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Responsiveness and Tolerance of Natural Killer Cells

by

Nataliya Shifrin

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor David H. Raulet, Chair
Professor Gregory Barton
Professor Russell Vance
Professor Richard Stephens
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Abstract

Tolerance and responsiveness of Natural Killer Cells

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Nataliya Shifrin

Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor David H. Raulet, Chair

A major role of natural killer cells is distinguishing between "self" and "non-self". This is accomplished through their ability to recognize MHC class I molecules and eliminate cells that have lost MHC I expression, a common feature of cancers and virus-infected cells. The interaction between NK receptors and MHC I on another cell is usually inhibitory and when MHC I is absent from target cells inhibition is relieved, leading to killing by NK cells (in a context of activating ligand expression). A subset of NK cells in wild-type mice does not express any MHC I specific inhibitory receptors and NK cells in MHC I-deficient (e.g. β2-microglobulin knockout) animals are never exposed to inhibition through MHC I. In both cases the NK cells do not attack MHC I deficient cells that are otherwise normal and exhibit a hyporesponsive phenotype when stimulated through their activating receptors, as evidenced by lack of inflammatory cytokine production. Work presented in this dissertation addresses the relationship of hyporesponsiveness and self-tolerance and demonstrates, for the first time, that the two phenomena are distinguishable. Additionally, the plasticity of NK cell education, the roles of hematopoietic vs. non-hematopoietic cells in this process, and finally the effects of inflammation on NK cell tolerance are also investigated.

In order to investigate whether responsiveness of NK cells is set once during their development or can be changed in mature NK cells based on their environment we employed an adoptive transfer model. We transferred splenocytes containing mature NK cells from WT mice into *β2m*-deficient mice, or vice-versa. 8 to 10 days after transfer we assessed the responsiveness of the transferred NK cells to activating receptor crosslinking, as well as the capacity of the cells to reject grafts of *β2m*$^{-/-}$ spleen cells. We found that upon transfer into *β2m* knockout hosts WT NK cells reset their responsiveness downward and lost the capacity to reject MHC I – negative grafts. Conversely, when *β2m*-deficient NK cells were transferred to MHC I – expressing animals their responsiveness was reset upwards. Interestingly – despite increased responsiveness – these NK cells did not acquire the ability to reject *β2m* – deficient grafts.

To address the roles or hematopoietic vs. non-hematopoietic cells in NK cell education we generated fetal liver chimeras by reconstituting WT or *β2m*$^{-/-}$ hosts with WT, *β2m*$^{-/-}$, or a 1:1 mixture of WT and *β2m*$^{-/-}$ fetal liver cells. We found that NK cells that developed in an MHC I-expressing host acquired normal responsiveness whereas those that developed in an MHC I –

negative host were hyporesponsive. Interestingly, however, NK cells that developed in the presence of *β2m* – deficient cells, of either non-hematopoietic or hematopoietic origin, acquired tolerance to *β2m*$^{-/-}$ grafts. Therefore, our data show that responsiveness of natural killer cells is mainly set by non-hematopoietic cells, while tolerance can be imposed either by hematopoietic or non-hematopoietic cells. Thus our data show for the first time that responsiveness of NK cells can be (partially) dissociated from tolerance of NK cells. These findings, taken together, lead to the proposal that two mechanisms operate to ensure NK cell self-tolerance, which are imposed by different cell types *in vivo*.

Finally, we investigated whether inflammation can affect the ability of NK cells to respond to "missing self". We infected mixed fetal liver chimeras with mouse cytomegalovirus and monitored the maintenance of *β2m*$^{-/-}$ donor cells. We found that *β2m*$^{-/-}$ donor cells were selectively rejected when the infected host was MHC I – sufficient, whereas no rejection was observed when the host was MHC I-deficient. These data indicate that different mechanisms of self tolerance are differentially regulated by inflammation, and that the form associated with hyporesponsiveness is more stable in the face of inflammation associated with infections.

Altogether, the data presented in this dissertation demonstrate that different interacting cell types play different roles in the process of NK cell education, with non-hematopoietic cells being especially critical for regulating the sensitivity of activating NK receptors. The data also show, however, that under steady state conditions (no infection) responsiveness of mature NK cells can be re-set upward or downward depending on corresponding changes in their MHC environment. This dissertation provides the first evidence that tolerance to MHC I – deficient cells can be decoupled from hyporesponsiveness. Finally, we show that infection can break tolerance of NK cells towards MHC I – negative grafts only a setting where it is accompanied by high responsiveness, whereas tolerance that is accompanied by low responsiveness is more stable and cannon be broken as a result of infection. These findings represent a substantial revision of current notions of NK cell self tolerance and hyporesponsiveness.

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"It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness…"

− Charles Dickens, "A Tale of Two Cities"

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Chapter 1: Introduction

The mammalian immune system

The function of the immune system is to protect the individual against disease. To do so the immune system has to possess four main capabilities: immunological recognition, