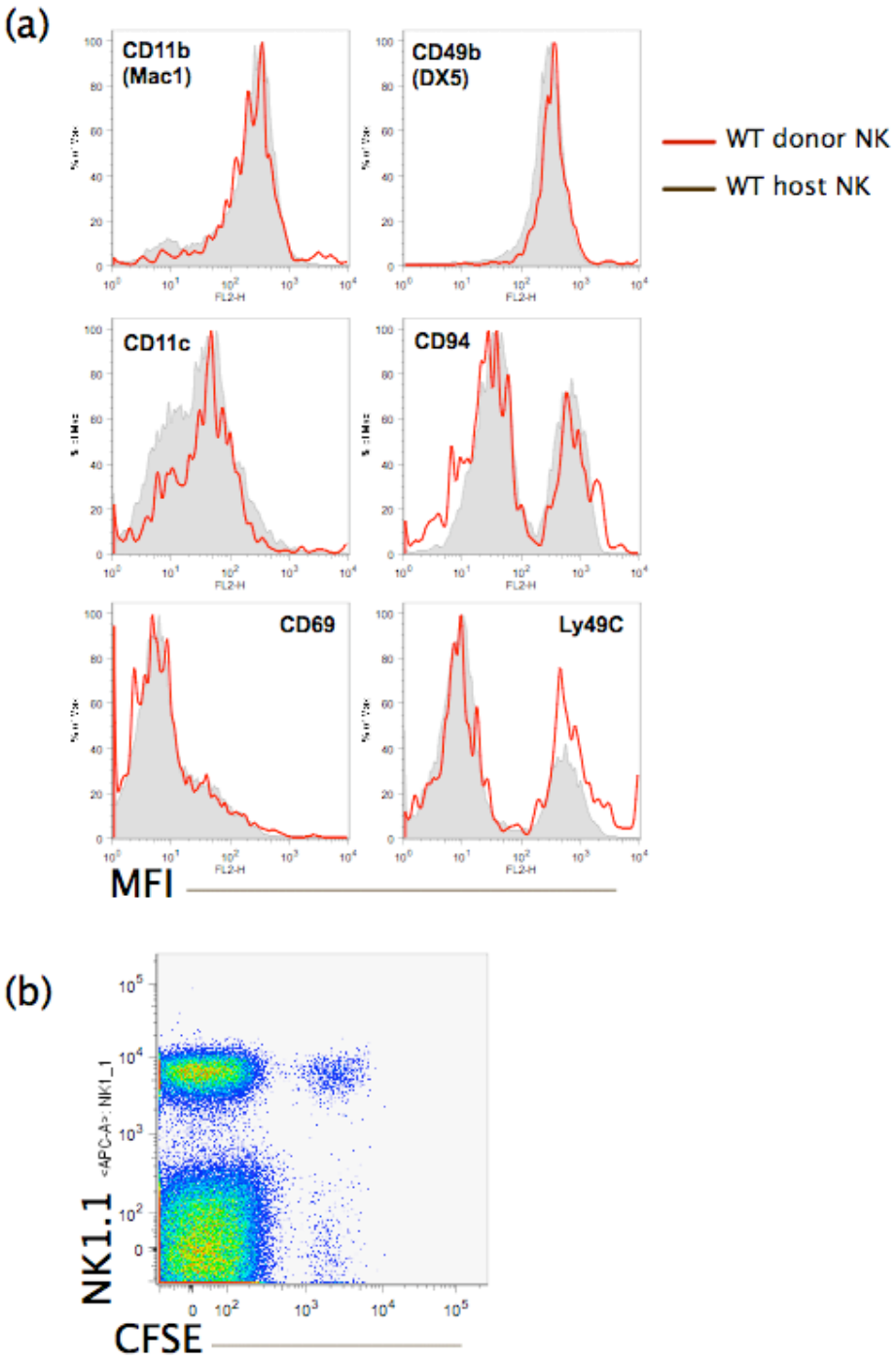

(a)
CD11b
(Mac1)
CD49b
(DX5)
CD11c
CD94
CD69
Ly49C
WT donor NK
WT host NK
MFI
(b)
NK1.1
CFSE

Figure 4. Adoptive transfer to an immunodeficient host activates WT NK cells. Splenocytes were purified from WT (C57BL/6) donors, CFSE-labeled and i.v. injected into unconditioned $Rag2^{-/-}\gamma_C^{-/-}$ and WT hosts. At 7d post-transfer, host spleens were harvested and NK cell phenotype was assessed via antibody staining for surface antigens, followed by flow cytometry. Cells are gated as NK1.1+CD3-CD19- and were examined for **(a)** surface phenotype, using CFSE+/- to discriminate donor and host cells, and (**b)** proliferation, as measured by CFSE dilution. Results are representative of 3 independent experiments.

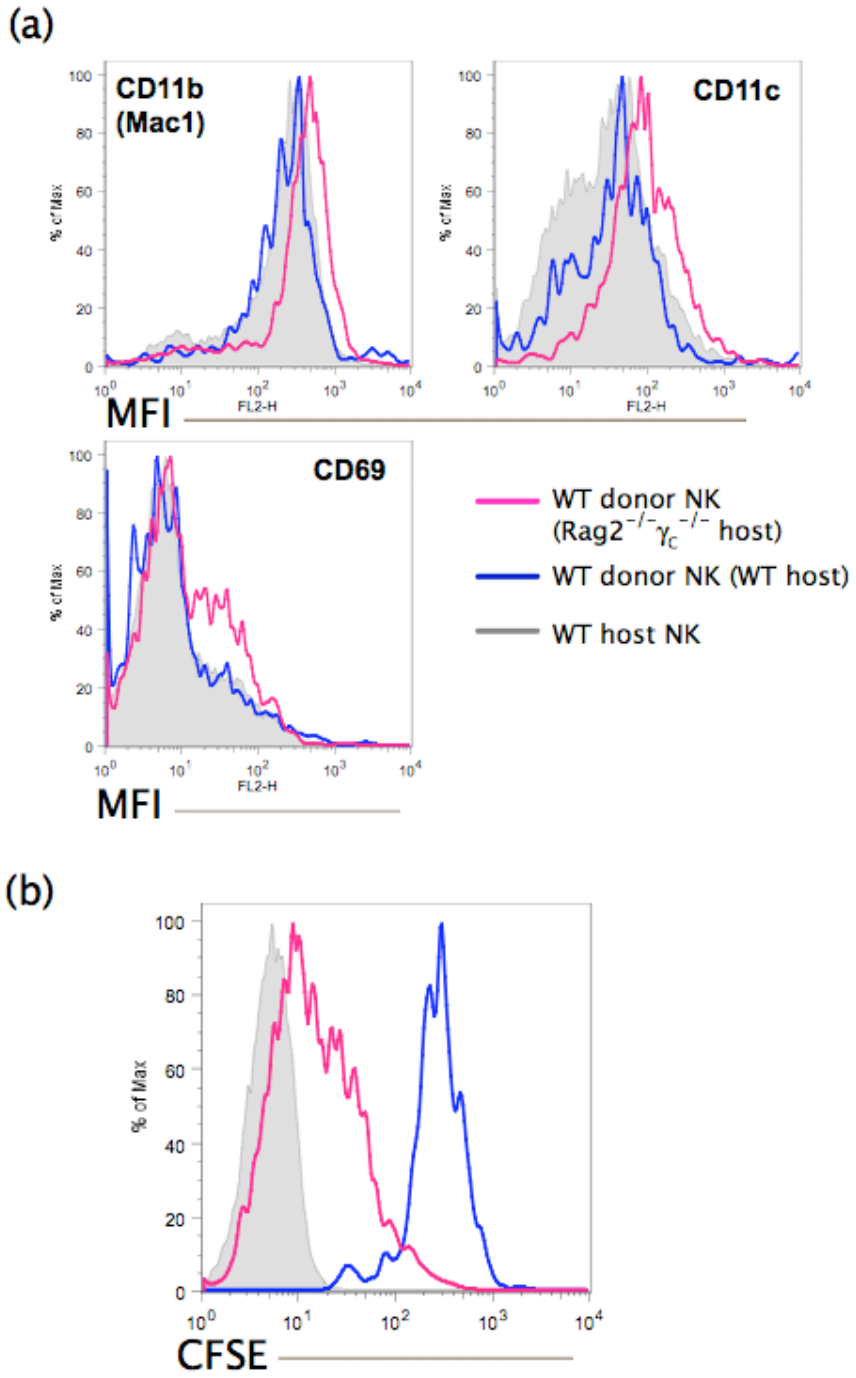

(a)
CD11b
(Mac1)
CD11c
CD69
% of Max
FL2-H
MFI
WT donor NK
(Rag2$^{-/-}\gamma_c^{-/-}$ host)
WT donor NK (WT host)
WT host NK
(b)
% of Max
CFSE

Figure 5. MHC I-deficient NK cells gain ability to make IFNγ in response to stimulation, following transfer to WT hosts.

$\beta_2m^{-/-}$ splenocytes were purified, CSFE-labeled and i.v. injected into unconditioned WT and $\beta_2m^{-/-}$ hosts. At 7d post-transfer, host spleens were harvested and total splenocytes stimulated via anti-NK1.1 antibody-mediated receptor crosslinking (PK136, 5μg/mL). Cells were stained for surface markers and intracellular IFNγ, then examined by flow cytometry. Plots depict IFNγ production versus Ly49C expression of donor (CSFE+) or host (CSFE-) NKp46+CD3- NK cells. Numbers indicate percentage of cells in each quadrant. Data are representative from 3 independent experiments.

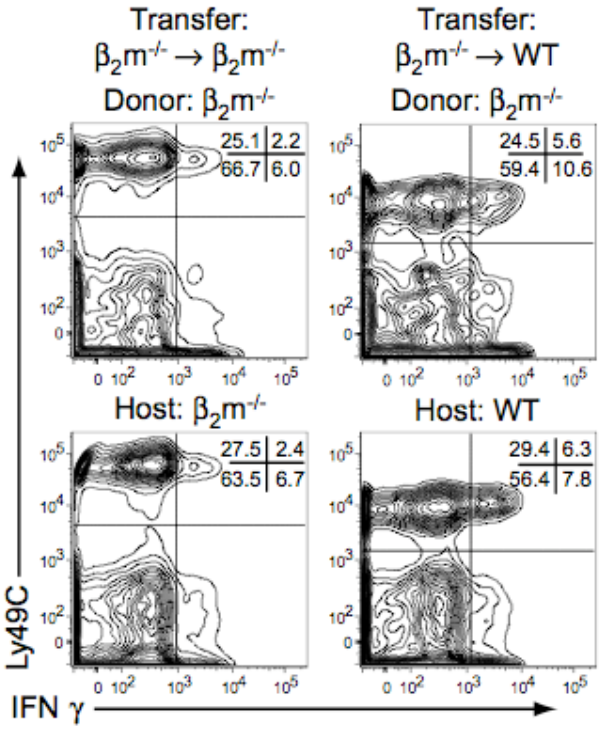

Transfer:
β2m-/- → β2m-/-
Donor: β2m-/-
25.1 2.2
66.7 6.0
Transfer:
β2m-/- → WT
Donor: β2m-/-
24.5 5.6
59.4 10.6
Host: β2m-/-
27.5 2.4
63.5 6.7
Host: WT
29.4 6.3
56.4 7.8
Ly49C
IFN γ

Figure 6. Gain of function by MHC I-deficient NK cells following activation through an alternate stimulus.

Summary of IFNγ production by Ly49C^{+} $\beta_2m^{-/-}$ NK cells recovered 7d post-transfer to WT or $\beta_2m^{-/-}$ hosts. On recovery, splenocytes were subjected to an 8hr *ex vivo* stimulation by activation receptor crosslinking via anti-Ly49H (3D10, 5μg/mL). Data are from 1 experiment, with 2-4 mice/group. Means $\pm$ standard error of the mean are shown.

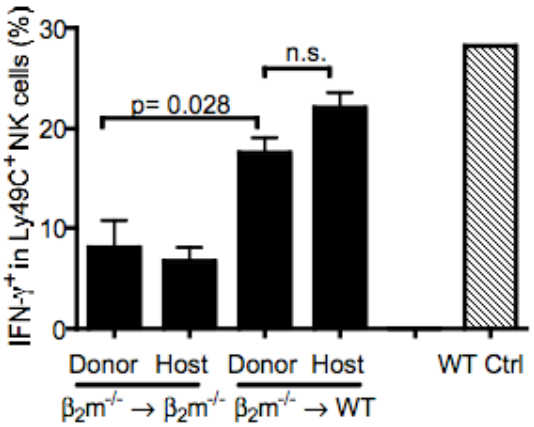

IFN-γ+ in Ly49C+ NK cells (%)
30
20
10
0
p= 0.028
n.s.
Donor
Host
Donor
Host
WT Ctrl
β2m-/- → β2m-/-
β2m-/- → WT

Figure 7. MHC I-deficient and WT NK cells retain distinct cytokine production phenotypes during splenocyte co-culture.

Freshly isolated splenocytes were purified from $\beta_2m^{-/-}$(Ly5.2) and Ly5.1 WT (C57BL/6) mice and plated at the indicated ratios. IFNγ production was assessed by flow cytometry after 8 hour *ex vivo* stimulation with plate-bound anti-NK1.1 (PK136, 5μg/mL), PMA + ionomycin (0.5μg/mL, 4μg/mL), or IL-12 + IL-18 (10ng/mL, 50ng/mL). $\beta_2m^{-/-}$ and WT cells were distinguished by anti-Ly5.2 antibody staining. Representative data from one of two experiments.

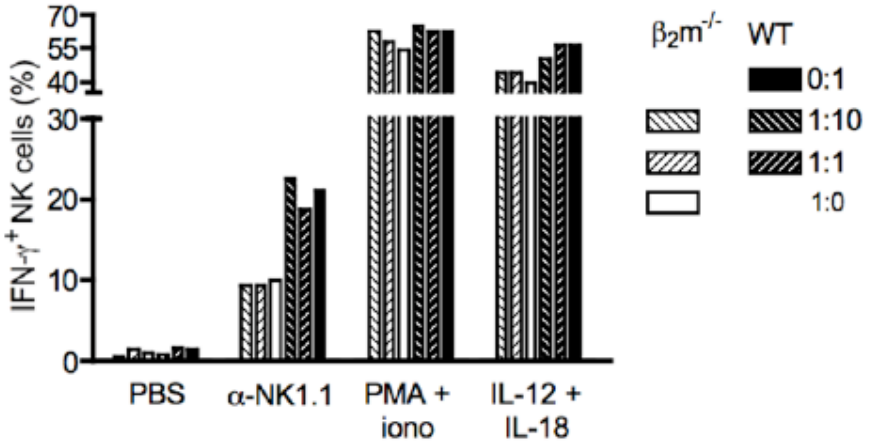

70
55
40
30
20
10
0
IFN-γ+ NK cells (%)
PBS
α-NK1.1
PMA + iono
IL-12 + IL-18
β2m-/-
WT
0:1
1:10
1:1
1:0

Figure 8. MHC I-deficient NK cells do not proliferate and do not constitutively produce IFNγ following adoptive transfer into WT hosts.

(a) Representative flow cytometry plot depicting CFSE intensity at day 7 post-transfer; cells were gated on forward and side scatter and NKp46^{+}. **(b)** Summary of spontaneous IFNγ production by NK cells at day 7 post-transfer after 8 hours *ex vivo* culture in untreated media. Data are from 3 independent experiments, 3-5 mice/group. Means $\pm$ standard error of the mean are shown.

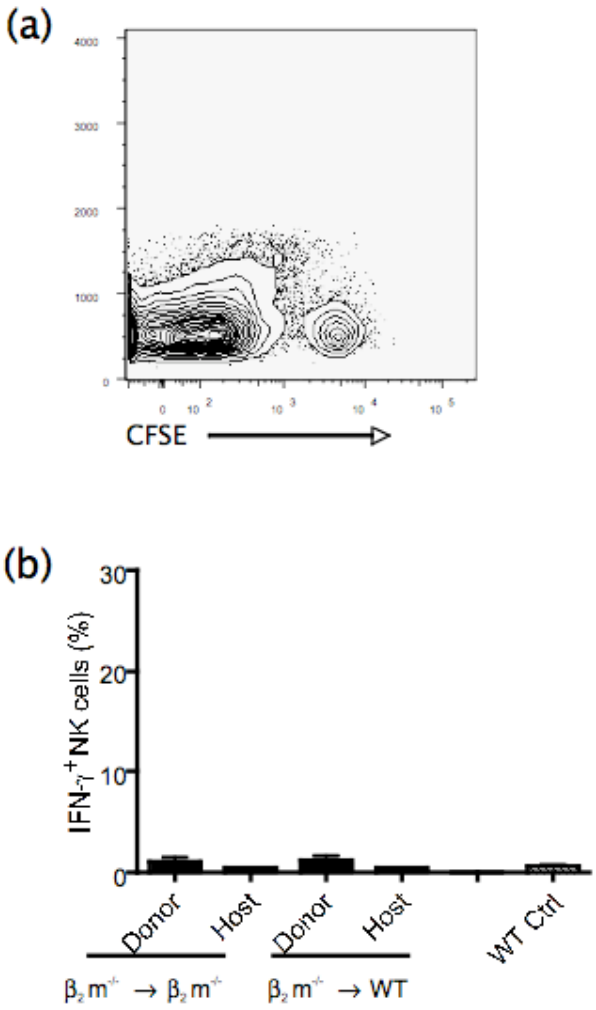
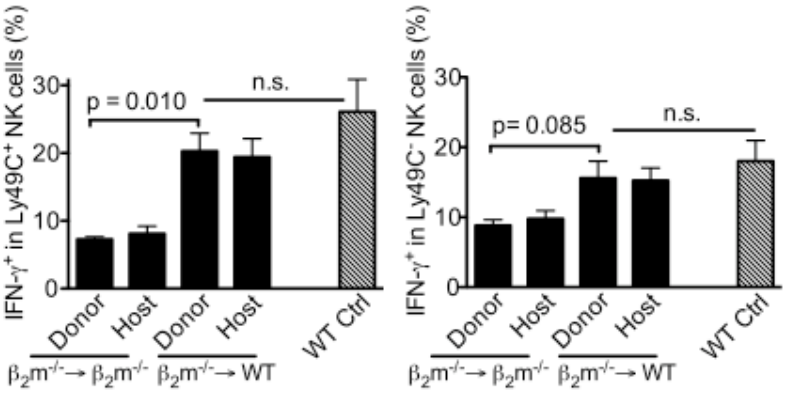

(a)
4000
3000
2000
1000
0
0
10 2
10 3
10 4
10 5
CFSE
(b)
IFN-γ+ NK cells (%)
30
20
10
0
Donor
Host
Donor
Host
WT Ctrl
β2m-/- → β2m-/-
β2m-/- → WT

Figure 9. MHC I-deficient Ly49C+ NK cells are more likely than MHC I-deficient Ly49C- NK cells to produce IFNγ after adoptive transfer to WT hosts.

(a) IFNγ production by Ly49C+ (left) and Ly49C- (right) $\beta_2m^{-/-}$ NK cells recovered from WT and $\beta_2m^{-/-}$ hosts, 7d post-transfer, following anti-NK1.1 stimulation. **(b)** Direct comparison of IFNγ production by $Ly49C^+$ and $Ly49C^-$ donor ($\beta_2m^{-/-}$) NK cells following anti-NK1.1 stimulation, 7d post-transfer to WT hosts. Data are pooled from 3 independent experiments, with 3-5 mice/group. Means are shown as horizontal lines with error bars representing standard error of the mean

(a)


IFN-γ+ in Ly49C+ NK cells (%)
30
20
10
0
p = 0.010
n.s.
Donor
Host
Donor
Host
WT Ctrl
β2m-/-→ β2m-/-
β2m-/-→ WT
IFN-γ+ in Ly49C- NK cells (%)
30
20
10
0
p= 0.085
n.s.
Donor
Host
Donor
Host
WT Ctrl
β2m-/-→ β2m-/-
β2m-/-→ WT

(b)

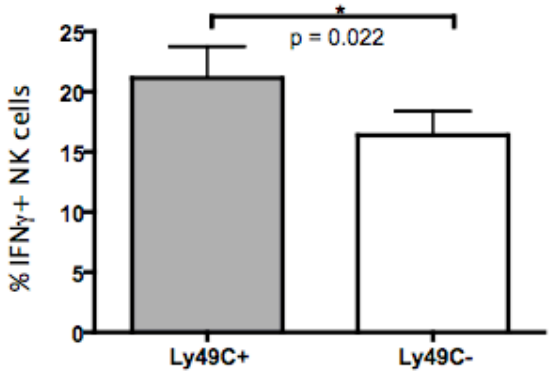

% IFNγ+ NK cells
25
20
15
10
5
0
*
p = 0.022
Ly49C+
Ly49C-

Figure 10. MHC I-deficient NK cells gain WT degranulation capacity following adoptive transfer to MHC I-sufficient hosts.

Splenocytes were purified from $\beta_2m^{-/-}$ donors and adoptively transferred into WT, $\beta_2m^{-/-}$ or Jinx hosts. On day 7 post-transfer, recipient splenocytes were harvested, co-incubated with YAC target cells and assayed for surface CD107a expression. **(a)** Representative contour plots showing CD107a expression on NK cells gated on $Ly5.1^+$(donor) or $Ly5.1^-$ (host) $CD3^-NK1.1^+$ cells. Figures show % of cells positive for surface CD107a staining. **(b)** Summary of CD107a surface expression by host and donor NK cells following incubation with target cells. Data are representative from 3 independent experiments, with 2-3 mice/group for adoptive transfer donor and host cells, and 1-2 mice/group for controls. Means are shown as horizontal lines with error bars representing standard error of the mean.

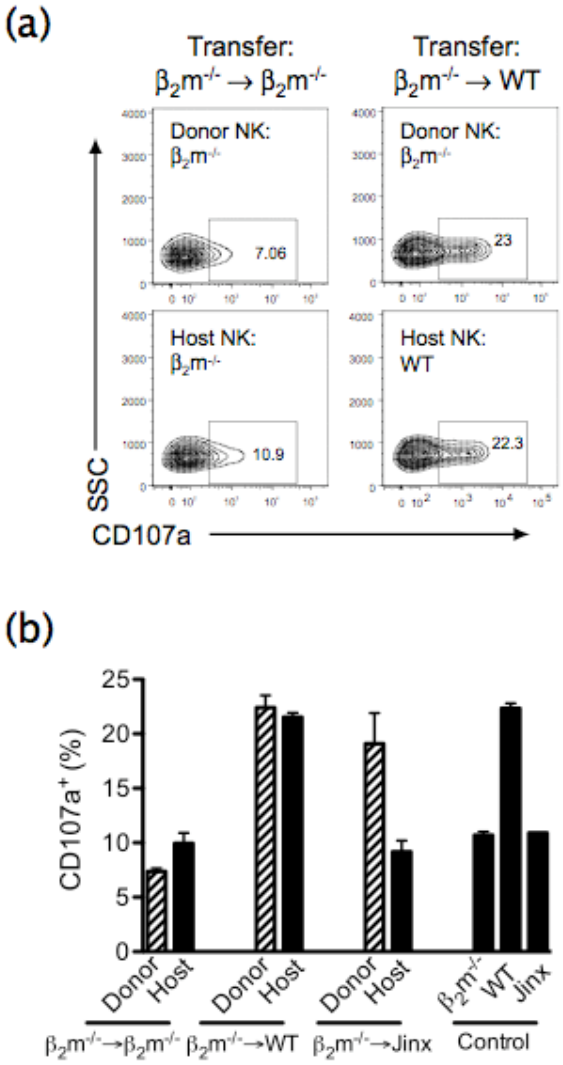

(a)
Transfer:
β2m-/- → β2m-/-
Transfer:
β2m-/- → WT
Donor NK:
β2m-/-
7.06
Donor NK:
β2m-/-
23
Host NK:
β2m-/-
10.9
Host NK:
WT
22.3
SSC
CD107a
(b)
CD107a+ (%)
25
20
15
10
5
0
Donor
Host
Donor
Host
Donor
Host
β2m-/-
WT
Jinx
β2m-/-→β2m-/-
β2m-/-→WT
β2m-/-→Jinx
Control

Figure 11. MHC I-deficient and WT NK cells retain distinct degranulation phenotypes during splenocyte co-culture.

Freshly isolated splenocytes were purified from $\beta_2m^{-/-}$(Ly5.2) and Ly5.1 WT (C57BL/6) hosts and plated at the indicated ratios. NK cells were assessed via flow cytometry for surface CD107a expression after 2 hour stimulation with YAC or RMA target cells (E:T, 1:1), or PMA + ionomycin (positive control), or PBS (negative control). $\beta_2m^{-/-}$and WT cells were distinguished with an anti-Ly5.2 antibody. Representative data from one of two experiments.

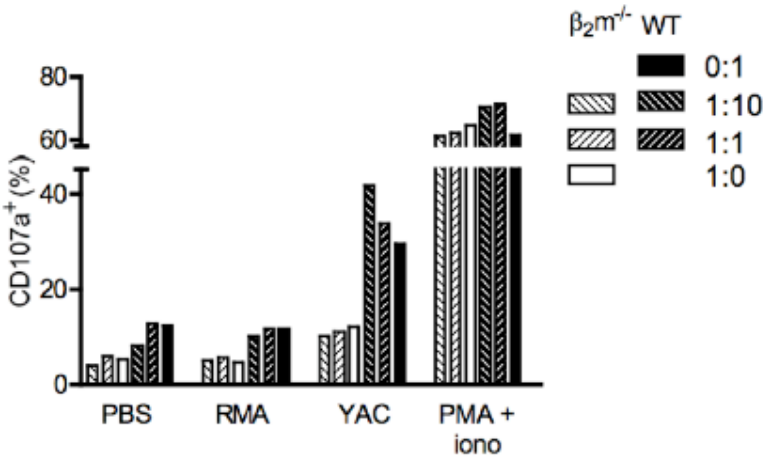

β2m-/- WT
0:1
1:10
1:1
1:0
CD107a+ (%)
80
60
40
20
0
PBS
RMA
YAC
PMA + iono

Figure 12. Adoptive transfer of competent NK cells has only a small impact on cytotoxicity in NK-dysfunctional, immunoreplete hosts.

WT splenocytes were adoptively transferred to Jinx hosts, and at 7d post-transfer, differentially labeled WT and $\beta_2m^{-/-}$ splenocytes (target cells) were i.v. injected in the previous adoptive transfer recipients, as well as into WT and Jinx controls. Recipient mice were sacrificed 16 – 24hr post-target injection, and spleens were assessed for remaining target cells, using flow cytometry. The ratio of $\beta_2m^{-/-}$:WT targets remaining is shown.

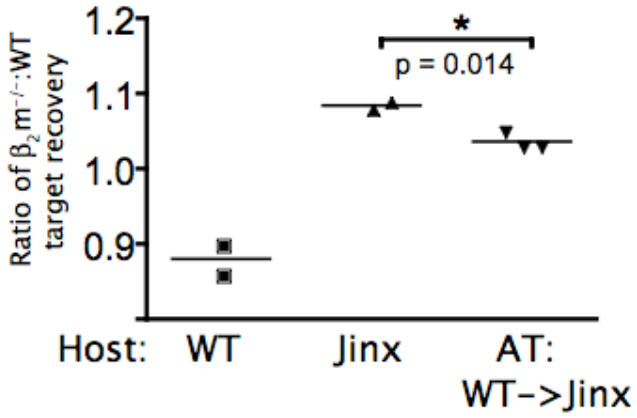

Ratio of $\beta_2m^{-/-}$:WT target recovery
1.2
1.1
1.0
0.9
*
p = 0.014
Host:
WT
Jinx
AT: WT->Jinx

Figure 13. MHC I-deficient NK cells gain function approximately 72 hours following transfer into WT hosts.

Summary of IFNγ production by NK cells recovered 1 – 7 days after adoptive transfer into WT hosts, following anti-NK1.1 stimulation. Data are pooled from 7 independent experiments, with 1 -3 mice/group ($\beta_2m^{-/-} \rightarrow \beta_2m^{-/-}$) and 2-8 mice/group ($\beta_2m^{-/-} \rightarrow$ WT). Means $\pm$ standard error of the mean are shown.

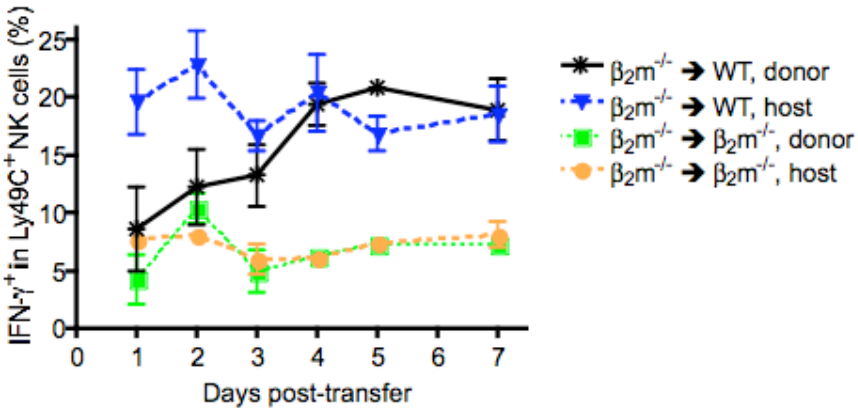
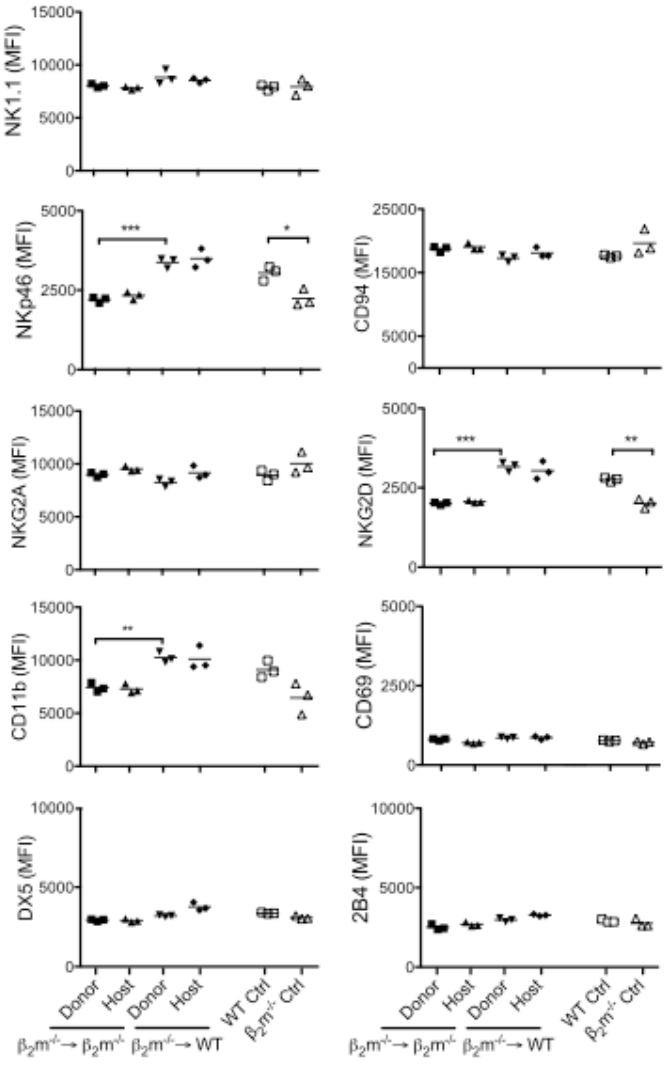

IFN-γ+ in Ly49C+ NK cells (%)
25
20
15
10
5
0
0
1
2
3
4
5
6
7
Days post-transfer
β2m-/- → WT, donor
β2m-/- → WT, host
β2m-/- → β2m-/-, donor
β2m-/- → β2m-/-, host

Figure 14. Minimal changes in surface phenotype of MHC I-deficient NK cells following adoptive transfer to WT hosts.

At day 7 following adoptive transfer of $\beta_2m^{-/-}$ splenocytes to WT hosts, unstimulated NK cells were assessed for NK receptor or marker expression by flow cytometry. Control staining of NK cells from unmanipulated WT or $\beta_2m^{-/-}$ mice is shown. Data are representative from 2 experiments per condition, with each point representing one individual spleen. Average MFI (mean fluorescence intensity) are shown as horizontal bars. $*p < 0.02$, $**p < 0.002$, $***p < 0.0005$.


NK1.1 (MFI)
NKp46 (MFI)
CD94 (MFI)
NKG2A (MFI)
NKG2D (MFI)
CD11b (MFI)
CD69 (MFI)
DX5 (MFI)
2B4 (MFI)
Donor
Host
Donor
Host
WT Ctrl
β2m-/- Ctrl
β2m-/- → β2m-/-
β2m-/- → WT

CHAPTER 4:

Identification and necessary localization of receptors and ligands required for NK cell licensing

Our initial results indicated that hyporesponsive, MHC I-deficient NK cells could become functional following exposure to MHC I molecules in WT hosts, in a mechanism that appeared to be licensing. We wished to confirm and extend these findings by investigating the effect of interaction between different pairs of inhibitory receptors and their cognate MHC I ligands on the licensing status of MHC I-deficient NK cells. Additionally, restriction of the location and identity of host-MHC I expression allows for the determination of tissues and cell types responsible for conferring function, as well as provides insight into the correct placement and physical orientation of receptors and ligands necessary to successfully impart the licensed phenotype.

Introduction

The education of NK cells has been observed for many different pairings of inhibitory receptors and their ligands [41, 71, 72], lending credit to the hypothesis that licensing is a universal mechanism by which NK cells gain functional competence. While specific identities of receptors and ligands comprising a complete functional pair have been documented, important questions remain regarding the physical orientation and localization of functional contact between these molecules. An important question concerns the identity of the cell which must express the educating MHC I ligand. While seemingly straightforward, this problem is complicated by the fact that NK cells themselves express both inhibitory receptors and their ligands, MHC I molecules. Therefore, there is potential that an NK cell may be licensed by contact between these molecules on the surface of the same cell (binding in *cis*), or by contact between the receptor and an MHC I molecule expressed on a different cell (binding in *trans*) [73-77].

In a related idea, if it is demonstrated that NK-intrinsic expression of MHC I is not sufficient to establish normal function, then one must question which cell types are required to express MHC I for the generation of licensed NK cells. These results have particular relevance within the field of human bone marrow transplantation, as tissue restriction of MHC I (HLA) in licensing could have important functional consequences when hematopoietic and non-hematopoietic compartments are of different origin (genetically non-identical).

Before this thesis work, MHC I-deficient NK cells had never been demonstrated to have the capacity to become functional. This precluded an isolated study of the individual contributions of *trans* or *cis* binding to licensing, and prevented precise identification of cell types which must express MHC I molecules for licensing, as the contribution of NK-intrinsic MHC I expression remained a complicating factor. Armed with evidence that MHC I-deficient NK cells can gain functional capacity in a mechanism believed to be licensing, we sought to firmly establish the gain of function as licensing, by extending the observed phenomenon to multiple pairings of inhibitory receptors and MHC I ligands. Subsequently, we utilized the transfer of MHC I-deficient NK cells to hosts with differential localization of MHC I expression, in order to definitively determine the relative contributions of *trans* and *cis* binding to NK cell licensing, as well as the tissue(s) responsible for conformation of the licensing phenotype.

Gain of function holds for multiple inhibitory receptor:MHC I ligand pairs

Our initial results indicated that following adoptive transfer of MHC I-deficient splenocytes into MHC I-expressing hosts, there was in increase in the ability of previously hyporesponsive MHC I-deficient NK cells to make IFNγ after stimulation through their activating receptors. Additionally, it appeared that acquisition of function was due to licensing of the transferred NK cells, as cells expressing an inhibitory receptor capable of interacting with host MHC I displayed a preferential gain of function.

To confirm that the observed gain of function was due to licensing, we expanded our search to alternate pairings of inhibitory receptors and their MHC I ligands, testing the ability of these interactions to induce function in MHC I-deficient NK cells. In the these experiments, we transferred $\beta_2m^{-/-}$ NK cells into KODO D8 hosts ($K^{b-/-}D^{b-/-}D^{d+}$), which lack $H2^b$ MHC class Ia heavy chains and display transgenic expression of $H2D^d$, a ligand for Ly49A [31]. KODO D8 host spleens were recovered 7d post-AT and splenocytes were stimulated via crosslinking of the NK1.1 receptor. On analysis, we noted a gain in production of IFNγ by $Ly49A^+$ MHC I-deficient NK cells (Figure 15), consistent with licensing of $Ly49A^+$ NK cells in $H2D^d$-expressing mice [41]. Thus, we confirmed that following adoptive transfer to an MHC I-expressing environment, there was a selective gain of function for transferred MHC I-deficient NK cells expressing another inhibitory receptor able to bind to a different host MHC I molecule. These results further supported our hypothesis that exposure to host MHC I results in licensing of MHC I-deficient NK cells.

Specific interaction between an inhibitory receptor and its cognate MHC I ligand is required for gain of function

Our results indicated that the subset of NK cells expressing an inhibitory Ly49 receptor capable of interacting with an expressed host MHC I molecule possess a greater capacity for cytokine production than NK cells lacking such a receptor. Interestingly, we also consistently observed that, although not statistically significant, inhibitory receptor-negative NK subsets (that is, Ly49C$^-$ NK cells in WT hosts and Ly49A$^-$ NK cells in KODO D8 hosts) trended towards greater IFNγ production than their counterpart subsets (identified as receptor-negative) from MHC class I-deficient mice (Figure 9a). It then became important to consider that exhaustive staining of all inhibitory receptors was not performed in these assays, and it remained possible that cells negative for the inhibitory receptor of interest expressed another, undetected, inhibitory receptor. Therefore, we hypothesized that, as inhibitory Ly49 receptors exhibit both redundancy as well as promiscuity in MHC I-binding [78-85], perhaps expression of other self-MHC-specific receptors may be responsible for the observed increase in function of 'receptor-negative' NK subsets in MHC I-expressing environments.

To investigate this hypothesis, we wished to restrict the expression of inhibitory Ly49 receptors and MHC I alleles in such a way that only one functional pairing was present. At present, there is no mouse with targeted deletion of the NKC (NK gene complex, the genetic locus of Ly49 alleles and other NK receptors) or components of the NKC, making it impossible to restrict inhibitory receptor expression. Therefore, in order to limit the number of functional pairings, we turned to restriction of the MHC I alleles

expressed. To accomplish this, we utilized the TKO SCT-K^b Tg mouse (triple knock-out, single chain trimer K^b-OVA transgenic), generated and generously provided by the laboratory of Dr. Ted Hansen (Washington University) [54, 86]. Through targeted deletion, these mice lack the genes necessary for endogenous MHC Ia expression (TKO = $K^{b-/-}D^{b-/-}\beta_2m^{-/-}$). Instead, these mice transgenically express, as a single peptide, the MHC I heavy chain $H2K^b$ covalently bound to β_2-microglobulin and the SIINFEKL peptide from ovalbumin, which occupies the peptide-binding cleft. It has been previously demonstrated that, among inhibitory receptors expressed in the C57BL/6 NKC, Ly49C is the sole inhibitory Ly49 receptor capable of binding to the SCT-K^b molecule [41] . Use of these mice as recipients in our adoptive transfer system thus allowed us to effectively restrict the inhibitory receptor:MHC I pairings to a single pair, Ly49C and SCT-K^b.

Following our standard protocol, $\beta_2m^{-/-}$ splenocytes were adoptively transferred to the SCT recipients. On recovery and stimulation at 7d post-AT, we observed that donor $Ly49C^+$ NK cells gained the ability to produce IFNγ at levels equivalent to that of host $Ly49C^+$ cells (Figure 16), as predicted. However, in contrast to the results from transfer to WT hosts, where it was suspected that multiple receptor:ligand pairs license, we now observed no increase in IFNγ production by either donor or host inhibitory receptor-negative (i.e. $Ly49C^-$) NK cells. These results allowed us to conclude that the interaction between an inhibitory Ly49 receptor and its cognate self-MHC I ligand is absolutely required for the gain of function observed in hyporesponsive MHC I-deficient NK cells following their adoptive transfer to MHC I-sufficient hosts, consistent with licensing of these cells.

MHC Ib molecules do not induce gain of function in MHC I-deficient NK cells

Thus far, we have limited our consideration of inhibitory receptor ligands to the classical MHC I alleles (i.e. MHC Ia). However, there is evidence that contact with non-classical, MHC Ib molecules, such as murine Qa-1 and human HLA-E, may endow NK cells with capacity for enhanced responsiveness, through interaction with the inhibitory NKG2A/CD94 receptor [49-51]. It is unclear if the apparent increased functional capacity following recognition of these MHC Ib ligands operates via the mechanism of licensing.

To investigate the role of MHC class Ib β_2m-associated molecules in gain of function of MHC I-deficient NK cells, $\beta_2m^{-/-}$ NK cells were transferred to KODO ($K^{b-/-}D^{b-/-}$) hosts, which lack expression of MHC Ia alleles, but do express β_2m-associated, MHC Ib molecules. Seven days post-AT, spleens were removed and NK cells were stimulated via crosslinking of NK1.1. In contrast to results obtained following transfer to WT hosts, here we found no significant increase in cytokine production by $\beta_2m^{-/-}$ NK cells upon recovery from hosts expressing only MHC Ib molecules (Figure 17a). Therefore, we can conclude that in the absence of MHC I heavy chains, MHC class Ib molecules likely do not contribute to licensing. Interestingly, in my hands, I also found no statistically significant difference in the ability of WT NKG2A+ and NKG2A- NK cells to produce IFNγ in response to anti-NK1.1 stimulation, further arguing against a licensing function for NKG2A, via contact with non-classical MHC I molecules, such as Qa-1 (Figure 17b).

Inverse correlation between gain of function and inhibitory receptor MFI

Although the phenotype of $\beta_2m^{-/-}$ and WT NK cells remained remarkably similar both before and following adoptive transfer (as described in Chapter 3), we did notice a distinct and reproducible decrease of Ly49C mean fluorescence index (MFI) on MHC I-deficient cells following adoptive transfer to WT hosts (Figure 18a). This effect was observed for multiple inhibitory Ly49 receptors in the presence of a known MHC I ligand (Figure 18a-c). The decrease in MFI was determined to be specific, as the MFI of Ly49C was decreased in $\beta_2m^{-/-}$ cells transferred to the TKO SCT-K^b Tg mice (Fig 18b), but the MFI of Ly49A, a receptor for which no MHC I ligand is present in this host, remained unchanged (Fig 18d). Decreased Ly49 MFI appears to reflect a specific interaction of an inhibitory receptor on a donor NK cell with a host MHC I molecule. The decrease in MFI could be due to an actual reduction in the amount of inhibitory receptor present at the surface of the NK cell, possibly via endocytosis of the inhibitory receptor following reception of signal. This has been observed for other NK cell receptors, such as NKG2A, which has been reported to recycle between the cell surface and early endosomes [87, 88].

Cis *'masking' hypothesis*

An alternative, though not mutually exclusive, explanation for the decreased inhibitory receptor MFI observed on MHC I-deficient NK cells following their adoptive transfer to MHC I-sufficient hosts is that of the 'masking' hypothesis. This hypothesis states that interactions between inhibitory receptors and their MHC I ligands can block access to the antibody-binding epitope on the inhibitory receptor, leading to a falsely low MFI when

receptor expression is quantified via flow cytometry [75, 89]. It is important to note that NK cell receptor-ligand interactions can occur in *trans* (on different cells) or in *cis* (on the same NK cell). Thus, while the normal orientation of most receptor:ligand interactions is assumed to be in the *trans* orientation, these 'masking' interactions are believed to occur in the *cis* orientation.

In support of the masking theory, crystal structures of the inhibitory Ly49 proteins reveal great flexibility in their membrane-proximal 'stalk' domains, making it theoretically possible for inhibitory receptors to bind MHC I in the *cis* orientation [90, 91]. It has been noted that in mice expressing an MHC I ligand for an inhibitory Ly49 receptor, the inhibitory receptor MFI is lower than in mice which do not express a ligand [76, 92-96], possibly due to the masking of inhibitory receptor epitopes in *cis*. Experimental evidence further supporting the masking hypothesis comes from studies in which, after a brief exposure to acid treatment, the inhibitory receptor MFI is shown to increase on NK cells from mice expressing the cognate MHC I ligand [75], presumably due to disruption of *cis* interactions.

As discussed, there is experimental evidence for *cis* binding between NK cell receptors and their MHC I ligands. However, the masking explanation is difficult to reconcile with the results of decreased inhibitory receptor MFI observed in our adoptive transfer system, as MHC I-deficient NK cells do not express surface MHC I and should therefore be incapable of establishing *cis* binding interactions between inhibitory receptors and MHC I molecules. Thus, the masking hypothesis alone is insufficient to account for the

decreased inhibitory receptor MFI observed on MHC I-deficient NK cells following their adoptive transfer to MHC I expressing hosts.

'Trogocytosis' of MHC I

While the masking hypothesis alone is insufficient to explain our previous result of inhibitory receptor downregulation, as donor cells do not have endogenous expression of MHC I, the hypothesis could become valid if an MHC I-deficient donor NK cell somehow acquired MHC I on its surface. Although this idea may seem implausible, numerous reports indicate that lymphocytes, including NK cells, can acquire plasma membrane components from other cells, in a process termed trogocytosis [93, 97-100]. We hypothesized then, that if through the mechanism of trogocytosis donor ($\beta_2m^{-/-}$) NK cells acquired surface MHC I 'expression', it might be possible that the decrease in inhibitory receptor MFI on MHC I-deficient NK cells following their transfer to MHC I-sufficient hosts was due, at least in part, to *cis*-masking of the inhibitory receptor epitopes. We predicted that the transfer of MHC I molecules could be accomplished by several, non-mutually exclusive mechanisms, including: 1) surface stabilization of donor ($\beta_2m^{-/-}$) MHC I heavy chains (K^b, D^b) by free β_2m present in host serum, 2) direct transfer of the β_2m molecule from the surface of a host (MHC I+) cell and stabilization of endogenous donor heavy chain, and/or 3) transfer of the entire, intact (host-derived) MHC I molecule to the surface of donor cells.

To explore the potential for transfer of MHC I molecules to the surface of MHC I-deficient NK cells, we examined the surface of $\beta_2m^{-/-}$ splenocytes for the presence of

MHC I molecules following their adoptive transfer to WT hosts. At 7d post-AT, splenocytes were recovered, stained with antibodies to the MHC Ia molecule H2K^b, and analyzed via flow cytometry. It was immediately apparent that following exposure to MHC I-expressing cells, MHC I-deficient splenocytes had acquired a small amount of surface MHC I (Figure 19a). This amount was calculated to be approximately 5% of the amount of MHC I present on the surface of WT cells (MFI = 913 vs. 26424). Interestingly, NK cells, which posses MHC I-specific receptors such as the Ly49s, displayed a greater amount of transferred MHC I than other lymphocytes (such as T cells), supporting the idea that MHC I transfer may occur via a receptor-specific transfer mechanism (Figure 19a).

While these data supported the idea that MHC I could be transferred to the surface of MHC I-deficient cells, the results did not allow us to discriminate which mechanism, transfer of β_2m molecules and/or transfer of intact MHC I, was responsible for the increase in surface expression. To further explore this question, we performed adoptive transfer of $\beta_2m^{-/-}$ splenocytes to TKO SCT-K^{b}OVA recipients, which lack expression of β_2m, K^b and D^b, but do express one MHC I molecule, K^b-OVA, as a covalently linked single peptide. Because these mice do not express free β_2m, they cannot stabilize endogenous MHC Ia heavy chain expression and any transfer of MHC I to $\beta_2m^{-/-}$ splenocytes must occur only through the transfer of intact, MHC I molecule. Upon recovery and analysis at 7d post-AT, we stained for the presence of surface K^b-OVA, using a monoclonal antibody that does not cross-react with either native K^b or D^b [101]. Our results clearly indicate that $\beta_2m^{-/-}$ splenocytes do display a small amount of K^b-OVA

on the cell surface, following their recovery from SCT hosts (Figure 19b), indicating that transfer of the entire, intact MHC I molecule had occurred. While these results do not absolutely preclude a role for the transfer of free β_2m to the surface of $\beta_2m^{-/-}$ cells after transfer to WT hosts, they do demonstrate that transfer of intact MHC I to the surface of MHC I-deficient splenocytes can occur.

Although our findings indicated that MHC I could be transferred to the surface of $\beta_2m^{-/-}$ splenocytes, we questioned whether this transfer may be an irregularity, owing to the high level of inhibitory receptor expression on the surface of MHC I-deficient cells (Figure 18). Therefore, to investigate the potential for transfer of MHC I molecules to cells expressing a full complement of endogenous MHC I, WT splenocytes were adoptively transferred to SCT mice. Following recovery at 7d post-AT, cells were stained with an antibody specific for K^b-OVA, as well an antibody against endogenous K^b, and analyzed by flow cytometry. The results indicated that WT cells had acquired a small but significant amount of surface K^b-OVA (Figure 19c), leading us to conclude that MHC I transfer is not restricted to only those cells lacking endogenous, surface MHC I expression.

Having firmly established the possibility of MHC I transfer, we wished to determine if this and ensuing *cis* interactions between inhibitory receptors and transferred MHC I molecules may account for the decrease in inhibitory receptor MFI observed following adoptive transfer of MHC I-deficient cells to MHC I+ hosts. To investigate, we established co-cultures of $\beta_2m^{-/-}$ splenocytes with WT and SCT splenocytes. In these

experiments, we included staining for simultaneous expression of surface MHC I and MHC I-specific inhibitory receptors. We found that although the highest observable level of MHC I transfer was achieved during co-culture (~5%), $\beta_2m^{-/-}$ inhibitory receptor MFI was only slightly diminished (Figure 20). In other words, the maximum level of MHC I transfer can occur in the absence of full inhibitory receptor downregulation. So, while MHC I transfer and resultant *cis* masking do appear to have a small impact on inhibitory receptor MFI, MHC I transfer alone cannot account for the decreased MFI observed on MHC I-deficient splenocytes, following their exposure to MHC I molecules.

While the concept of 'masking' likely does not explain the decreased inhibitory receptor MFI, it does raise some important questions regarding the interaction of the inhibitory receptors and their MHC I ligands within our adoptive transfer system, leading to an interesting consideration of the functional implications of the physical orientation of these molecules during their contact.

Trogocytosis of MHC I occurs in adoptive transfer, but does not correlate with increased functional capacity

Although we initially anticipated only the existence of *trans* interactions between donor inhibitory receptors and host MHC I within our adoptive transfer system, *cis* interactions had been demonstrated feasible, through the mechanism of MHC I transfer. Thus, while the results primarily indicated that MHC I-deficient cells became licensed by interactions with host MHC I in *trans*, the importance of interactions in *cis* could not be discounted. By concurrently examining the gain in function of MHC I-deficient NK cells and their

acquisition of host MHC I, we could determine if there was a correlation between surface MHC I expression and function, thereby directly assessing the significance of *cis* interactions in the process of NK cell licensing.

In order to address the importance of *cis* interactions in licensing, we performed an adoptive transfer of $\beta_2m^{-/-}$ splenocytes to SCT hosts, as previously described. Splenocytes were harvested, stimulated, and prepared for flow cytometry analysis by staining for intracellular IFNγ, as well as surface MHC I and other markers. The results from these analyses demonstrated that there was no correlation between expression of surface MHC I (i.e. MHC I transfer), and functional capacity, in the form of cytokine production (Figure 21). MHC I-deficient donor NK cells that had acquired host MHC I molecules were no more or less likely to produce IFNγ than donor cells that had not acquired surface MHC I, or host NK cells with normal MHC I expression. Thus, adoptive transfer studies indicate that *cis* interactions do not appear to establish function in MHC I-deficient NK cells, and that *trans* interactions are likely sufficient to fully license NK cells, as exposure of MHC I-deficient cells to an MHC I-sufficient environment imparts normal function.

MHC I expression is required on both hematopoietic and non-hematopoietic cells for gain of function following adoptive transfer

Results from adoptive transfer studies using MHC I-deficient NK cells indicated cell-intrinsic MHC I expression was not required for licensing. While it was determined that some component of the environment must display MHC I for licensing to occur, the

adoptive transfer studies did not reveal the identity of the cell type in which MHC I expression was required. We hypothesized that for proper establishment of NK cell licensing, MHC I expression may be required on a hematopoietic cell, a non-hematopoietic cell, or both.

In order to test these hypotheses, we revisited the historical system of bone marrow chimeras. Although data from bone marrow chimeras had previously been unable to provide clear answers regarding the contribution of MHC I expression to NK cell function, we believed that armed with methods of single-cell analysis, chimeras could be an informative experimental system. Using this model, we proceeded to investigate which compartment(s) must express MHC I for NK cells to become licensed.

In order to determine if MHC I expression is necessary on hematopoietic, non-hematopoietic or both types of tissue to confer NK licensing, bone marrow chimeras with MHC I-expression in either hematopoietic or stromal compartments ($\beta_2m^{-/-} \rightarrow$ WT and WT $\rightarrow \beta_2m^{-/-}$) were generated. Analyses of the chimeras indicate that expression of MHC class I on either compartment alone is insufficient to generate functional NK cells, necessitating MHC I expression on both hematopoietic as well as non-hematopoietic cells for proper NK cell function (Figure 22). Additionally, results from mixed chimeras, in which WT BM is titrated at increasing ratios with $\beta_2m^{-/-}$ BM and engrafted in WT hosts, indicate that NK cells appear sensitive to the total amount of MHC I in the environment. Both WT as well as $\beta_2m^{-/-}$ NK cells display greater function as the amount of MHC I is increased. Importantly, these results refute the theory of 'dominant tolerance' [56], which

predicts only a decrease in WT function, but does not predict or account for the concurrent gain of function observed in MHC I-deficient cells upon mixing with WT cells, as is experimentally observed.

Summary

The results described in this chapter demonstrate that the gain of function following adoptive transfer appears to be a general mechanism, like licensing, as it holds for a variety of inhibitory receptor:MHC Ia ligand pairs. Also, following transfer of MHC I-deficient NK cells to MHC I+ hosts, we showed that specific contact between an inhibitory receptor and cognate MHC I ligand is absolutely required for gain of function, consistent with licensing of these cells. In a setting where the pairings of inhibitory receptor and MHC I were restricted to a single pair (Ly49C and SCT-K^b), only those NK cells with expression of Ly49C showed normal functional capacity following stimulation.

Interestingly, we did not find the general mechanism of licensing to be applicable to the interaction of inhibitory receptors with non-classical, MHC Ib ligands. However, there is one notable caveat within these particular experiments: Qa-1, the MHC Ib molecule predicted to act as the MHC Ib licensing ligand, is fairly dependent upon the presence of K^b/D^b leader peptide fragments for its expression [20, 102]. It is possible then that KODO mice may have abnormally low expression of Qa-1, as they are lacking the genes for K^b and D^b (and therefore expression of the leader peptides). In our experiments, normal Qa-1 surface expression was not verified by flow cytometry. Therefore, a more stringent and accurate test of the role of Qa-1 in NK cell licensing could be obtained using, as hosts,

KODO mice with Tg expression of K^b and/or D^b leader peptides, or $\beta_2m^{-/-}$ hosts with Tg expression of a Qa1-β_2m-peptide single chain trimer molecule. Regardless, data comparing the function of NK cells from a WT environment (i.e. Qa-1+) indicated that NK cells with expression of NKG2A, the inhibitory receptor for Qa-1, did not appear to possess greater functional capacity than NK cells lacking this receptor, arguing against a role for this interaction in licensing.

Results from adoptive transfer and co-culture provide new insight into the requirement for specific physical orientation of the contact between NK cell inhibitory receptors and MHC I molecules to induce licensing. Following exposure to MHC I+ environments, transfer of MHC I molecules to the surface of MHC I-deficient donor cells is detectable, though this alone does not account for the observed downregulation in inhibitory receptor expression. While MHC I transfer could allow for *cis* interactions between inhibitory receptors and MHC I on the surface of donor NK cells, it appears that interactions in *trans* are sufficient to license, as we observed no correlation between 'surface' MHC I expression on donor ($\beta_2m^{-/-}$) NK cells and their ability to produce IFNγ after recovery from MHC I+ hosts.

As it cannot be fully explained by MHC I transfer and subsequent interactions in *cis*, the mechanism of inhibitory receptor downregulation following adoptive transfer of MHC I-deficient cells to MHC I+ hosts remains an interesting subject for investigation. In accordance with the behavior of many other signaling receptors [103-105], it is likely that the decrease in inhibitory receptor MFI reflects a real decrease in the amount of protein

present at the cell surface, due to receptor internalization following ligand engagement. Using techniques such as immunoprecipitation, western blot, and surface labeling (biotinylation), it may be possible to quantify the actual amount of inhibitory receptor present at the cell surface. Additionally, confocal microscopy of NK cells utilizing co-staining of markers of intracellular trafficking, such as the Rab proteins, may be useful to determine in which vesicles inhibitory Ly49 receptors are located. Results could provide information regarding the trafficking and/or recycling patterns of these receptors, possibly revealing aspects of the regulation of their surface expression. The fluctuation in NK cell inhibitory receptor expression is conceptually interesting, particularly when one considers that these receptors, unlike many other signaling receptors, may have constant access to their ligand. In this case, maintenance of a functional receptor repertoire at the NK cell surface is far more than trivial. Investigations into the mechanisms responsible for the downregulation of NK inhibitory receptor expression following exposure to MHC I would certainly be of value.

Finally, our results indicate that neither stromal nor hematopoietic expression alone of MHC I is sufficient for generation of licensed NK cells. Bone marrow chimeras reveal a 'bidirectional' effect of mixing WT and MHC I-deficient NK cells, resulting in an intermediate phenotype. These results refute the theory of 'dominant tolerance', which would predict only a decrease in WT NK function but not an increase in function of $\beta_2m^{-/-}$ NK cells, which is observed. Also, we observed that even a small decrease in the amount of MHC I present in the host appeared to have a large negative impact on the function of the entire NK cell population.

These results may allow for the generation of hypotheses regarding the temporal aspects of MHC I contact required for licensing. For example, if licensing is a permanent event established by a single contact (or contact event) with an MHC I molecule, a small decrease in MHC I expression may have very little effect on the licensed status of the entire population. One could predict that the time required to license an entire population might increase if access to 'resources' (i.e. MHC I) is decreased, but given long enough, the population will complete the transition. An analogous situation would be that of a chemical reaction, with the formula A + B → C. Here, 'A' represents an NK cell with an inhibitory receptor specific for MHC I, 'B' represents MHC I and 'C' represents a licensed NK cell. If one contact with MHC I is sufficient to license, this becomes an irreversible reaction and we will never see C → A (+B), or cells becoming unlicensed. Over time (provided there are sufficient molecules of 'B' or 'B' is not consumed), all 'A' will be consumed. In other words, if there is MHC I in the environment, all NK cells will become licensed.

However, if continuous or repeated contact with MHC I is required to maintain the licensed phenotype, even a small decrease in available MHC I would negatively affect the entire population. This is best conceptualized as a function of mathematical probability over time. At each instant, there is some probability that an NK cell will encounter MHC I and become licensed. If environmental MHC I is decreased, the probability for that particular time point is also decreased. If we examine the population of NK cells over a longer period of time and we assume they must make repeated contacts with MHC I to become/stay licensed, the probability they will be licensed at the

end of this time is the product of all probabilities over time. Therefore, even a small decrease in the probability at one instant has a massive impact on the final outcome, as it is multiplied over time.

In the next chapter, the requirement for continuous contact with MHC I molecules is explored when we examine the components necessary to maintain the licensed phenotype, as well as the potential for its reversal.

Figure 15. Gain of function holds for another inhibitory receptor:self-MHC I pair. Summary of IFNγ production by Ly49A^{+} (left panel) and Ly49A^{-} (right) donor ($\beta_2m^{-/-}$) and host NK cells recovered 7d post-adoptive transfer from KODO D8 (KD8) or $\beta_2m^{-/-}$ hosts, following *ex vivo* anti-NK1.1 stimulation. Results from anti-NK1.1 stimulation of unmanipulated KODO D8 NK cells are shown. Data were pooled from 4 independent experiments, with 3-8 mice/group. Means are shown as horizontal lines with error bars representing standard error of the mean.

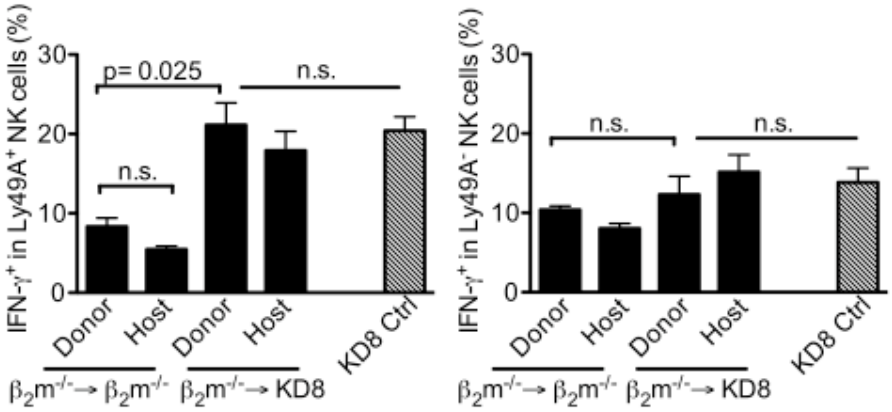

IFN-γ+ in Ly49A+ NK cells (%)
30
20
10
0
p= 0.025
n.s.
n.s.
Donor
Host
Donor
Host
KD8 Ctrl
β2m-/-→ β2m-/-
β2m-/-→ KD8
IFN-γ+ in Ly49A- NK cells (%)
30
20
10
0
n.s.
n.s.
Donor
Host
Donor
Host
KD8 Ctrl
β2m-/-→ β2m-/-
β2m-/-→ KD8

Figure 16. Inhibitory receptor and cognate MHC I ligand are required for gain of function.

Summary of IFNγ production by Ly49C^{+} (left panel) or Ly49C^{-} (right) donor ($\beta_2m^{-/-}$) and host NK cells recovered 7d post-adoptive transfer from TKO SCT-K^{b} Tg (SCT) or $\beta_2m^{-/-}$ hosts, following *ex vivo* stimulation via anti-NK1.1. Results from anti-NK1.1 stimulation of unmanipulated SCT NK cells are shown. Data were pooled from 3 independent experiments with 3-7 mice/group. Means are shown as horizontal lines with error bars representing standard error of the mean.

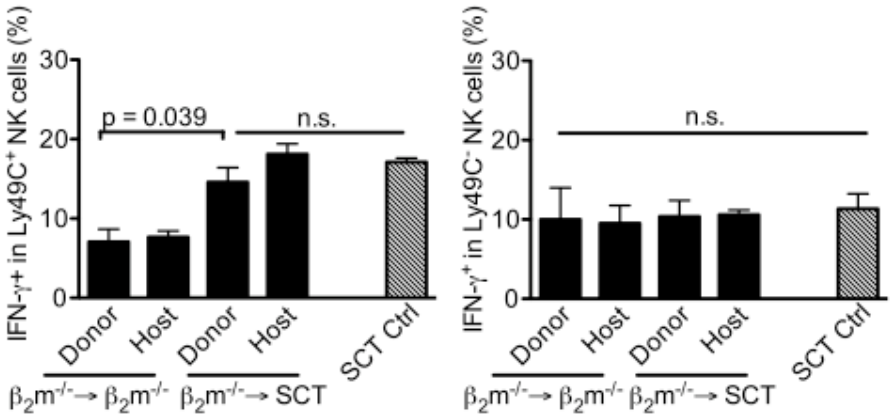
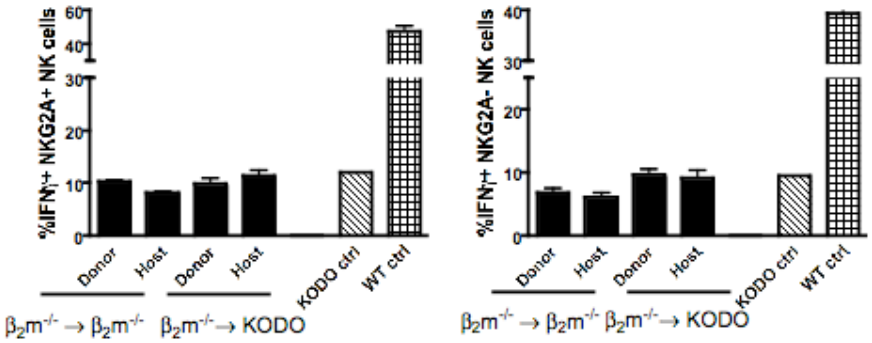

IFN-γ+ in Ly49C+ NK cells (%)
30
20
10
0
p = 0.039
n.s.
Donor
Host
Donor
Host
SCT Ctrl
β2m-/-→ β2m-/-
β2m-/-→ SCT
IFN-γ+ in Ly49C- NK cells (%)
30
20
10
0
n.s.
Donor
Host
Donor
Host
SCT Ctrl
β2m-/-→ β2m-/-
β2m-/-→ SCT

Figure 17. MHC Ib ligands do not appear to license NK cells.

(a) Summary of IFNγ production by NKG2A+ (left panel) or NKG2A- (right) donor ($\beta_2m^{-/-}$) or host NK cells recovered 7d post-adoptive transfer from KODO hosts, following *ex vivo* stimulation via anti-NK1.1. Results from anti-NK1.1 stimulation of unmanipulated KODO and WT NK cells are shown. Data were pooled from 2 independent experiments with 2-4 mice/group. Means are shown as horizontal lines with error bars representing standard error of the mean. **(b)** Summary of IFNγ production by NKG2A+ (left panel) or NKG2A- WT NK cells, following *ex vivo* stimulation via anti-NK1.1. Data are pooled from 2 experiments, with 3 mice/group.

(a)


%IFNγ+ NKG2A+ NK cells
60
40
30
20
10
0
Donor
Host
Donor
Host
KODO ctrl
WT ctrl
β2m-/- → β2m-/-
β2m-/- → KODO
%IFNγ+ NKG2A- NK cells
40
30
20
10
0
Donor
Host
Donor
Host
KODO ctrl
WT ctrl
β2m-/- → β2m-/-
β2m-/- → KODO

(b)

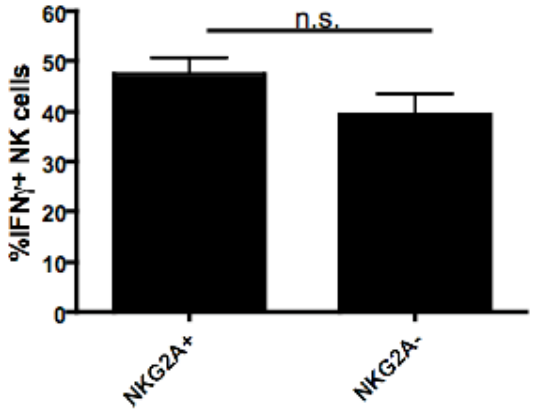

n.s.
%IFNγ+ NK cells
60
50
40
30
20
10
0
NKG2A+
NKG2A-

Figure 18. Specific reduction in inhibitory Ly49 receptor expression on MHC I-deficient donor NK cells following adoptive transfer into MHC I-expressing hosts. Splenocytes from $\beta_2m^{-/-}$ donors were adoptively transferred into indicated hosts. At day 7, unstimulated recipient splenocytes were assessed for **(a, b)** Ly49C or **(c, d)** Ly49A surface expression. Control staining from unmanipulated mice are shown, as indicated. Data are from 2-3 experiments per condition, with each point representing one individual spleen. Average MFI (mean fluorescence intensity) are shown as horizontal bars. $^{**}p < 0.004$, $^{***}p < 0.0001$.

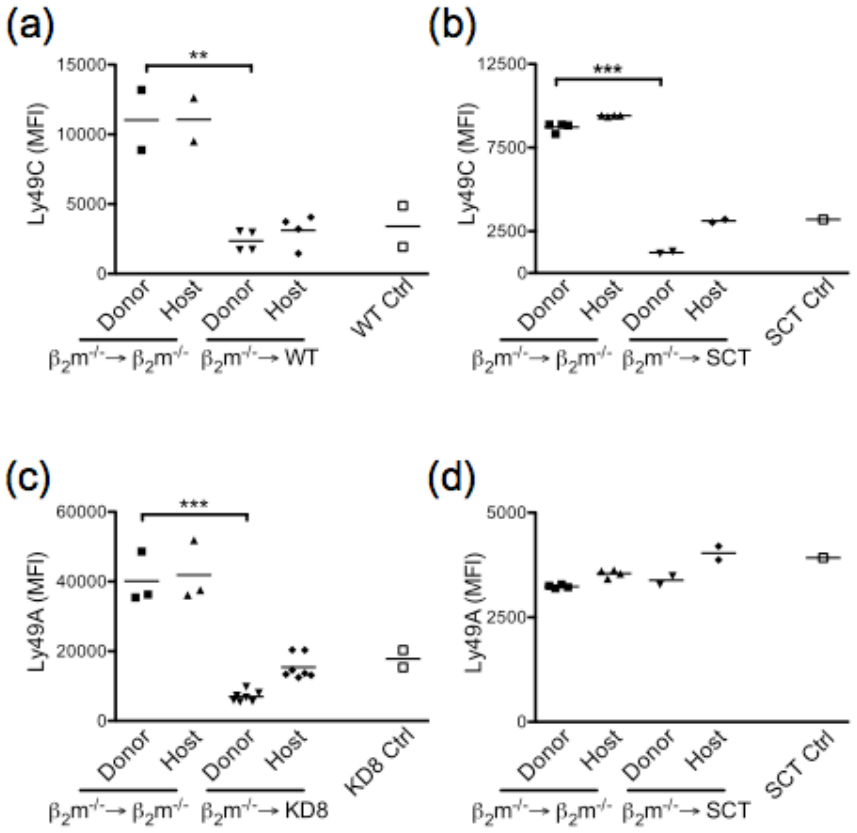

(a)
Ly49C (MFI)
15000
10000
5000
0
**
Donor
Host
Donor
Host
WT Ctrl
β2m-/-→ β2m-/-
β2m-/-→ WT
(b)
Ly49C (MFI)
12500
7500
2500
0
***
Donor
Host
Donor
Host
SCT Ctrl
β2m-/-→ β2m-/-
β2m-/-→ SCT
(c)
Ly49A (MFI)
60000
40000
20000
0
***
Donor
Host
Donor
Host
KD8 Ctrl
β2m-/-→ β2m-/-
β2m-/-→ KD8
(d)
Ly49A (MFI)
5000
2500
0
Donor
Host
Donor
Host
SCT Ctrl
β2m-/-→ β2m-/-
β2m-/-→ SCT

Figure 19. NK cells acquire surface MHC I from surrounding cells.

$\beta_2m^{-/-}$ or WT splenocytes were CFSE-labeled and adoptively transferred to indicated hosts. Surface MHC I expression was assessed via flow cytometry 7d post-transfer. **(a)** Surface expression of $H2K^b$ on $\beta_2m^{-/-}$ donor and WT host splenocytes. **(b)** Surface expression of K^b-OVA on $\beta_2m^{-/-}$ donor and TKO SCT K^b-OVA host splenocytes. **(c)** Surface expression of K^b-OVA on WT donor and TKO SCT K^b-OVA host splenocytes.

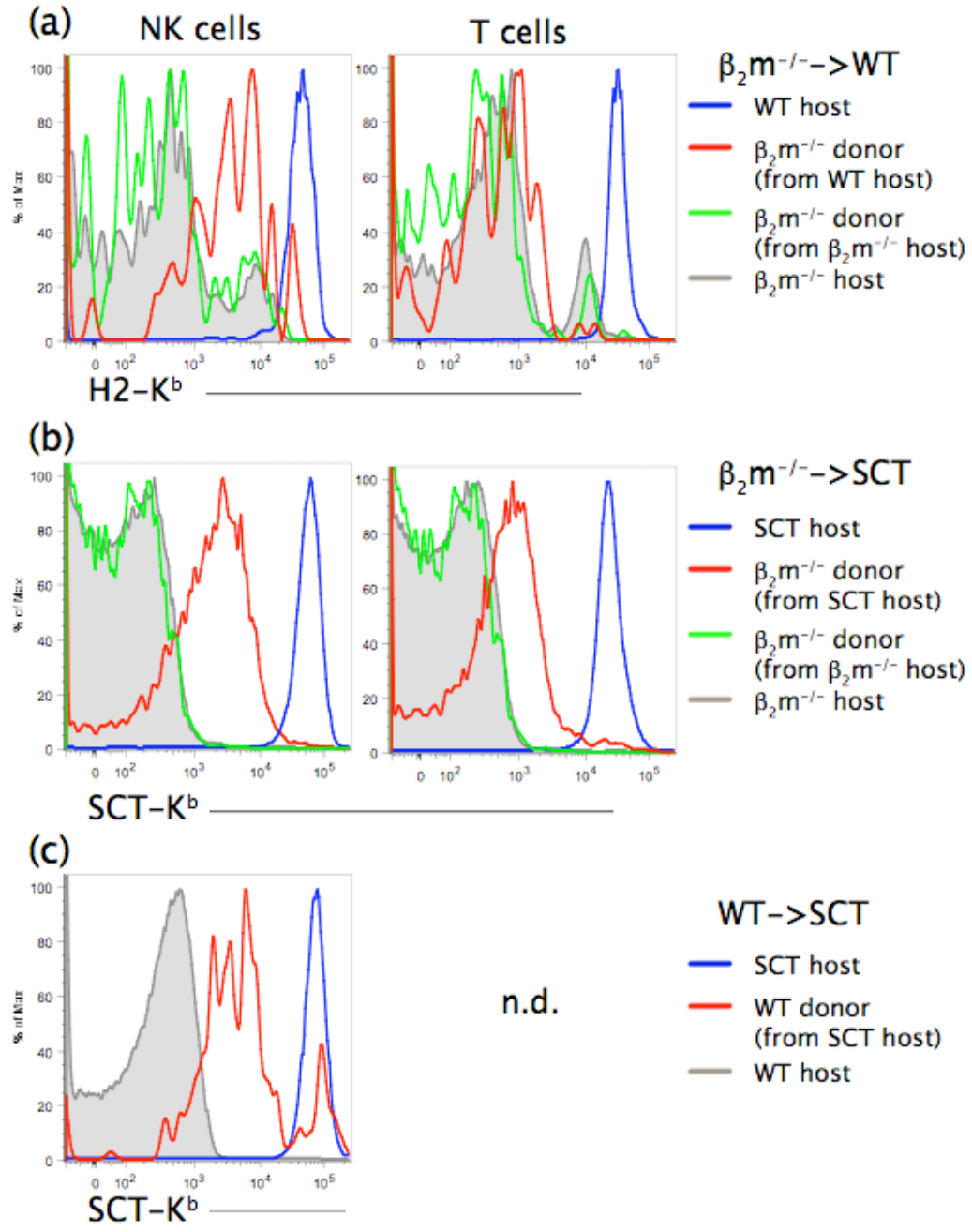
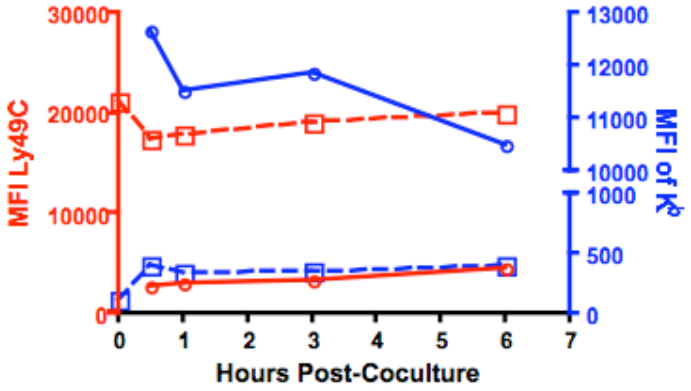

(a)
NK cells
T cells
β2m−/−->WT
WT host
β2m−/− donor (from WT host)
β2m−/− donor (from β2m−/− host)
β2m−/− host
% of Max
H2-Kb
(b)
β2m−/−->SCT
SCT host
β2m−/− donor (from SCT host)
β2m−/− donor (from β2m−/− host)
β2m−/− host
SCT-Kb
(c)
WT->SCT
SCT host
WT donor (from SCT host)
WT host
n.d.
SCT-Kb

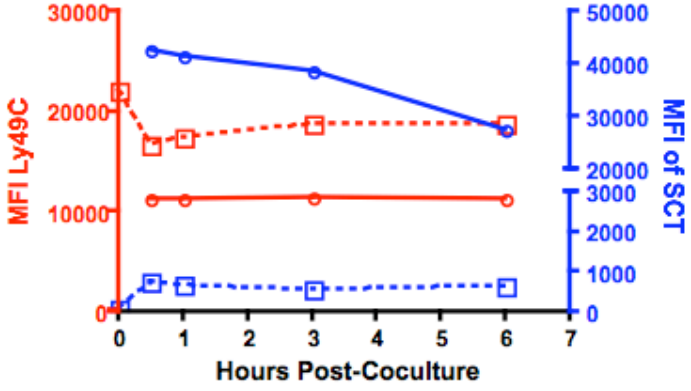
Figure 20. Acquisition of surface MHC I does not correlate with inhibitory Ly49 receptor downregulation on MHC I-deficient NK cells.

Freshly isolated **(a)** $\beta_2m^{-/-}$ and WT Ly5.1 splenocytes or **(b)** $\beta_2m^{-/-}$ and TKO SCT K^b-OVA splenocytes were co-incubated in normal medium for the times indicated. After incubation, cells were stained for surface expression of MHC I and Ly49C, and analyzed via flow cytometry.

(a)



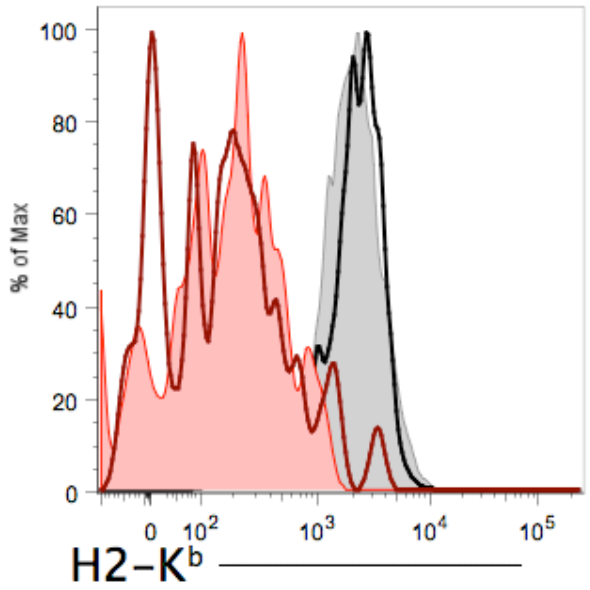
(b)



Dashed line = $\beta_2 m^{-/-}$
Solid line = MHC I+

Figure 21. Acquisition of surface MHC I does not correlate with gain of function in MHC I-deficient NK cells.

Splenocytes from $\beta_2m^{-/-}$ donors were adoptively transferred into WT hosts. Upon harvest, host and donor NK cell populations (gated as CD3-NKp46+CFSE+/-) were stained for surface $H2K^b$ expression and intracellular IFNγ, following anti-NK1.1 stimulation. Histograms of surface K^b expression for host and donor NK cell populations determined to be positive or negative for intracellular IFNγ are shown.

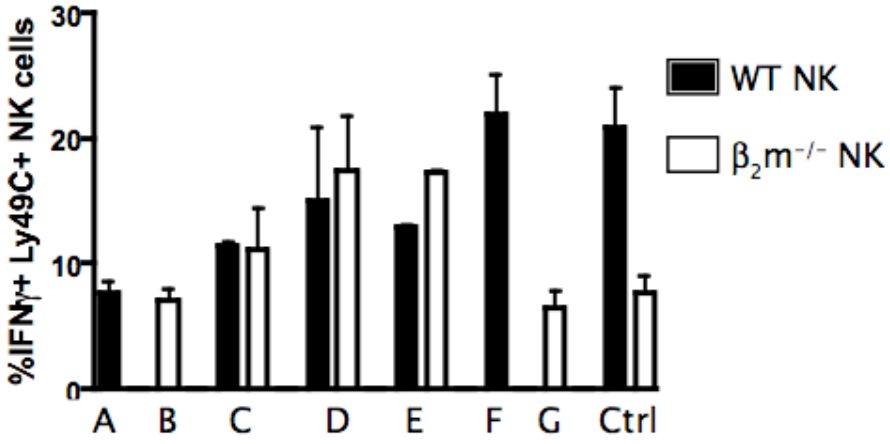


Red = $\beta_2 m^{-/-}$ donor NK (from WT host)
Black = WT host NK

Open = IFNγ positive NK
Filled = IFNγ negative NK

Figure 22. MHC I expression is required on both hematopoietic and non-hematopoietic cells for NK cell function.

Eight to ten weeks after their generation, NK cell function was assessed in bone marrow chimeras with varying hematopoietic and non-hematopoietic MHC I expression (as indicated). Splenocytes were stimulated via anti-NK1.1 treatment and stained for intracellular expression of IFNγ. WT and $\beta_2m^{-/-}$ cells were individually identified by allelic variation at the CD45 locus (Ly5.1 vs. Ly5.2).



Chimera A: WT -> $\beta_2m^{-/-}$

Chimera B: $\beta_2m^{-/-}$ -> WT

Chimera C: WT+ $\beta_2m^{-/-}$ (1:1) -> $\beta_2m^{-/-}$

Chimera D: WT+ $\beta_2m^{-/-}$ (1:1) -> WT

Chimera E: WT+ $\beta_2m^{-/-}$ (10:1) -> WT

Chimera F: WT -> WT

Chimera G: $\beta_2m^{-/-}$ -> $\beta_2m^{-/-}$

CHAPTER 5:

Factors contributing to maintenance of the licensed phenotype and potential for its reversal

Initial experiments of this thesis project focused on determining the potential for normal function by MHC I-deficient NK cells, as well as identity and localization of the environmental components necessary for induction of normal function, following transfer to MHC I-sufficient hosts. With these parameters established, we turned our investigation to factors that contribute to the preservation of the licensed phenotype in WT NK cells, as well as the potential for the reversal of the phenotype. The results described in this chapter indicate that NK cell licensing requires continued contact with MHC I molecules for its maintenance, and is reversible following loss of contact with MHC I, resulting in the induction of a hyporesponsive state.

Introduction

Upon initiation of this project, little was known regarding the plasticity of NK cell licensing. Questions as to whether licensing is a permanent phenotype, akin to lineage differentiation, or if it requires constant input to be maintained were not addressed at the time the licensing hypothesis was originally proposed [41]. However, while the permanency of the licensed phenotype had not been experimentally determined, extrapolation from published data led to predictions that NK cell licensing, once established, is permanent and fixed, with little to no potential for reversal. For example, many reports demonstrate that exposure of NK cells to MHC I-deficient targets results in NK cell activation and target cell cytolysis, not NK anergy [27, 28, 33, 35, 63, 106]. In a clinically relevant analogy, following bone marrow transplantation in the treatment of leukemic patients, it is reported that donor-derived NK cells can exert a beneficial anti-tumor effect against residual host cells lacking normal surface expression of HLA alleles

[107-111]. Were the licensed phenotype easily reprogrammed or reversible, one might predict that contact with MHC I- or HLA-deficient cells would decrease NK cell responsiveness, not incite effector function as is observed. Therefore, these results support the idea that licensing is fixed.

While experimental evidence generally did not substantiate the idea that licensing is reversible, our own results describing the gain of licensing by mature, MHC I-deficient NK cells following adoptive transfer to MHC I+ hosts led us to suspect that licensing might be more flexible than previously assumed. Therefore, we began a series of experiments to determine if licensing is reversible and which factors, if any, are necessary for the maintenance of the licensed phenotype.

Loss of all contact with MHC I results in complete loss of NK cell function

In order to examine the potential for loss of the licensed phenotype and effector functions conferred by licensing, splenocytes were purified from WT donors and adoptively transferred into $\beta_2m^{-/-}$ recipients, or WT recipients as a control. At 7d post-transfer, host spleens were harvested and assessed for IFNγ production, via intracellular flow cytometry. When spontaneous cytokine production was examined, neither host nor donor NK cells were positive for IFNγ (Figure 23a). These results were initially surprising as, based on previously published data citing NK activation following exposure to MHC I-deficient targets, we anticipated that WT NK cells might become activated in the $\beta_2m^{-/-}$ hosts and demonstrate signs of activation upon harvest, such as spontaneous cytokine

production. However, our negative results clearly indicated that WT donor NK cells were not in a state of activation when recovered from MHC I-deficient hosts.

Next, we conducted measurements of NK cell responsiveness to activation receptor cross-linking. We observed that WT donor NK cells recovered from $\beta_2m^{-/-}$ hosts no longer responded normally to stimulation, making significantly less IFNγ than WT control NK cells (Figure 23b). In contrast, WT donor NK cells recovered from the control (WT) recipients produced a normal amount of IFNγ following stimulation, indicating that the decrease in cytokine production observed by WT cells recovered from $\beta_2m^{-/-}$ hosts was not merely a consequence of adoptive transfer. These results demonstrated that WT NK cells recovered from MHC I-deficient hosts had specifically adopted a hyporesponsive phenotype.

It remained possible that WT NK cells had become activated at time points early after transfer, and demonstrated low cytokine production in our assays as a result of 'exhaustion', a phenomenon whereby chronic stimulation induces an anergic phenotype, best understood in the context of an antiviral T cell response [112, 113]. However, following stimulation with PMA (phorbol mistryl acetate) and ionomycin, agents which act on distal elements of intracellular signaling pathways [114], both WT donor as well as $\beta_2m^{-/-}$ host NK cells retained the ability to produce large amounts of IFNγ (Figure 23c). Therefore, neither the lack of spontaneous IFNγ production by WT donor NK cells, nor their poor responses to receptor cross-linking could be explained by 'exhaustion'.

Taken together, the results of these analyses indicate that following transfer to an environment devoid of MHC I expression WT NK cells appear to lose the potential for responsiveness and adopt a phenotype which corresponds to that of 'unlicensed' cells.

Partial loss of contact with MHC I results in reduced, but not abrogated, function

Although new data suggested that total loss of contact with MHC I could 'unlicense' WT NK cells, we considered an alternative explanation for the hyporesponsive phenotype displayed by WT NK cells recovered from $\beta_2m^{-/-}$ hosts. We hypothesized that some minimal contact with MHC I might be necessary to maintain NK cell effector function in licensed cells, and that total loss of contact may completely disable a cell, resulting in a similar hyporesponsive phenotype as observed in unlicensed cells, but governed by a different mechanism. It could be possible that partial loss of NK cell inhibitory receptor contact with MHC I, for example following transfer to an environment lacking some but not all MHC I expression would not initiate the disabling program and would result in a different experimental outcome, such as activation of WT donor NK cells.

Therefore, adoptive transfer experiments were devised to test the hypothesis that exposure to environments with expression of fewer MHC I alleles, as opposed to environments totally lacking MHC I expression, would result in activation of WT NK cells. Splenocytes from WT mice were purified and transferred to mice lacking expression of one MHC I allele, either $H2K^b$ or $H2D^b$ (i.e. $K^{b-/-}D^{b+}$ or $K^{b+}D^{b-/-}$). At 7d post-transfer, host spleens were recovered and cytokine production assessed. Identical to results from transfer to MHC I-null environments, we found no spontaneous production

of IFNγ by either host or donor NK cells upon recovery from single-MHC I hosts. Following activation receptor cross-linking, WT NK cells recovered from single-MHC I hosts demonstrated low cytokine production, similar to that of their single-MHC I counterparts (Figure 24a). The functionality of single-MHC I NK cells is close to, but slightly better than, that of NK cells from mice completely lacking MHC I. It is interesting to note that the loss of the $H2K^b$ allele appears to have a greater negative impact on functionality than does loss of the $H2D^b$ allele (Figure 24a). We hypothesize that this is due to greater licensing of C57BL/6 NK cells by $H2K^b$, which has been reported to be a better ligand for inhibitory receptors expressed in the C57BL/6 NKC [82]. Interestingly, following activation via PMA/ionomycin, it appears that there may be a slight defect in IFNγ production by donor WT NK cells recovered from single-MHC I hosts (Figure 24b). However, this result is not statistically significant.

These results demonstrate that exposure of WT, licensed NK cells to environments expressing fewer MHC I alleles results in induction of a hyporesponsive phenotype, and is consistent with the idea that loss of contact with MHC I results in reversal of NK cell licensing. Additionally, these results, together with results from the transfer of WT NK cells to MHC I-deficient hosts, allow us to conclude that the loss of functionality appears gradated, with loss of contact with additional MHC I alleles resulting in greater loss of function. Assuming that loss of function operates analogously to gain of function, these data provide evidence that licensing does indeed appear to operate in the fashion of a rheostat, with a greater number of MHC I contacts resulting in an increase in the potential

for NK cell effector function, and fewer contacts resulting in decreased potential for function.

Inhibitory receptor expression is only modestly increased on WT NK cells following loss of contact with MHC I

Previously, we had demonstrated that the gain of function by $\beta_2m^{-/-}$ NK cells following their transfer to MHC I+ hosts was accompanied by a specific decrease in inhibitory receptor expression for those receptors with an MHC I ligand expressed in the host (Figure 18). We therefore sought to determine if the inverse were also true: increase in inhibitory receptor expression (coincident with loss of function) following adoptive transfer of WT NK cells to MHC I-deficient hosts.

To investigate the potential for increase in inhibitory receptor expression during loss of contact with MHC I (and loss of NK cell function), we performed adoptive transfer of WT NK cells to $\beta_2m^{-/-}$ hosts and examined NK cell phenotype using flow cytometry. Upon harvest of host spleens at 7d post-AT, splenocytes were stained for surface markers, including inhibitory Ly49 receptor expression. Surprisingly, we found that the Ly49 inhibitory receptor expression on WT NK cells had increased only slightly following their transfer to MHC I-deficient hosts (Figure 25). The increased expression was still much lower than the expression of inhibitory receptors on the surface of MHC I-deficient cells. Therefore, we may conclude that loss of function in WT NK cells following their adoptive transfer to MHC I-deficient environments can occur independently of a full increase in inhibitory receptor expression, suggesting that

inhibitory receptor surface expression may not be the (sole) mechanism governing NK cell function or licensing status.

Change in MHC I-specificity or 'relicensing' of NK cells

Having acquired data that supported the idea it is possible to reverse licensing in WT NK cells, we next questioned the flexibility of the MHC I-specificity in mature, licensed NK cells. New experiments were designed to explore the possibility that WT NK cells could become reprogrammed, or 're-licensed', on MHC I alleles different from those on which they were originally licensed.

Experiments to test the ability of WT NK cells to become re-licensed were initiated utilizing a system of adoptive transfer and MHC I-mismatch. Two genotypes of mice were used: C57BL/6 and B10.D2, which are congenic with C57Bl/6 mice, varying at the MHC I locus. Instead of expressing the $H2^b$ alleles $H2K^b$ and $H2D^b$ as in C57BL6, B10.D2 mice express $H2^d$ alleles, $H2K^d$, $H2D^d$ and $H2L^d$ at the MHC I locus.

Although in our previous experiments we had not directly addressed the presence of T cells in either host or donor splenocyte preparations, this series of experiments required greater consideration of such issues. It is known that in the absence of irradiation transfer of MHC I-disparate cell populations containing mature T cells can initiate an allospecific T cell response, leading to T cell activation and graft rejection, as well as widespread inflammation of the host [115, 116]. Therefore, in these experiments, donor NK cells were always sort-purified prior to transfer.

Sorted B10.D2 NK cells were transferred to Rag1$^{-/-}$ hosts (who are T cell deficient). (The inverse transfer could not be completed, as we lacked T cell-deficient B10.D2 hosts.) The results of these experiments showed that, even in the absence of alloreactive T cells, MHC I-disparate NK cells were eliminated (Figure 26). We hypothesize that this rejection may be due to the engagement of activating NK cell receptors with specificity for MHC I alleles, such as Ly49D [117, 118].

Therefore, although conceptually interesting, this experimental system failed to provide interpretable results and we are currently unable to definitively conclude whether NK cells can become 'relicensed' on different MHC I alleles.

MHC I expression is required on both hematopoietic and non-hematopoietic cells for maintenance of function

Our results indicated that contact with MHC I is required in mature licensed NK cells in order to maintain their licensed state (Figure 22). Data utilizing bone marrow chimeras with restriction of MHC I expression to hematopoietic or non-hematopoietic compartments (described in Chapter 4) had shown that full MHC I expression in both compartments is necessary for the initial acquisition of the licensed phenotype. Using a similar system of bone marrow chimeras coupled with the technique of adoptive transfer, we determined that it would be possible to examine the importance of MHC I expression in these compartments for maintenance of the licensed phenotype.

Although one may initially predict that full MHC I expression is required in both compartments for maintenance of licensing as it is for acquisition of licensing, it is possible that the requirements for contact with MHC I are different in mature, licensed NK cells. There are other examples of differential requirement for contact with essential ligands depending on the maturation or activation status of the cell, particularly in the T and B cell lineages. For example, B cells become more dependent on signaling through the BLyS family receptors for survival as they mature [119]. In a converse example, memory T cells appear to become less dependent on contacts between TCR and MHC for survival and homeostatic proliferative capacity, opposed to their naïve counterparts [120-122]. Additionally, it is possible that NK cells are licensed by one cell type, but are dependent on interactions with a different cell type for their maintenance in a licensed state.

Therefore, to test the requirement for continued MHC I expression in hematopoietic and non-hematopoietic compartments in the maintenance of NK cell licensing, we performed adoptive transfer of WT NK cells to bone marrow chimeras, in which the hematopoietic or non-hematopoietic system lacked MHC I expression. We found that MHC I expression is required in both compartments to retain the licensed phenotype, as WT donor NK cells recovered from either chimera appeared hyporesponsive (Figure 27). Thus, the results indicated that MHC I is required in both compartments for maintenance of NK cell licensing and functional capacity, as it is required in both for initial acquisition of the licensed phenotype.

Conclusions

Here we have investigated the potential for reversal of the licensed phenotype, as well as factors required for its maintenance. Experiments were conducted in which WT NK cells were transferred to hosts singly or totally deficient in MHC I and examined for their ability to produce IFNγ, spontaneously and following stimulation via activating receptor cross-linking or exposure to chemical stimulants.

Results from the transfer of WT NK cells to MHC I-deficient hosts indicated that after a period of residence in an environment lacking MHC I expression, WT NK cells demonstrated a hyporesponsive phenotype, identical to that of unlicensed NK cells. Additionally, following adoptive transfer of WT to single MHC I-expressing hosts (either $K^{b-/-}$ or $D^{b-/-}$), WT cells acquire the (hyporesponsive) phenotype of the host environment. These data suggest that, contrary to previous assumptions, NK licensing is reversible and can be lost if contact with MHC I is disrupted. Additionally, we believe these results suggest that the rheostat model is that which best describes the licensing process, as there is a quantized loss of function upon sequential loss of MHC I contact.

Previously, we had demonstrated that transfer of $\beta_2m^{-/-}$ NK cells to MHC I+ hosts resulted in a gain of function in the in MHC I-deficient donor cells, as well as a decrease in the expression of inhibitory receptors specific for MHC I molecules expressed in the host environment. We hypothesized that this decrease in inhibitory receptor expression may be related to the gain of function, and might even be an underlying mechanism. Forming a complementary hypothesis, we then predicted that if inhibitory receptor

expression were inversely related to NK cell function, we would observe an increase in inhibitory receptor expression on the surface of WT NK cells following their transfer to MHC I-deficient hosts, corresponding to the observed loss of function. However, to our surprise, we did not find this to be completely true. WT NK cells recovered from $\beta_2m^{-/-}$ hosts demonstrated only a partial increase in inhibitory receptor expression. These results suggest that surface expression of inhibitory receptors alone may be insufficient to regulate the functional phenotype of the host. Alternatively, the results may suggest that WT loss of function could be governed by a mechanism different from that which controls hyporesponsiveness in $\beta_2m^{-/-}$ NK cells, or that the threshold for receptor calibration is much narrower than previously anticipated.

Attempts to define the capacity of NK cells to become 'relicensed' on different MHC I alleles were unsuccessful. Perhaps they may be attempted in a host lacking cytotoxic capacity, such as the Jinx mouse, in order to preserve the grafted cells. Experiments probing the requirements for MHC I expression in maintenance of the licensed phenotype demonstrate that MHC I expression must be maintained in both hematopoietic as well as non-hematopoietic compartments in order for NK cells to remain functional. These results have interesting implications for the ideas of NK cell tolerance, as well as for implications of NK function in settings of chronic MHC I deficiency, such as the tumor microenvironment.

Additionally, these data provide potentially important information regarding the necessity and sufficiency of *trans* and *cis* binding in establishment and maintenance of the licensed phenotype. Adoptive transfer of WT NK cells to $\beta_2m^{-/-}$ hosts resulted in loss of WT NK

cell function, demonstrating that interactions with MHC I in *trans* are necessary to maintain the licensed phenotype. This experiment also demonstrates that *cis* interactions are insufficient to maintain WT NK cells in a licensed state. Also, previous experiments in which $\beta_2m^{-/-}$ NK cells displayed increased function following transfer to WT hosts demonstrate that *cis* interactions are not necessary to induce licensing, and *trans* interactions are sufficient. Therefore a complete conclusion is that *trans* interactions are both necessary and sufficient to induce and maintain licensing, while *cis* interactions are neither necessary nor sufficient.

Interestingly, we often noted that recovery of WT NK cells from MHC I-deficient hosts was relatively poor, compared to recovery from WT hosts. Co-identification of WT donor cells via CFSE-label and staining for a congenic marker (Ly5.1) did not reveal proliferation in the WT donor cell population, suggesting the cells were not 'lost' due to label dilution after division. We hypothesize that perhaps once licensed, there is a role for contact with MHC I in NK cell survival. As mentioned previously, data demonstrating dependence on contact with MHC I for survival has been presented for other immune subsets. Further experiments to investigate this hypothesis could involve examination of WT donor cells for surface markers of apoptosis upon recovery from MHC I-deficient hosts. If found to be elevated, perhaps mice deficient in components of the apoptotic pathway, or mice with transgenic expression of anti-apoptotic molecules (such as Bcl-2), could be used as the source of donor NK cells. In an alternative, though not mutually exclusive hypothesis, WT NK cells may be differentially localized following injection

into MHC I-deficient hosts. Extensive tissue distribution analyses were not performed and further investigation into this issue may prove interesting.

Our results appear to indicate that NK cells may transition between functional and non-functional states. In the next chapter, the biological relevance of this flexibility is investigated, with an examination of the *in vivo* relevance of free transition between licensed and unlicensed states.

Figure 23. WT NK cells are not activated and adopt a hyporesponsive phenotype following transfer to MHC I-deficient hosts.

WT splenocytes were CFSE-labeled and adoptively transferred to $\beta_2m^{-/-}$ hosts. At 7d post-transfer, NK cell production of IFNγ was assessed via intracellular flow cytometry. **(a)** Spontaneous IFNγ production by host and donor NK cells (gated on CD3-NKp46+CFSE+/-), after 8hr *ex vivo* culture in normal medium. **(b)** IFNγ production by WT donor and $\beta_2m^{-/-}$ host NK cells following anti-NK1.1 activation. **(c)** IFNγ production after treatment with PMA and ionomycin. Data are representative from 2 independent experiments, with 1-3 mice/group.

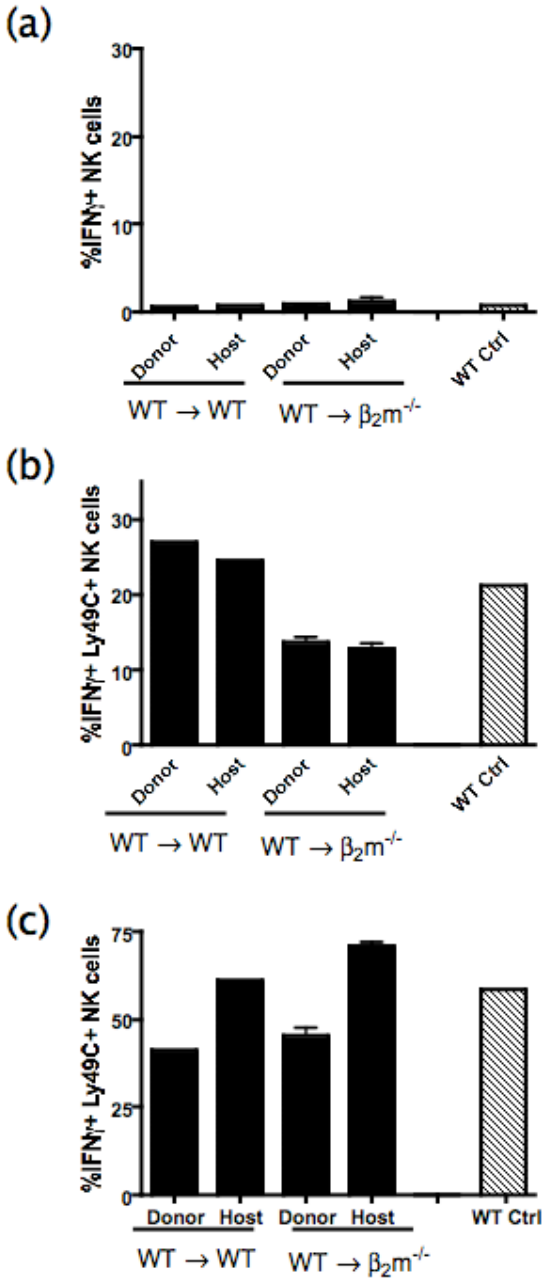

(a)
%IFNγ+ NK cells
30
20
10
0
Donor
Host
Donor
Host
WT Ctrl
WT → WT
WT → β2m-/-
(b)
%IFNγ+ Ly49C+ NK cells
30
20
10
0
Donor
Host
Donor
Host
WT Ctrl
WT → WT
WT → β2m-/-
(c)
%IFNγ+ Ly49C+ NK cells
75
50
25
0
Donor Host
Donor Host
WT Ctrl
WT → WT
WT → β2m-/-

Figure 24. WT NK cells display a graded loss of function following transfer to hosts expressing a single MHC I allele.

WT splenocytes were CFSE-labeled and adoptively transferred to hosts expressing a single MHC I allele. NK cell production of IFNγ was assessed 7d post-transfer via intracellular flow cytometry. **(a)** IFNγ production by WT donor and single MHC I host NK cells (CD3-NKp46+CFSE+/-) following 8hr *ex vivo* anti-NK1.1 activation. **(b)** IFNγ production after treatment with PMA and ionomycin. Data are pooled from 2 independent experiments, with 2-4 mice/group for experimental and 1-2 mice/group for controls.

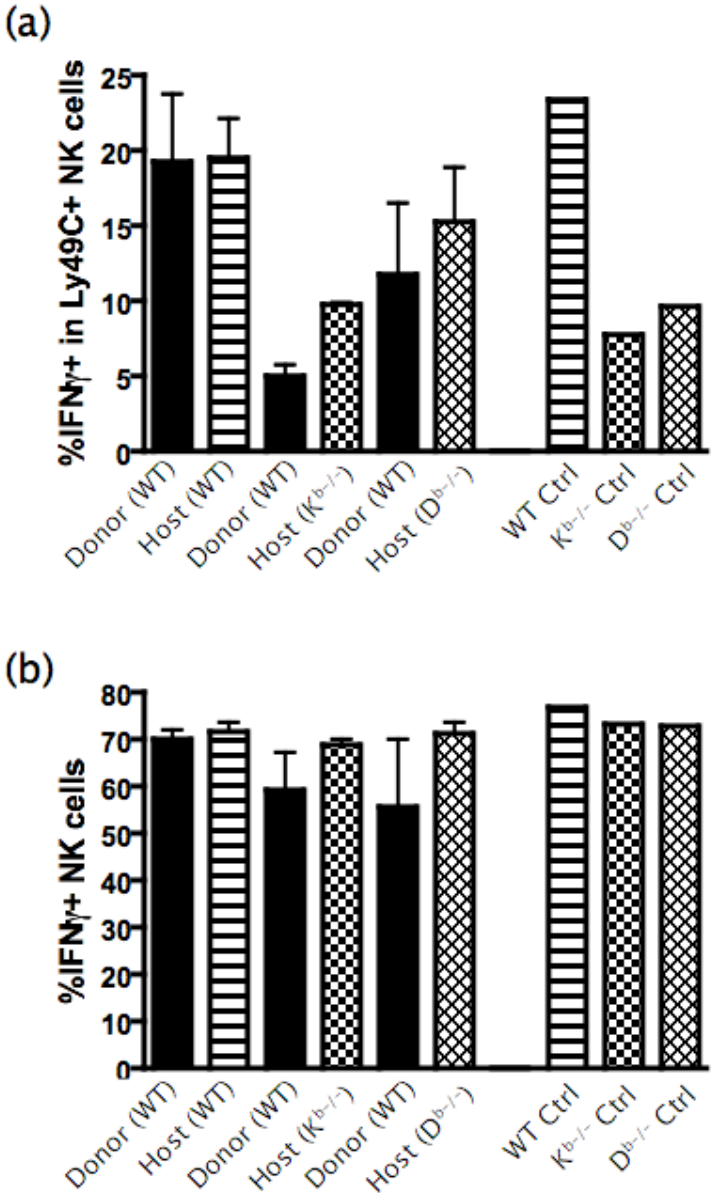

(a)
%IFNγ+ in Ly49C+ NK cells
25
20
15
10
5
0
Donor (WT)
Host (WT)
Donor (WT)
Host (Kb−/−)
Donor (WT)
Host (Db−/−)
WT Ctrl
Kb−/− Ctrl
Db−/− Ctrl
(b)
%IFNγ+ NK cells
80
70
60
50
40
30
20
10
0
Donor (WT)
Host (WT)
Donor (WT)
Host (Kb−/−)
Donor (WT)
Host (Db−/−)
WT Ctrl
Kb−/− Ctrl
Db−/− Ctrl

Figure 25. Inhibitory receptor expression on WT NK cells does not match level on MHC I-deficient NK cells following transfer to MHC I-deficient hosts.

WT splenocytes were CFSE-labeled and adoptively transferred to $\beta_2m^{-/-}$ hosts, then recovered 7d post-transfer. Inhibitory receptor surface expression on WT donor and $\beta_2m^{-/-}$ host NK cells (CD3-NKp46+CFSE+/-) was quantified by antibody surface staining and flow cytometry. Data are representative of 4 independent experiments, with each dot representing a single spleen.

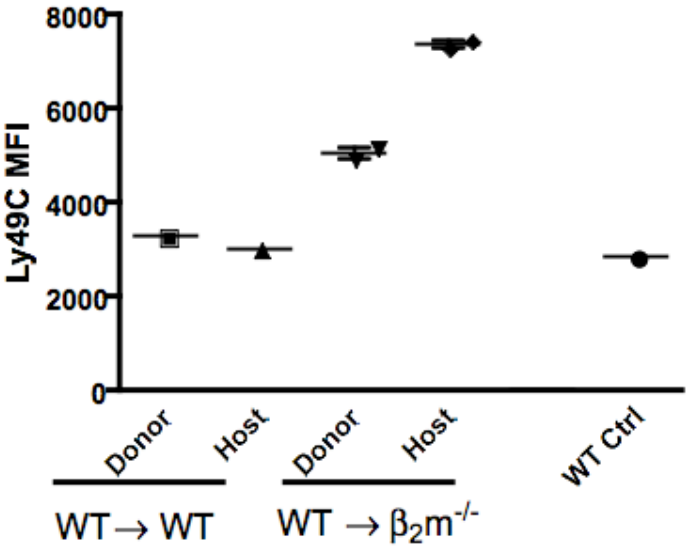

8000
6000
4000
2000
0
Ly49C MFI
Donor
Host
Donor
Host
WT Ctrl
WT → WT
WT → β2m-/-

Figure 26. MHC I mismatched grafts are eliminated, in a T-independent manner. NK cells were sort-purified from B10.D2 splenocytes, CFSE-labeled and adoptively transferred to C57Bl/6 $Rag1^{-/-}$ or B10.D2 recipients. At 7d post-transfer, host spleens were harvested and analyzed for the presence of CFSE+ donor NK cells remaining.

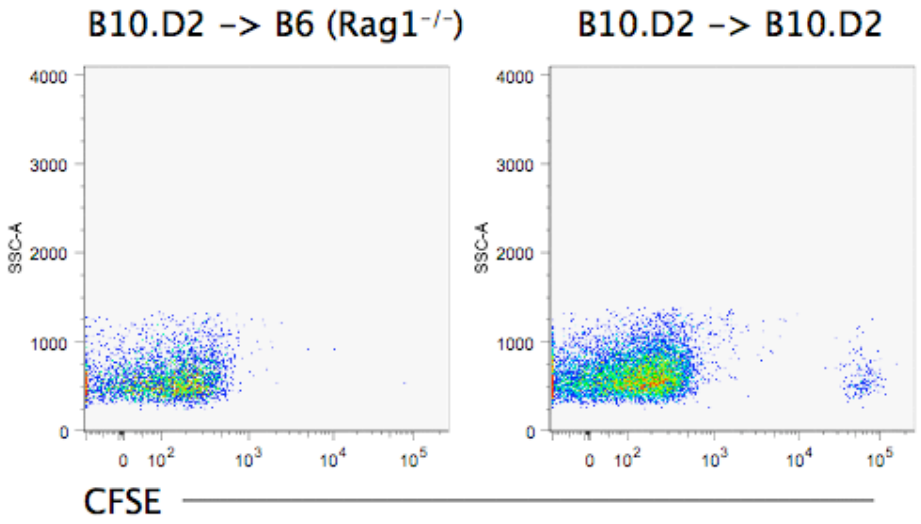
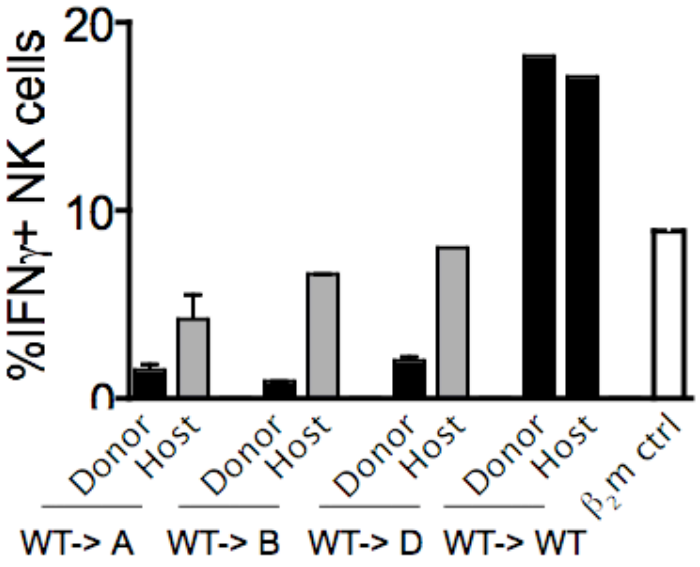

B10.D2 -> B6 (Rag1-/-)
B10.D2 -> B10.D2
SSC-A
4000
3000
2000
1000
0
0
10^2
10^3
10^4
10^5
CFSE

Figure 27. MHC I expression is required in both hematopoietic and non-hematopoietic compartments for maintenance of licensing.

Bone marrow chimeras with varying hematopoietic and non-hematopoietic MHC I expression (as indicated) were generated. Chimeras were rested 8 – 10w, then given an adoptive transfer of CFSE-labeled WT splenocytes. Host spleens were recovered 7d post-transfer and splenocytes were stimulated via anti-NK1.1 treatment. WT donor NK cells and host NK cells (containing a mixture of WT and $\beta_2m^{-/-}$ cells) were assessed for IFNγ production via intracellular flow cytometry.



Chimera A: WT -> $\beta_2m^{-/-}$

Chimera B: $\beta_2m^{-/-}$ -> WT

Chimera D: WT+ $\beta_2m^{-/-}$ (1:1) -> WT

CHAPTER 6:

Bidirectional manipulation of licensing *in vivo*:

murine parabiosis

To more fully explore the contributions of MHC I density and localization to the generation and maintenance of functional NK cells, as well as to study the potential for unique patterns of NK cell distribution given simultaneous access to different environments, we created an experimental system utilizing parabiosis of mice differing in their expression of MHC I. These experiments indicate that parabiosis of MHC I-deficient mice to WT partners is fundamentally and specifically detrimental to the MHC I-deficient partner, resulting in the induction of a syndrome with symptoms such as weight loss and extreme splenic atrophy, and resulting in death of parabionts 14 – 21 days post-surgery. This phenotype appears to be at least partially NK-dependent, although there may be a role for T and B lymphocytes, as well. Interestingly, in MHC I-deficient:WT pairs, unequal lymphocytic chimerism was consistently observed, with efficient engraftment of MHC I-deficient cells in WT partners, but very poor engraftment of WT cells in MHC I-deficient partners. These results provide new insight into the flexibility of NK cell licensing and the biological relevance of this property, *in vivo*.

Introduction

While adoptive transfer has proven a useful tool for studying acquisition and maintenance of NK cell function, this experimental system does have limitations. In particular, inherent restrictions on the number of donor NK cells able to successfully engraft in an unconditioned host environment limit the number and types of post-transfer analyses that may be performed on donor cells. Additionally, during preparation of the donor cell population, unintentional selection effects may occur. Furthermore, enforced localization of transferred cells by the chosen route of administration (i.v. injection) may artificially

dictate the final distribution of donor cells. Finally, when studying the behavior or functions of cells following their transfer to genetically deficient hosts, potentially unknown effects of the genetic deficiency on other cell types within the environment may lead to misinterpretation of results.

In light of these potential difficulties associated with the use of adoptive transfer, we sought an alternative experimental system in which to accomplish lymphocyte exchange. One such system is that of parabiosis, or shared circulation. Historically, parabiosis has been used within the field of immunology to study immunological tolerance [123, 124] and immune cell trafficking [125-127], as well as in the study of physiological processes like metabolism [128]. With a relatively simple surgical technique, parabiotic animals are generated via connection of complementary surgical incisions. Vascular anastomosis is achieved as the incisions heal, resulting in a shared circulatory system and, theoretically, free and unlimited exchange of all blood components, including lymphocytes [125, 129]. Therefore, parabiosis appeared to meet our requirements for a new experimental system in which to study the flexibility of NK cell licensing, without the use of adoptive transfer.

Parabiosis surgical protocol

A protocol permitting the generation of and experimentation with parabiotic mice was drafted and was approved by the Washington University animal studies committee. Utilizing training obtained under the supervision of post-doctoral fellows in the laboratory of Dr. Michel Nussensweig (Rockefeller University), I established an experimental procedure of parabiosis in our lab. Briefly, female mice, 8-16w (average

12w), were surgically joined using 3 dissolvable sutures (two discontinuous sutures, one around each tibia and one around each ulna, as well as one continuous suture between the patellar ligaments) and approximately 15-20 wound clips (9mm stainless steel), located dorsally and ventrally along the entire length of the incision. Animals were supplied accessible gel nutrition for 2 weeks post-SX and were maintained on antibiotic drinking water from days -1 thru sacrifice.

Gross external pathology reveals specific illness of $\beta_2m^{-/-}$ when parabiosed to WT

There were few outward differences in the appearance of $\beta_2m^{-/-}$ and WT mice during days 1 – 14 post-SX. However, between days 14 – 21 post-SX, $\beta_2m^{-/-}$ mice parabiosed to WT partners begin to appear very ill, adopting a hunched posture and ruffled fur (Figure 28). Individual weights were taken before the surgical procedure and at the time of sacrifice. At weeks 1-2 post-SX, all mice appeared to lose some weight, typically less than 20% of body weight (on average around 10%), possibly due to factors such as dehydration and decreased food consumption. At times past 2w post-SX, $\beta_2m^{-/-}$ mice parabiosed to WT partners appeared to rapidly lose a significant amount of weight (Figure 29a). However, total weight loss was difficult to determine definitively, as the pairs often died suddenly between weeks 2 and 3 post-SX, precluding a quantitative assessment. Co-housing of $\beta_2m^{-/-}$ and WT mice in the absence of surgical joining did not induce weight loss, indicating that the mechanism of weight loss is unlikely related to infection or transmission of endogenous microbiota (Figure 29b). At no time were skin lesions or sores noticed on the tails or ears of any of the mice, as is commonly noted in settings of acute and chronic GVHD (graft vs. host disease) [130].

Necropsy results do not readily reveal mechanism of illness in $\beta_2m^{-/-}$ mice parabiosed to WT partners

Six pairs of mice ($\beta_2m^{-/-}$:WT and WT:WT at 1, 2 and 3w post-SX) were submitted for necropsy to Dr. Suellen Greco, D.V.M. (Washington University, Division of Comparative Medicine). She examined gross anatomy, hydration and hematology, as well as histopathology of major organ systems, including: brain, heart, spleen, kidney, liver, lung, and intestines. No major cellular infiltration was noted in any organ of any of the experimental animals. Negative histopathology was later independently confirmed by Dr. Thad Stappenbeck. Although abscesses were often noted at the points of attachment (knees, elbows), all attempts to culture bacteria from these sites, as well as organs (heart, spleen, lymph nodes) were negative. PCR analyses of fecal pellets showed the animals to be negative for most pathogens tested, including: *Citrobacter rodentium, Clostridium piliforme, Klebsiella oxytoca, Klebsiella pneumoniae, Mycoplasma pulmonis, Pneumocystis* sp., *Pseudomonas aeruginosa, Salmonella* sp., *Staph aureus, Shigella, Strep moniliformis, S. pneumoniae, Cryptosporidium, Giardia* and pinworms. The animals did test positive for: MNV (murine norovirus), *Helicobacter* genus and *Staph xylosus*. However, these positive results are unlikely to account for the observed phenotype.

Atrophy of MHC I-deficient spleen is the major pathological finding

There were no gross differences noted in the lungs, kidneys, intestines, thymi or lymph nodes. Occasionally some livers, in both $\beta_2m^{-/-}$ and WT mice when paired together,

appeared pale, suggesting anemia. This finding was not noted in control pairs (WT:WT, $\beta_2m^{-/-}$:$\beta_2m^{-/-}$). Additionally, there appeared to be a trend of cardiac atrophy in $\beta_2m^{-/-}$ mice parabiosed to WT partners although, when normalized to body weight, the trend does not appear significant. The most striking, significant and reproducible finding upon gross internal examination was atrophy of the $\beta_2m^{-/-}$ spleen, following parabiosis to a WT partner.

When analyzed at 2w post-SX, the $\beta_2m^{-/-}$ spleen in $\beta_2m^{-/-}$:WT pairs consistently appeared dramatically atrophied (Figure 30a). Cell counts confirm a decrease in cellularity, with changes evident as early as 1w post-SX (Figure 30b). By 3w post-SX, the $\beta_2m^{-/-}$ spleen is almost completely acellular, with cell counts averaging less than 1×10^6 splenocytes. In $\beta_2m^{-/-}$:WT pairs, no increase in cellularity of the WT spleen was noted at any time point, suggesting that the decrease in cellularity of the $\beta_2m^{-/-}$ spleen is not simply due to trafficking to and accumulation of $\beta_2m^{-/-}$ cells in the WT partner. There is a slight decrease in cellularity of both spleens from control pairs, and we hypothesize this may be due to emigration of immune cells during the inflammation and healing processes following the surgical procedure.

Increased cell death is one mechanism that could account for the decrease in cellularity of the $\beta_2m^{-/-}$ spleen following parabiosis to a WT partner. Therefore, splenic apoptosis was evaluated by staining with AnnexinV (a protein that binds phosphotidylserine (PS) moieties, increased on the surface of cells during apoptosis) and 7-AAD (an intravital dye), followed by flow cytometry. We observed a measurable increase in apoptosis of

cells recovered from the $\beta_2m^{-/-}$ spleen, indicating that the decrease in splenic cellularity may be due, at least in part, to increased $\beta_2m^{-/-}$ splenocyte death after parabiosis to a WT partner (Figure 31). Interestingly, the increased apoptosis observed in $\beta_2m^{-/-}$ splenocytes appears to be organ-specific. Cells from the bone marrow of mice in $\beta_2m^{-/-}$:WT pairs were harvested and similarly evaluated for apoptosis. However, no increase in apoptosis of bone marrow was observed for either parabiont, at any time 1-3w post-SX (Figure 31 and data not shown).

Splenic atrophy appears partially NK-dependent

We next wished to determine if NK cells contributed to the striking atrophy of $\beta_2m^{-/-}$ spleens following parabiosis to WT partners, possibly through mechanisms of degranulation or direct cytotoxicity. Preliminary experiments were conducted using two approaches: antibody-mediated depletion of NK cells, and a second approach of $\beta_2m^{-/-}$ parabiosis to partners genetically deficient in immune cell components, including NK cells and T/B cells. With these experimental manipulations, we hoped to determine if NK cells are necessary and/or sufficient for the development of splenic atrophy, and if necessary, the mechanism by which they generate the phenotype.

Antibody-mediated NK cell depletion was accomplished by injection of parabionts with anti-NK1.1 monoclonal antibody (PK136), once before surgery and at regular intervals (usually every 3-4d) until sacrifice. At 2w post-SX, we noted that some $\beta_2m^{-/-}$ spleens from NK-depleted pairs appeared to retain cellularity equivalent to that of WT partners, implying that the destruction of the $\beta_2m^{-/-}$ spleen is at least partially NK-dependent

(Figure 32). However, the number of $\beta_2m^{-/-}$ which were 'protected' (3/9) did not appear to be much greater than the random number of $\beta_2m^{-/-}$ mice which did not exhibit full splenic atrophy following parabiosis to WT partners, in the absence of NK-depletion (4/15). It is interesting to note the appearance of a bimodal distribution of splenic cellularity in $\beta_2m^{-/-}$ spleens, following NK-depletion. Preliminary data do suggest that NK-depletion was not totally efficient in the parabionts, perhaps due to factors such as disruption in circulation or abnormal distribution of cells responsible for antibody-mediated depletion, such as splenic macrophages. Further experiments examining antibody dosage and depletion efficiency are warranted.

In a second method to discern NK contribution to $\beta_2m^{-/-}$ splenic atrophy following parabiosis to WT partners, we generated new parabionts utilizing mice with known genetic deficiencies in immune cell components. For our first experiment, we hoped to join $\beta_2m^{-/-}$ mice to MHC I+ mice deficient in NK cells. Unfortunately (until very recently with the discovery of E4bp4), truly NK-specific transcription factors have remained elusive. Therefore, currently there is no 'NK knock-out' mouse available. Other mouse models in which mature NK cells are not generated, such as the common signaling chain knock-out ($\gamma_C^{-/-}$), have deficiencies in multiple immune cell lineages. Still other NK-deficient models, such as IL-$15^{-/-}$, lack components necessary for the generation and maintenance of NK cells and thus cannot support an exogenous (transferred) NK population. Therefore, in place of an NK cell knock-out mouse, we utilized NK-deficient mice (NKD strain, generated in the Yokoyama lab and described in [131]). Although these mice do posses immature NK precursors, they completely lack mature NK cells, a

phenotype attributed to a transgene insertion effect. Importantly, the immature precursor NK cells are not functional and these mice have documented defects in cytotoxicity.

$\beta_2m^{-/-}$:NKD parabionts were generated and splenic cellularity was analyzed at 2w post-SX. Interestingly, $\beta_2m^{-/-}$ spleens did not appear significantly less cellular than their NKD counterparts (Figure 33a). However, parabiotic pairs utilizing NKD mice, both WT:NKD and $\beta_2m^{-/-}$:NKD, appeared less cellular overall. NKD mice have not been documented to have abnormal splenic cellularity, so the reason for this particular finding is unknown. The reduced cellularity of all parabionts in this series of experiments precludes definitive interpretation of these results. A more detailed examination of the normal cellularity in NKD mice, as well as NKD:NKD parabionts, would be of value. However, these preliminary results do suggest a role for mature WT NK cells in the manifestation of $\beta_2m^{-/-}$ splenic atrophy, following parabiosis to WT partners.

As preliminary data suggested a role for NK cells in $\beta_2m^{-/-}$ splenic atrophy within parabiosis, we next wished to determine if NK cells are sufficient to induce this phenotype. To accomplish this, we produced parabionts by joining $\beta_2m^{-/-}$ mice to mice deficient in Rag1, which lack both T and B cells. We hypothesized that if NK cells are sufficient to induce splenic atrophy in $\beta_2m^{-/-}$ mice following parabiosis to WT partners, the atrophy phenotype would be reproduced in $\beta_2m^{-/-}$ mice joined to $Rag1^{-/-}$, as these partners maintain a mature and functional NK cell population. $\beta_2m^{-/-}$:$Rag1^{-/-}$ pairs were generated and splenic cellularity was determined at 2w post-SX. We found that in contrast to our predictions, $\beta_2m^{-/-}$ mice did not display decreased splenic cellularity

following parabiosis to Rag1$^{-/-}$ partners (Figure 33b). In fact, $\beta_2m^{-/-}$ splenic cellularity actually appeared increased, a novel finding within our experimental system.

In the simplest interpretation, these results implicate a direct role for T and/or B cell function in $\beta_2m^{-/-}$ splenic atrophy, following parabiosis to WT partners. However, it is important to note that detailed flow cytometry was not performed on these splenocytes. Therefore, it remains possible that the elevated gross cellularity of the $\beta_2m^{-/-}$ spleen is achieved through a mechanism other than the preservation of $\beta_2m^{-/-}$ splenocytes, such as increased colonization by Rag1$^{-/-}$ cells. Alternatively, perhaps the increase in cellularity is indeed accomplished via the preservation of endogenous $\beta_2m^{-/-}$ splenocytes, and this result reflects an indirect role for T or B cells, through the support of proper WT NK cell function. Further work must be done to clarify this issue. Overall, these findings do suggest that NK cells alone are not sufficient to induce splenic atrophy in $\beta_2m^{-/-}$ mice, following parabiosis to MHC I+ partners.

Preliminary experiments utilizing mice genetically deficient in immune cell components revealed a necessary, though seemingly insufficient, role for NK cells in induction of splenic atrophy within parabiosis, but the mechanism for their action remained unclear. To examine the contribution of direct cytotoxicity to $\beta_2m^{-/-}$ splenic atrophy within parabiosis, we utilized mice deficient in components necessary for degranulation, a process required for cytotoxic function. Jinx mice (previously described in Chapter 3, and in [55]) were parabiosed to $\beta_2m^{-/-}$ mice, and splenic cellularity determined at 2w post-SX. Upon analysis, we found that $\beta_2m^{-/-}$ mice had retained normal splenocyte counts (Figure

33c). Interestingly, we also noted that the spleens from Jinx mice parabiosed to $\beta_2m^{-/-}$ partners appeared to be slightly more cellular than expected. It is proposed that direct cytotoxicity contributes to the resolution of the immune response, through elimination of activated lymphocytes, and we propose that the increase in cellularity of Jinx spleens may reflect an excess of activated immune cells, due to a defect in cytotoxic elimination of these cells.

As all immune subsets in the Jinx mouse, including both NK and T cells, are disabled by the Jinx mutation, it is not possible to use this data to definitively discern the identity of the effector lymphocyte. However, the absence of $\beta_2m^{-/-}$ splenic atrophy following parabiosis to Jinx mice does indicate that granule exocytosis, and resultant cytotoxicity, is likely to be a mechanism by which the atrophy is induced.

Several different experimental techniques, including antibody mediated NK cell depletion as well as parabiosis to genetically deficient mice, were utilized to examine the role of NK cells in the induction of $\beta_2m^{-/-}$ splenic atrophy, following parabiosis of these mice to WT partners. Although not entirely conclusive, NK depletion may point to a necessary role for NK cells. Parabiotic experiments with NKD mice appear to support this conclusion. Results from parabiosis of $\beta_2m^{-/-}$ mice to $Rag1^{-/-}$ partners indicate that NK cells, while perhaps necessary, are not sufficient to induce splenic atrophy in MHC I-deficient partners. Mechanistically, direct cytotoxicity appears to play an important role in the phenotype of splenic atrophy, as parabiosis of $\beta_2m^{-/-}$ mice to partners defective in degranulation, and therefore cytotoxicity, did not result in the atrophy of $\beta_2m^{-/-}$ spleens.

Overall, the role for NK cells in induction of splenic atrophy in MHC I-deficient mice appears complex and remains incompletely understood, but warrants further investigation, as it offers insight into fundamental processes governing NK cell tolerance.

Unequal chimerism in spleen and blood of $\beta_2m^{-/-}$:WT parabionts

Parabionts differing in their expression of the CD45 alleles (i.e. Ly5.1, Ly5.2) were generated and sacrificed at weeks 1-3 post-SX. Lymphoid chimerism, as well as NK-specific chimerism of spleen, blood and bone marrow were established using antibody staining and flow cytometry.

Analysis of lymphocytic chimerism in control pairs (WT:WT Ly5.1) revealed maximal splenic chimerism of around 40%, by about 1w post-SX (Figure 34a). The kinetics of this chimerism are faster than some previously published, which report maximal splenic chimerism at 3w post-SX [132]. In experimental pairs ($\beta_2m^{-/-}$:WT Ly5.1), $\beta_2m^{-/-}$ lymphocytes achieve high levels of splenic chimerism in their WT partners, peaking at a maximal chimerism of around 50% (Figure 34a). In general, the kinetics of chimerism of MHC I-deficient cells in WT partners appear similar to the kinetics of chimerism observed in WT:WT Ly5.1 control pairs.

Cellular composition of the $\beta_2m^{-/-}$ lymphocyte population present in the WT spleen was evaluated by staining with antibodies specific for lineage-specific markers (e.g. CD3, CD4, CD8, CD19). Among $\beta_2m^{-/-}$ lymphocytes, we found that each cell lineage was represented at approximately the same frequency as in the endogenous WT cell

population. When NK-specific chimerism was examined, we noted that $\beta_2m^{-/-}$ NK cells appear to survive well in the WT partner, achieving an NK-specific chimerism of over 50% by 2w post-SX (i.e., of all NK1.1+CD3- cells in the WT spleen at 2w post-SX, 50% are $\beta_2m^{-/-}$-derived) (Figure 34b). From these data, we can conclude that establishment of $\beta_2m^{-/-}$chimerism in WT hosts is not due to an outgrowth of one MHC I-deficient cell type in particular, and that no single lymphoid lineage appears to possess a clear competitive advantage or disadvantage in colonization of the WT host.

In contrast to the efficient chimerism of $\beta_2m^{-/-}$ lymphocytes in WT spleens, we found that WT lymphocytes displayed poor chimerism in the spleens of their $\beta_2m^{-/-}$ partners, reaching a maximum of less than 20% at 2w post-SX (Figure 34a). NK-specific chimerism of WT cells in $\beta_2m^{-/-}$ hosts is particularly poor, with a maximum of less than 15% at 2w post-SX (Figure 34b). It is possible that the decreased cellularity of the $\beta_2m^{-/-}$ spleen following parabiosis to a WT partner does not allow for the normal chimerism of WT cells in $\beta_2m^{-/-}$ hosts. However, the mechanism for the decreased chimerism of WT lymphocytes in the MHC I-deficient spleen remains unclear.

Blood chimerism of parabionts was similarly evaluated. Unlike splenic chimerism, in which WT lymphocytes appeared with low frequency in their $\beta_2m^{-/-}$ partners, WT and $\beta_2m^{-/-}$ lymphoid chimerism of blood appeared more similar in both directions, ranging from 30 to 40% (Figure 35a), although there was considerable variability. Interestingly, although WT lymphocytes as a whole appeared to be well-represented in the blood of

both partners, WT NK cells were notably absent from $\beta_2m^{-/-}$ blood, achieving chimerism of less than 10% (Figure 35b).

A simple mechanism of exclusion could account for the deficiency of WT NK cells in $\beta_2m^{-/-}$ partners, and would be predicted to result in an accumulation of WT NK cells within the WT host. However, spleens of WT mice parabiosed to $\beta_2m^{-/-}$ partners contained normal absolute numbers of NK cells (Figure 35c), and blood from WT partners was not demonstrated to be enriched for NK cells (Figure 35d). These results suggest that simple exclusion of WT NK cells from the $\beta_2m^{-/-}$ partner does not best describe why WT NK cells are found at low numbers in the $\beta_2m^{-/-}$ partner. Instead, perhaps WT NK cells are displaced to an organ not yet examined, or experiencing disrupted homeostasis due to accelerated death or decreased production.

In addition to examining splenic and blood chimerism, we also evaluated parabiotic bone marrow chimerism. We found that in contrast to the appreciable lymphoid chimerism observed in blood and spleen, chimerism of bone marrow in WT:WT Ly5.1 control pairs is very low (Figure 35e). Although we occasionally noted slightly increased chimerism in the bone marrow of $\beta_2m^{-/-}$ mice from $\beta_2m^{-/-}$:WT experimental pairs (Figure 35e), there was very little difference in the percentage of NK cells within the bone marrow (Figure 35f), indicating that the small increase in chimerism is not due to an NK-specific infiltration. The small number of mature NK cells that could be recovered from bone marrow were analyzed for surface expression of phenotypic markers of activation, such as CD69 and CD11b, but no major differences were observed. Additionally, Dr. Jeff Klco

(Washington University), confirmed normal marrow histology, including precursor frequency, in parabionts.

NK cell licensing appears intermediate in parabionts

Murine parabiosis was proposed in the context of this thesis work as an experimental method for manipulating the exposure of NK cells to elements required for licensing, specifically MHC I. Chimerism data demonstrated the presence of partner-derived NK cells in the spleen of each parabiont confirming that, through circulation of the cells to a new host, parabiosis results in an alteration of NK cell exposure to MHC I. We next wished to determine the effect of residence in the new environment on the licensing status of these immigrant NK cells.

In order to determine the basal activation status of NK cells recovered from the parabionts, splenic NK cells from each parabiont were assessed for spontaneous IFNγ production. In all cases, no spontaneous IFNγ production was observed in NK cells from either parabiont, in either spleen (Figure 36a). Additionally, flow cytometry revealed no difference in surface expression of activation markers, such as CD69. These results indicate that within the setting of parabiosis, NK cells do not become activated upon exposure to an environment differing in MHC I expression. These data agree with results obtained within the adoptive transfer experimental system, as neither WT NK cells transferred to $\beta_2m^{-/-}$ hosts nor $\beta_2m^{-/-}$ NK cells transferred to WT hosts demonstrated spontaneous cytokine production, nor increased surface expression of activation markers.

Next, we measured IFNγ production following receptor cross-linking, in order to determine the NK cell licensing status. With the exception of $\beta_2m^{-/-}$ NK cells recovered from the $\beta_2m^{-/-}$ spleen, we found that all NK cells in both hosts from $\beta_2m^{-/-}$:WT pairs appeared to have an intermediate licensing phenotype (Figure 36b). This phenotype is reminiscent of that from mixed bone marrow chimeras (Chapter 4, Figure 22), lending further support to the hypothesis that NK cell function (licensing) is sensitive to the total amount of environmental MHC I, including that contributed by the hematopoietic compartment. The licensing status of NK cells from WT:WT control chimeras appeared normal, indicating that parabiosis alone does not impact NK cell functionality.

Several possibilities may account for the variation observed in licensing status of $\beta_2m^{-/-}$ NK cells recovered from the $\beta_2m^{-/-}$ spleen, following parabiosis. It is possible that differential chimerism of microenvironments within the spleen may result in differential ability to promote NK cell licensing. Perhaps these NK cells, expected to become licensed but determined to have remained unlicensed, were sequestered by a large number of MHC I-deficient hematopoietic cells. In this case, it is possible that the $\beta_2m^{-/-}$ cells could not make a sufficient number of contacts with MHC I on hematopoietic cells, and remained unlicensed. Splenic sectioning and staining for markers of origin (Ly5.1, Ly5.2) would be useful.

Another potential explanation for the intermediate results of licensing in parabiotic NK cells may be attributed to time. Variation in NK cell residence time within each host could result in newly immigrated cells retaining normal or near normal function, while

cells with longer residence time are able to adopt the new phenotype. In support of this, kinetic analyses from adoptive transfer experiments clearly indicate that the gain of function of $\beta_2m^{-/-}$ NK cells in WT hosts occurs at least 48hr after residence in the host. Therefore, it is possible that variation in our results is introduced by unsynchronized lymphocyte influx and efflux. Unfortunately, although mechanisms exist for marking cells, such as BrdU (bromodexoyuridine) labeling, these labels are not useful for determining host-specific residence in a parabiotic system, as free diffusion allows dissemination of the label from one mouse to the other.

Overall, we are able to state that NK cells do not appear activated within the parabiotic system, indicated by the absence of spontaneously produced cytokine. Additionally, we found that the licensing status of NK cells within MHC I-disparate parabionts is intermediate.

Conclusions

These experiments indicate that parabiosis of MHC I-deficient mice to WT mice results in decreased health of the MHC I-deficient partner. Severe splenic atrophy of the MHC I-deficient partner was noted, with no corresponding increase in the cellularity of the spleen of the WT partner, suggesting that $\beta_2m^{-/-}$ cells may be lost through a mechanism of death or relocalization, as opposed to emigration to or altered retention within the WT partner.

Parabiosis to genetically-deficient partners and antibody-mediated depletion studies point to a necessary, but insufficient role for NK cells in the induction of this splenic atrophy, likely through a mechanism of cytotoxicity. To further dissect the mechanism of splenic atrophy, experiments pairing $\beta_2m^{-/-}$ mice to mice deficient in only T, B or NK cells (using mice such as $Tcr\alpha^{-/-}$, $Ig\alpha^{-/-}$ or $E4bp4^{-/-}$) would be informative. Additionally, via generation of a novel mixed bone marrow chimera (Jinx marrow + $Tcr\alpha^{-/-}$ into a WT host) and parabiosis of this chimera to a $\beta_2m^{-/-}$ partner, it would be possible to examine an indirect role for T cell support of NK function. (This chimera would generate functional NK cells but only non-cytolytic T cells, so the influence of T cell presence on NK cell function could be assessed in the absence of cytotoxic T cell contributions.) Finally, it would be desirable to test the parabiosis of an NK-deficient, MHC I-deficient animal model (e.g. $\beta_2m^{-/-}$ NKD or $\beta_2m^{-/-}E4bp4^{-/-}$) to NK-deficient and –sufficient, MHC I+ partners, in order to evaluate the potential for pathogenesis by MHC I-deficient NK cells.

Interestingly, we consistently observed that in $\beta_2m^{-/-}$:WT parabionts, chimerism of $\beta_2m^{-/-}$ cells was efficient in WT partners, but chimerism of WT cells in $\beta_2m^{-/-}$ hosts was lower than expected. The exact cause for this is unknown. However, it is documented that $\beta_2m^{-/-}$ mice can develop a small population of functional CD8+ T cells, even in the absence of normal MHC I expression and proper positive and negative selection T cell selection [133, 134]. These cells have been documented to be highly reactive to antigenic peptides derived from MHC I [135]. Therefore, we hypothesize that the inefficient chimerism of WT cells into $\beta_2m^{-/-}$ hosts may reflect the elimination of WT cells by anti-MHC I CD8+ T cells present in the $\beta_2m^{-/-}$ partner. Although flow cytometry did not reveal a clear increase

in splenic or blood CD3+ or CD8+ lymphocytic populations, perhaps these actions occur locally, in tissues at the site of anastomosis. To test this hypothesis, one could attempt CD8+ T cell depletion in the context of $\beta_2m^{-/-}$:WT parabiosis, and look for increased chimerism of WT cells in the $\beta_2m^{-/-}$ partner.

The low level of chimerism observed in parabiotic marrow was somewhat surprising. We suspected that MHC I-deficient bone marrow cells may be particularly vulnerable to WT NK-mediated cytolysis, as $\beta_2m^{-/-}$ bone marrow cells, even more so than $\beta_2m^{-/-}$ splenocytes, are readily eliminated by WT NK cells. Perhaps the limited access of WT NK cells to $\beta_2m^{-/-}$ bone marrow prevents the initiation of an immune response. Alternatively, WT NK cells may be tolerized to $\beta_2m^{-/-}$ bone marrow, owing to their exposure to other $\beta_2m^{-/-}$ cells, as is seen in the setting of adoptive transfer.

Finally, results from functional assessments of parabiotic NK cells proved ambiguous. The licensing status of these cells is difficult to definitively determine, possibly due to uncoordinated movement of cells between hosts and differential residence times. To 'resynchronize' NK cell populations, a survival surgery to separate parabionts could be performed, and splenic NK cell function analyzed at 3 or more days following separation.

Figure 28. MHC I-deficient mice parabiosed to WT partners appear unwell at 2w post-SX.

Age-matched, 9 – 12 week-old female WT mice were parabiosed to **(a)** WT or **(b)** $\beta_2m^{-/-}$ partners. At 2w post-SX, $\beta_2m^{-/-}$ mice paired with WT partners (dorsal view, $\beta_2m^{-/-}$ mouse is on right) display an overall unwell appearance, including hunched posture, flattened ears and ruffled fur. WT mice in the same pairs (dorsal view, left mouse), or in control pairs (WT:WT), demonstrate no obvious phenotype. Pictures depict 3 distinct pairs of mice and are representative of >15 pairs, over multiple experiments.

(a)

WT:WT

(b)

WT: $\beta_2m^{-/-}$

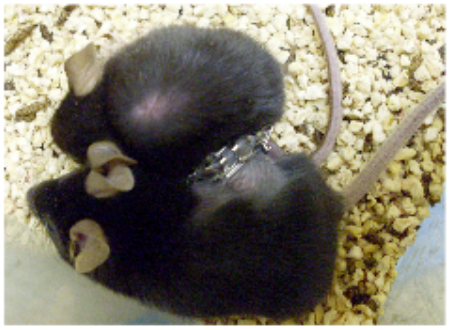
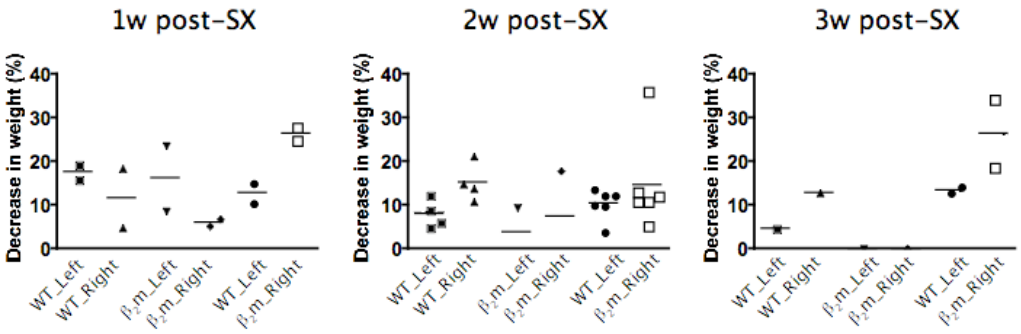


Figure 29. Weight loss in parabiotic mice.

Age-matched, 9 – 12 week-old female $\beta_2m^{-/-}$ mice were parabiosed to WT mice or $\beta_2m^{-/-}$ (as control). **(a)** Individual weights were recorded at day -1 (before SX) and upon sacrifice and separation. The difference was calculated and is plotted as function of change in percent of initial weight, for times 1 – 3 weeks post-SX. Data are pooled from multiple experiments. Each dot represents a single mouse. **(b)** WT and $\beta_2m^{-/-}$ mice were co-housed without surgical joining and weights were recorded. Data are from three pairs of mice, each pair housed in a separate cage.

(a)


1w post-SX
2w post-SX
3w post-SX
Decrease in weight (%)
0
10
20
30
40
WT_Left
WT_Right
β2m_Left
β2m_Right
WT_Left
β2m_Right

(b)

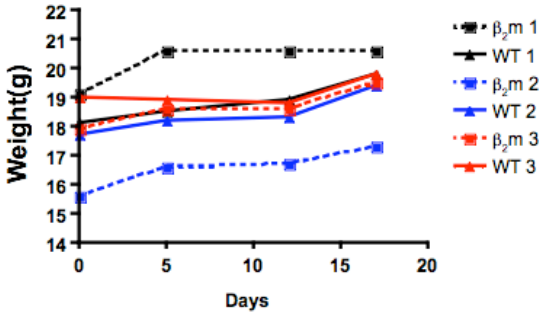
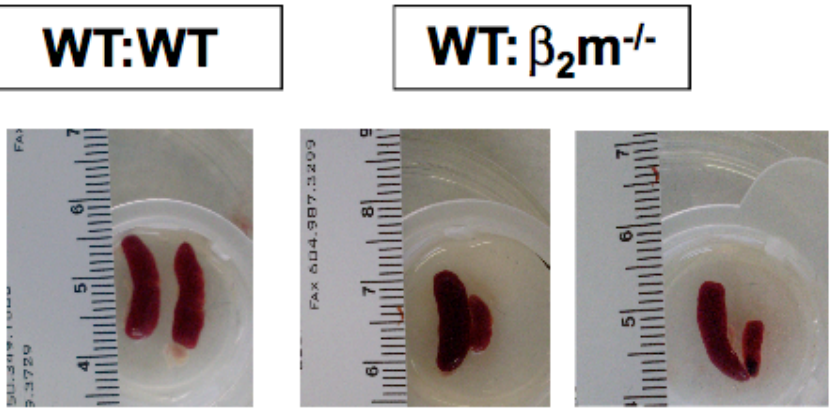

Weight(g)
14
15
16
17
18
19
20
21
22
0
5
10
15
20
Days
β2m 1
WT 1
β2m 2
WT 2
β2m 3
WT 3

Figure 30. Major splenic atrophy due to decrease in cellularity of MHC I-deficient spleen after parabiosis to WT.

Age-matched, 9 – 12 week-old female WT mice were parabiosed to $\beta_2m^{-/-}$ mice, or WT (as control). **(a)** Spleens harvested from parabionts at 2w post-SX. Pictures are equivalently scaled and representative of >15 pairs, over multiple experiments. **(b)** Splenic cellularity of parabionts was calculated at harvest, 1 – 3w post-SX. The blue and green lines represent $\beta_2m^{-/-}$ and WT mice in a pair, while the black and grey lines represent WT mice paired with WT mice (as control).

(a)


WT:WT
WT: β2m-/-

(b)

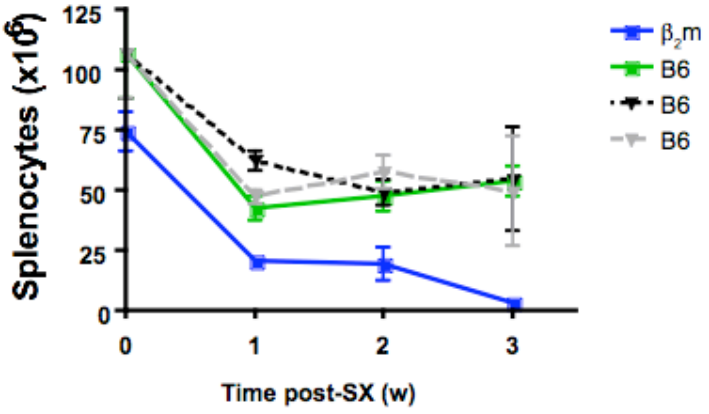

Splenocytes (x10⁶)
125
100
75
50
25
0
0
1
2
3
Time post-SX (w)
β2m
B6
B6
B6

Figure 31. Increased apoptosis in spleen, but not bone marrow, of MHC I-deficient partner after parabiosis to WT.

Age-matched, 9 – 12 week-old female mice were joined in parabiotic pairs of $\beta_2m^{-/-}$:WT, $\beta_2m^{-/-}$:$\beta_2m^{-/-}$ (control) and WT:WT (control). At 2w post-SX, spleens and bone marrow were recovered from each parabiont, stained with AnnexinV and 7-AAD, then analyzed via flow cytometry. Each dot represents one mouse and data are representative of 3 independent experiments.

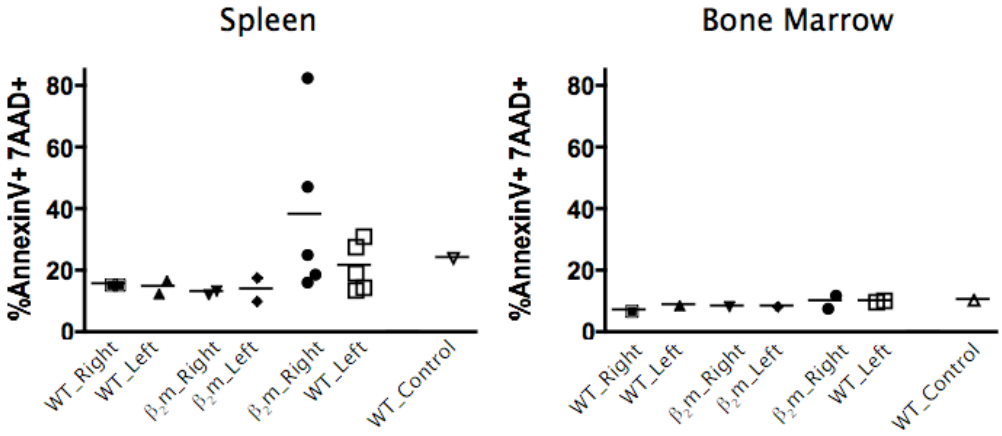

Spleen
%AnnexinV+ 7AAD+
80
60
40
20
0
WT_Right
WT_Left
β2m_Right
β2m_Left
β2m_Right
WT_Left
WT_Control
Bone Marrow
%AnnexinV+ 7AAD+
80
60
40
20
0
WT_Right
WT_Left
β2m_Right
β2m_Left
β2m_Right
WT_Left
WT_Control

Figure 32. NK depletion in MHC I-disparate parabiotic mice.

Age-matched, 9 – 12 week-old female WT mice were parabiosed to $\beta_2m^{-/-}$ mice, or WT (as control). Mice in the NK-depleted group were injected i.p. with anti-NK1.1 monoclonal antibody (PK136) at day -1 pre-SX and at regular intervals (every 3-4d) until sacrifice. Splenic cellularity of parabionts was calculated at harvest, 2w post-SX. Data are pooled from multiple experiments; each data point represents the spleen from one mouse.

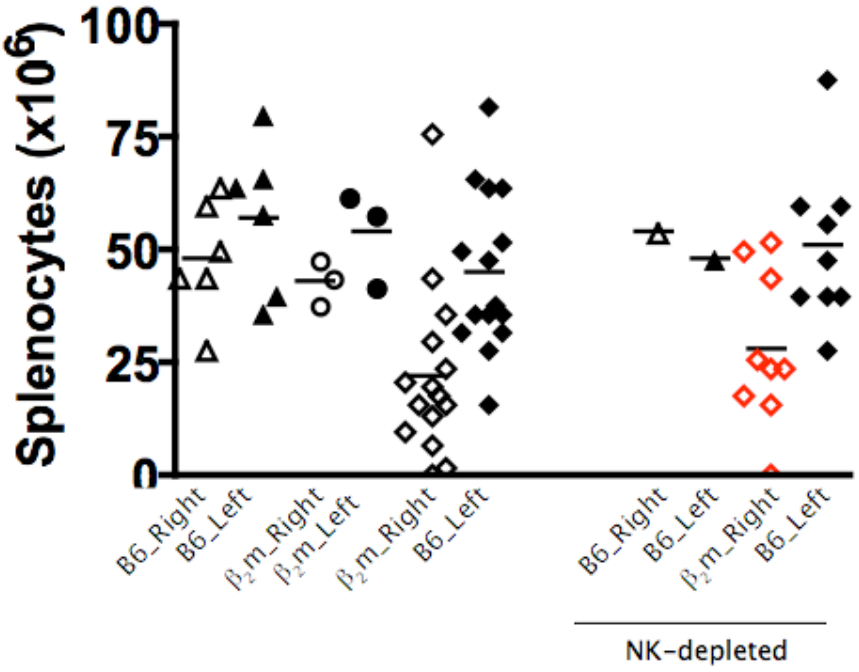

Splenocytes (x10^6)
100
75
50
25
0
B6_Right
B6_Left
β2m_Right
β2m_Left
β2m_Right
B6_Left
B6_Right
B6_Left
β2m_Right
B6_Left
NK-depleted

Figure 33. Parabiosis of MHC I-deficient mice to partners with various genetic deficiencies to reveal mechanism of splenic atrophy.

Age-matched, female $\beta_2m^{-/-}$ mice were parabiosed to partners genetically deficient in various immune cell lineages and functions, including **(a)** NK-deficient **(**NKD), **(b)** T and B cell deficient ($Rag1^{-/-}$), and **(c)** cytotoxicity-deficient (Jinx). Splenic cellularity was determined 2w post-SX. Splenic cellularity from $\beta_2m^{-/-}$:WT pairs (obtained in separate experiments) is included for reference.

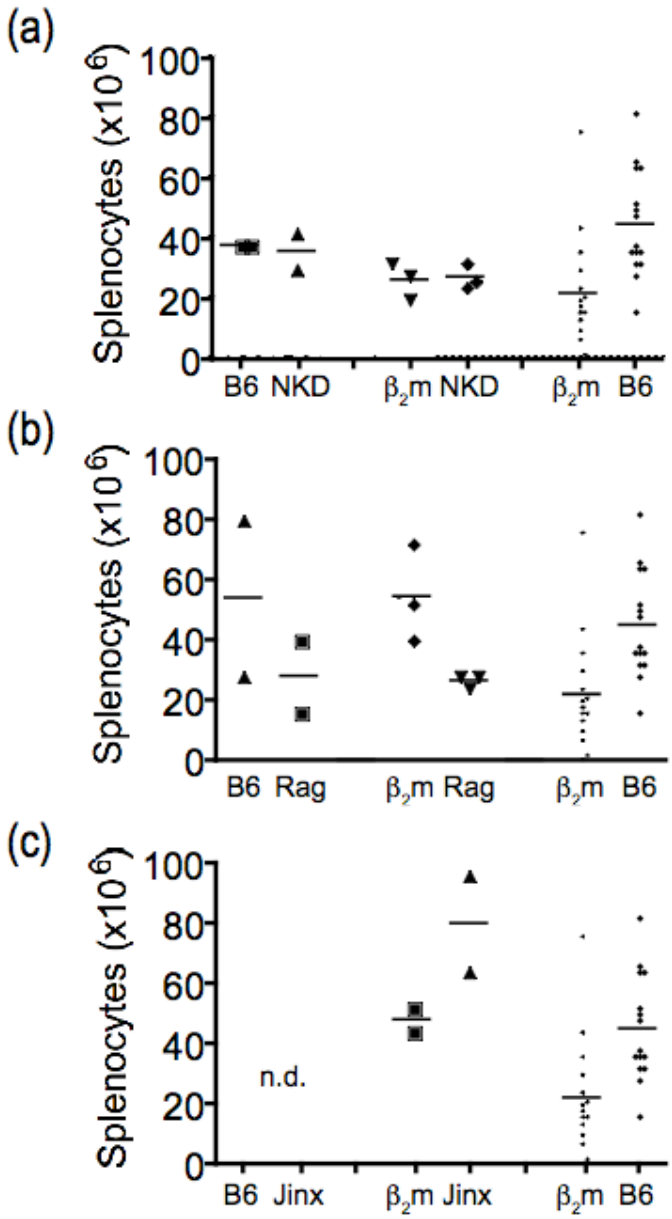
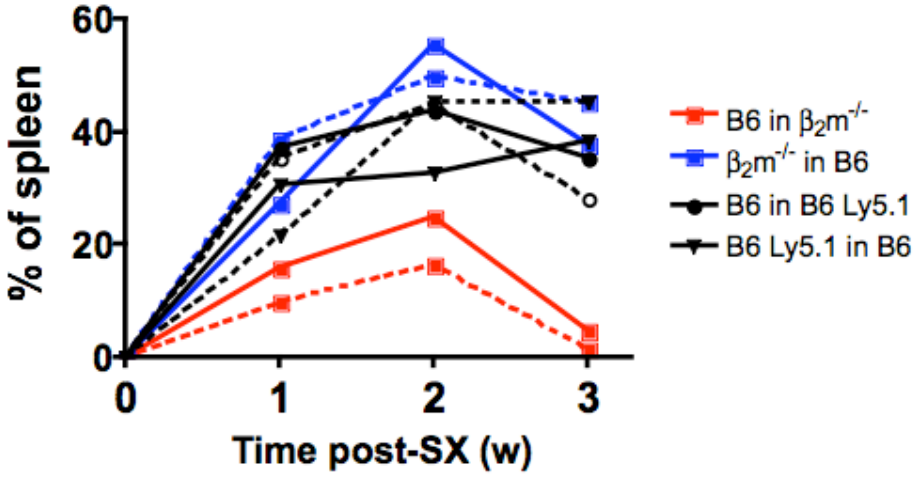

(a)
Splenocytes (x10^6)
100
80
60
40
20
0
B6
NKD
β2m
NKD
β2m
B6
(b)
Splenocytes (x10^6)
100
80
60
40
20
0
B6
Rag
β2m
Rag
β2m
B6
(c)
Splenocytes (x10^6)
100
80
60
40
20
0
n.d.
B6
Jinx
β2m
Jinx
β2m
B6

Figure 34. Unequal splenic chimerism in MHC I-disparate parabionts.

Parabiotic pairs of $\beta_2m^{-/-}$:WT Ly5.1 and WT:WT Ly5.1 were generated using age-matched female mice. Splenic chimerism was determined by staining splenocytes for the differential CD45 allele. **(a)** Total lymphocytic chimerism (solid lines), and NK-specific chimerism, at times 1 – 3w post-SX**.** Data are pooled from multiple experiments, and each data point represents an average with 2-8 mice/point. **(b)** Individual representation of NK-specific splenic chimerism at 2w post-SX. Each dot represents an individual mouse. Data are pooled from 2 independent experiments.

(a)


60
40
20
0
% of spleen
0
1
2
3
Time post-SX (w)
B6 in β2m-/-
β2m-/- in B6
B6 in B6 Ly5.1
B6 Ly5.1 in B6

(b)

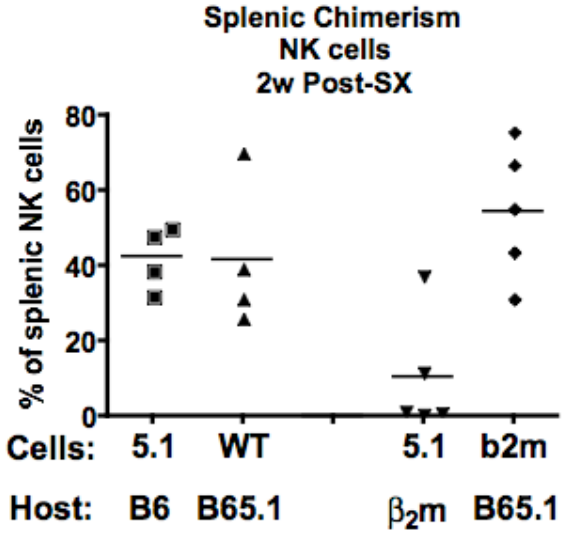

Splenic Chimerism
NK cells
2w Post-SX
% of splenic NK cells
80
60
40
20
0
Cells: 5.1 WT 5.1 b2m
Host: B6 B65.1 β2m B65.1

Figure 35. Differential NK chimerism of blood but not bone marrow in MHC I-disparate parabionts at 2w post-SX.

Parabiotic pairs of $\beta_2m^{-/-}$:WT Ly5.1 and WT:WT Ly5.1 were generated using age-matched female mice. Blood and marrow chimerism were determined by staining splenocytes for the differential CD45 allele, followed by flow cytometry. **(a)** Total lymphocytic chimerism of peripheral blood (obtained by cardiac puncture)**. (b)** NK-specific chimerism of peripheral blood. **(c)** Percentage of splenocytes identified as NK cells (CD3-NK1.1+). **(d)** Percentage of lymphocytes from peripheral blood identified as NK cells (CD3-NK1.1+). **(e)** Total lymphocytic chimerism of bone marrow. **(e)** Percentage of bone marrow identified as NK cells (CD3-NK1.1+). Data are pooled from multiple experiments; each data point represents an individual mouse.

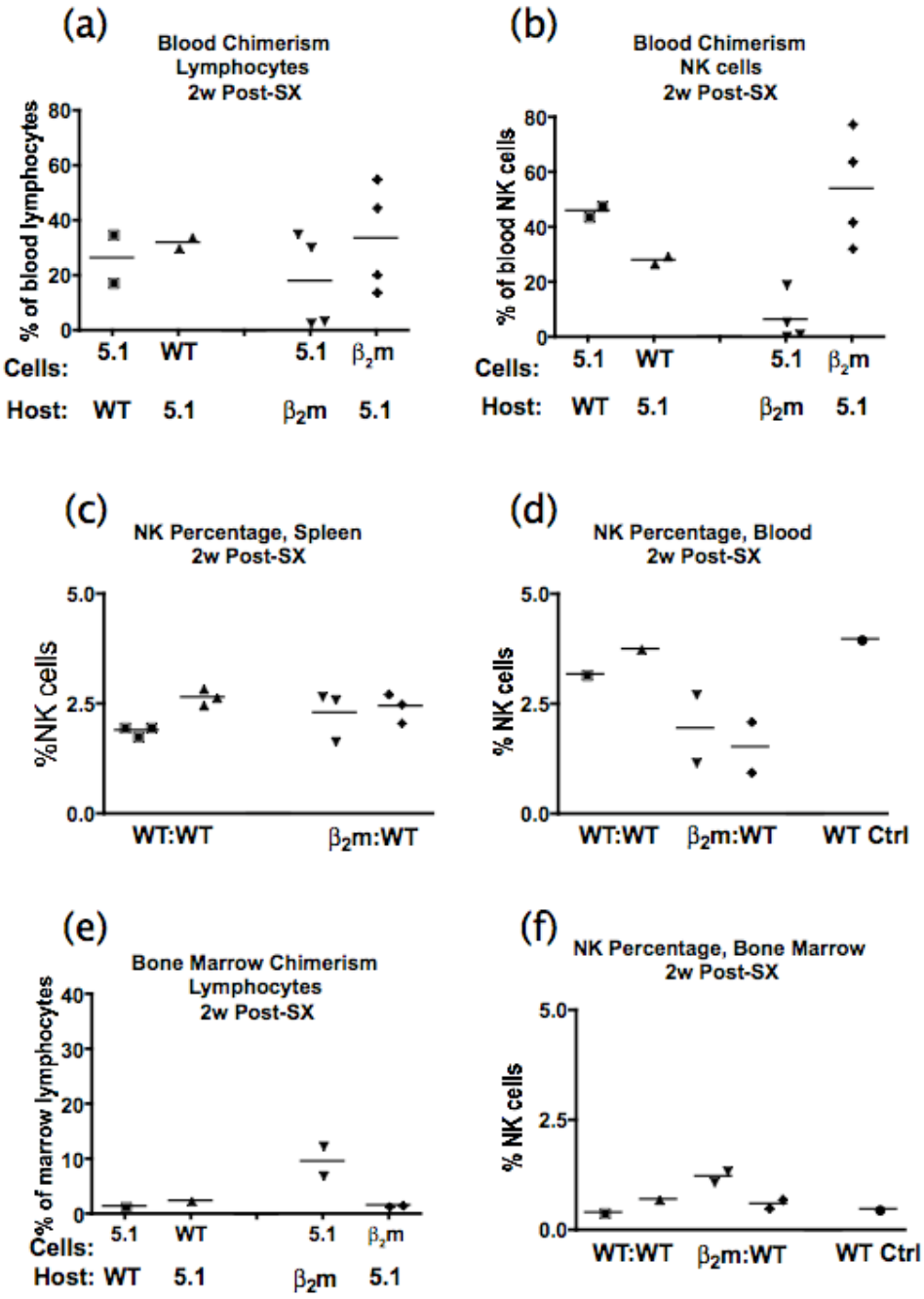
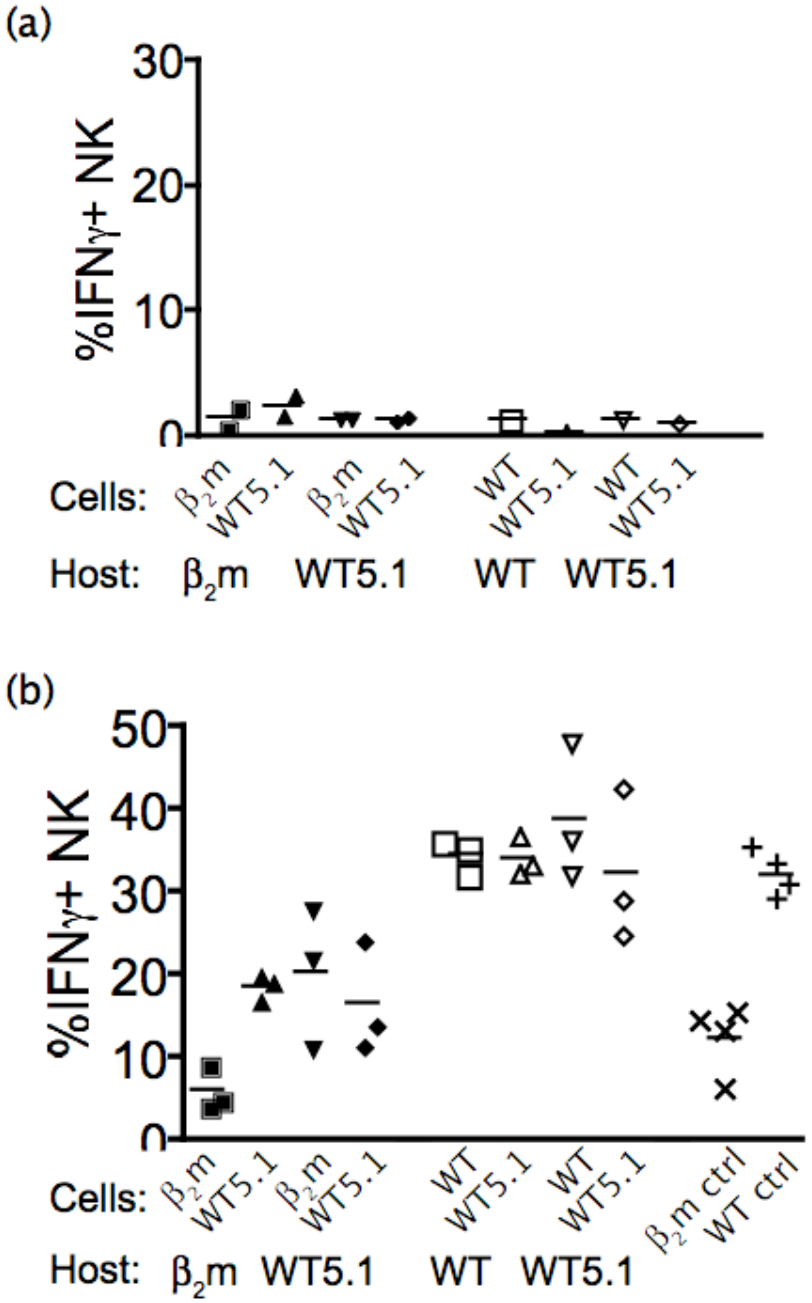

(a)
Blood Chimerism
Lymphocytes
2w Post-SX
% of blood lymphocytes
80
60
40
20
0
Cells: 5.1 WT 5.1 β2m
Host: WT 5.1 β2m 5.1
(b)
Blood Chimerism
NK cells
2w Post-SX
% of blood NK cells
80
60
40
20
0
Cells: 5.1 WT 5.1 β2m
Host: WT 5.1 β2m 5.1
(c)
NK Percentage, Spleen
2w Post-SX
%NK cells
5.0
2.5
0.0
WT:WT
β2m:WT
(d)
NK Percentage, Blood
2w Post-SX
% NK cells
5.0
2.5
0.0
WT:WT
β2m:WT
WT Ctrl
(e)
Bone Marrow Chimerism
Lymphocytes
2w Post-SX
% of marrow lymphocytes
40
30
20
10
0
Cells: 5.1 WT 5.1 β2m
Host: WT 5.1 β2m 5.1
(f)
NK Percentage, Bone Marrow
2w Post-SX
% NK cells
5.0
2.5
0.0
WT:WT
β2m:WT
WT Ctrl

Figure 36. NK cell licensing in parabionts.

Parabiotic pairs of $\beta_2m^{-/-}$:WT Ly5.1 and WT:WT Ly5.1 were generated using age-matched female mice. At 2w post-SX, spleens were harvested and NK cells stained for **(a)** spontaneous production of IFNγ and **(b)** production of IFNγ following NK1.1 crosslinking. Data are from one experiment (a) or pooled from 2 independent experiments (b). Each point represents one mouse.


(a)
30
20
10
0
%IFNγ+ NK
Cells:
β2m
WT5.1
β2m
WT5.1
WT
WT5.1
WT
WT5.1
Host:
β2m
WT5.1
WT
WT5.1
(b)
50
40
30
20
10
0
%IFNγ+ NK
Cells:
β2m
WT5.1
β2m
WT5.1
WT
WT5.1
WT
WT5.1
β2m ctrl
WT ctrl
Host:
β2m
WT5.1
WT
WT5.1

CHAPTER 7:

Discussion

Major findings

In this thesis work, we present several novel and interesting findings, which greatly advance our understanding of fundamental NK cell biology. The first of these findings is that MHC I-deficient NK cells can acquire normal function given access to MHC I, in a process which appears to be licensing. The capacity of MHC I-deficient NK cells to be influenced by a change in their environment disputes the idea that the signal responsible for licensing is cell-intrinsic, as has been previously proposed [74]. We conclude that the licensing signal is most likely not delivered in *cis*, and that engagement of NK inhibitory receptors by MHC I ligands presented in *trans* is sufficient to induce the licensed phenotype. Additionally, we find that there does not appear to be a critical window during development in which licensing must occur, perhaps further permitting the separation of licensing from the process of development.

The second major finding of this work is that WT NK cells can be made hyporesponsive or 'unlicensed' following their transfer to environments devoid of MHC I expression. These results reveal that the licensed phenotype is not fixed, and further support the idea that licensing is dominated by contacts with MHC I in *trans*, as *cis* interactions alone are insufficient to maintain the licensed phenotype.

Results from mixed bone marrow chimeras as well as from parabionts differing in MHC I expression reveal the potential for an intermediate functional phenotype of the NK cell population, refuting the longstanding theory of 'dominant tolerance' [56, 136]. Instead, we propose that all NK cells, regardless of intrinsic MHC I expression, are equally

sensitive to the amount of environmental MHC, and that these are the signals which regulate and maintain the responsiveness, or licensing, of an NK cell population.

Advancements in theories on the mechanism of licensing

While much progress has been made in understanding the role of MHC-specific receptors in NK cell education, there remain many unanswered questions. Primarily, the exact molecular mechanisms dictating licensing, including the identities of intracellular signaling molecules and their targets, remain unknown. We believe that our data may provide additional insight into the accuracy of some of the current theories regarding the mechanism of NK cell licensing.

Receptor 'tuning'

In the theory of 'receptor tuning', it is postulated that NK cell responsiveness is influenced by the number of inhibitory receptors present at the NK cell surface (or by the access of these receptors to their ligands) [46, 48]. Following contact with an MHC I ligand, the surface expression level of inhibitory receptors is decreased (possibly via internalization). Expression of fewer inhibitory receptors translates to a decrease in the capacity to be inhibited, thus allowing NK cells to be more easily activated. This is the reasoning presented to explain the increased functionality (licensing) of NK cells from MHC I-expressing hosts. Incidentally, some researchers proposing that licensing occurs in the *cis* orientation have also supported the receptor tuning hypothesis. However, in their model the decreased MFI is explained by interactions between inhibitory NK cell receptors and their MHC I ligands, in *cis*. In any case, sequestration of inhibitory

receptors serves the same function as internalization would, which is to limit the capacity of receptors to contact their ligands, thereby limiting transmission of inhibitory signals and allowing for easier activation.

Receptor tuning as the mechanism for NK cell licensing is an attractive theory for several reasons: experimentally, hypofunctional NK cells from MHC I-deficient hosts have been demonstrated to have high inhibitory receptor expression, while inhibitory receptor expression is documented to be lower on NK cells from hosts with expression of MHC I ligand, where NK cells are functional. Therefore, inhibitory receptor expression and NK cell functionality appear to be related. We reproduce these findings in our experiments, with the additional finding that gain of function in MHC I-deficient NK cells is accompanied by a corresponding decrease in cognate inhibitory receptor expression, again seeming to support the hypothesis that inhibitory receptor expression is related to functionality. Furthermore, in our *in vitro* experiments, simple mixing and co-culture of MHC I-deficient cells with WT cells was not shown to result in inhibitory receptor downregulation on MHC I-deficient cells, and these cells did not gain functional capacity, implying that a decrease in inhibitory receptor expression is necessary to acquire the gain in function.

The theory of receptor tuning is also appealing as an explanation for the mechanism underpinning NK cell licensing, because it is allows for the reconciliation of inhibitory receptor function during licensing with traditional inhibitory receptor function. If signaling through the inhibitory receptor serves to remove it from the cell surface (or

sequester it), repeated contact with self MHC I ligands results in a net *gain* in NK cell functionality, through removal of a negative signal or influence (i.e., the double negative 'minus a minus' results in a positive outcome).

Although data exist to support receptor tuning as the mechanism for NK cell licensing, other data, including results of our own experiments, challenge this theory. The primary experimental piece of evidence that does not appear to completely agree with the theory of receptor tuning concerns inhibitory receptor expression on WT NK cells, following their transfer to MHC I-deficient hosts. In these studies, we noted that although WT cells lost functional capacity after transfer to MHC I-deficient hosts, their surface expression of inhibitory receptors only partially increased, to a level less than that expressed by MHC I-deficient NK cells. These results may be interpreted to mean: 1) the level of inhibitory receptor expression on MHC I-deficient NK cells is higher than the threshold necessary to interfere with activating signaling, or induce hyporesponsiveness, and/or, 2) another mechanism exists to induce hyporesponsiveness in WT NK cells, one which may be complementary to partial inhibitory receptor upregulation, or one which is unrelated; 3) the changes in inhibitory receptor expression and NK cell functional capacity are true, true, and unrelated.

To further explore the possibility of receptor tuning as a mechanism for NK cell licensing, I propose the creation of a mutant inhibitory receptor that cannot be internalized. This receptor cannot simply be a tailless mutant, as such a mutant would also lack the ITIM sequence, which has been demonstrated to be necessary for licensing

[41]. Instead, the receptor should be a deletion mutant, lacking other portions of the intracellular tail, or a mutant with sequence variation at regions outside of the ITIM. Our *in vitro* experiments indicate that, for unknown reasons, contact with MHC I in co-culture alone is insufficient for inhibitory receptor downregulation. Therefore, to test the ability of mutant receptors to downregulate following contact with MHC I, the mutants could be transfected into NK cells *in vitro*, then transferred to MHC I-expressing hosts. (As primary NK cells exhibit low transfection efficiency, a more realistic experiment would involve transfection into bone marrow and analysis of NK cells allowed to develop in bone marrow chimeras reconstituted with transfected bone marrow). Once a deficiency in internalization has been confirmed, the licensing status of these NK cells could be examined, in order to directly test the hypothesis that inhibitory receptor internalization is required to achieve licensing.

In a final thought on receptor tuning as a mechanism for NK cell licensing, semantically, one must propose that unengaged receptors signal, or otherwise interfere with positive signaling. Otherwise, the high level of inhibitory receptor expression on MHC I-deficient NK cells would be of no consequence. In the next section, we will explore this idea in further detail, as we consider a second mechanism proposed to explain licensing.

Spatial reorganization of NK cell receptors

Licensing appears to alleviate repression of signaling through multiple activating receptors, which utilize different signaling chains (i.e. NK1.1/$F_CR\gamma$ and Ly49H/Dap12, data presented in Chapter 3). This suggests that licensing may serve to act on a common

mechanism shared by all receptors, or that it operates at a point downstream where signaling for multiple activating receptors converges. However, although much effort has been expended, differential transcription of licensed and unlicensed cells has not been demonstrated, suggesting that licensing is not accomplished by transcriptional regulation of downstream signaling components, including kinases, phosphatases and transcription factors. This begs the question, "Is there a mechanism to generate disparity between two identical sets of items?" The answer is, yes: differential spatial organization.

Recent work on NK cell inhibition (mostly by the Long group), demonstrates that inhibition likely does not function by a one-to-one dephosphorylation of activation receptors, but instead is accomplished through dephosphorylation of cytoskeletal components, necessary to reorganize and cluster activating receptors [26]. Theoretically, clustered activating receptors have more power to activate, due to factors such as transactivation and increased kinetic potential through greater probability of contact with downstream components, due to decreased travel through space. So, if inhibition works by preventing cytoskeletal rearrangements, perhaps licensing works by interfering with this inhibitory mechanism, and/or encouraging clustering of activating receptors, through a mechanism of exclusion. It is possible that transmembrane inhibitory receptors may achieve high density upon MHC I engagement, forcing activating receptors out, and together. Interestingly, this theory fits with the idea of receptor calibration by promoting a function for the presence of inhibitory receptors at the cell surface, without requiring these receptors to signal in the absence of ligand engagement. Recent literature reports describe what appears to be differential clustering of inhibitory and activating receptors at

the surface of licensed versus unlicensed NK cells [137], further lending credit to this theory as the mechanism by which NK cell licensing is achieved. Certainly, advances in this field of investigation will prove interesting.

Physiological role of licensing

While results from this thesis work further define parameters surrounding acquisition and retention of NK cell licensing, questions remain regarding its physiological function. Certainly it may be argued that the establishment of self-tolerance is a beneficial outcome of NK cell licensing, particularly in an immune lineage that does not appear to undergo deletion or receptor editing. However, recent publications challenge the notion that licensing is advantageous, citing the finding that licensed NK cells proliferate less and offer less protection in the context of viral infection [138].

We hypothesize that restraint in activation, a consequence of NK cell licensing, may better serve a real (non-experimental) host, where multiple pathogenic insults are encountered sequentially. If following infection there truly is a refractory period, perhaps the time required for generation of new NK cells, limits on NK cell activation imposed by licensing may prevent the induction of a temporary immunodeficiency. The idea that immunodeficiency arises following activation of the entire NK cell population may be experimentally tested: one could infect Ly49H Tg mice with MCMV and, at the phase in which activated NK cells contract, infect the mice with another pathogen (or conduct a tumor elimination challenge). It is likely that the previously infected Ly49H Tg mice will not fare well in the second challenge, compared to Tg mice not receiving a primary infection. Regardless of potential liabilities NK cell licensing may impart in the context

of infection, its beneficial functions of self-tolerance and self-recognition for the elimination of unhealthy cells are clearly recognized.

Clinical relevance and applications

Our data indicate that NK cell function with respect to host MHC environment is more plastic than originally anticipated, as *in vivo* exposure of a peripheral cell to the presence of an inhibitory ligand can alter the functional capacity of the NK cell. As such, these results may have important clinical implications. In particular, patients undergoing BM transplantation experience greater donor-mediated anti-leukemic effect when donor NK cells express KIRs specific for HLA lacking in the patient (reviewed in [108]). Therefore, within the context of the transfer of peripheral NK cells, such as NK immunotherapy, donor NK cells unlicensed by HLA alleles absent in the donor may become licensed by host HLA alleles, leading to a beneficial gain in reactivity of donor NK cells against host tumors lacking HLA expression. Perhaps prospective pairing of donor KIR along with donor and host HLA to anticipate gain of function by otherwise unlicensed donor NK cells may enhance patient outcomes.

Additionally, our finding that NK cell licensing can be compromised following loss of contact with MHC I molecules holds potential for the use of NK cells in therapies to treat solid tumors. In our current understanding, NK cells have not been demonstrated to be particularly efficient at solid tumor elimination [139]. It is known that this is, at least in part, due to downregulation of activating receptors (particularly NKG2D), following contact with soluble activating ligands, shed from the tumor surface [140]. In addition to

ligand shedding as an immunoevasive strategy, tumors also demonstrate decreased expression of MHC I molecules. Perhaps NK cells are also rendered non-responsive to solid tumors because they become unlicensed in the local tumor microenvironment. Attempts to reestablish licensing could involve injection of single-chain MHC I molecules or cells transfected to express high levels of surface MHC I directly into the tumor. It is possible that the delivery of exogenous MHC I into the tumor may bolster NK cell anti-tumor activity to a level that would provide clinical benefit. Preliminary experiments are warranted.

Concluding remarks

Our data provide new insight into the flexibility of the licensing process, as we have shown that mature natural killer cells have the ability to become both licensed and unlicensed, depending on the expression of MHC I molecules in the host environment. Data demonstrating that expression of MHC I is required on hematopoietic and non-hematopoietic cells, but not NK cells themselves, to produce and maintain the licensed phenotype reveal the correct physical orientation of the contact between these receptors and ligands during the licensing process. The findings of differential chimerism and phenotype in parabiosis of MHC I-deficient mice to WT partners suggest the presence of unappreciated factors that contribute to the maintenance of NK cell licensing, *in vivo*. Finally, we suggest that our results may translate to improvements in human NK cell therapies, particularly those for the treatment of hematological malignancies, with possibilities extending to treatment of solid tumors, as well.

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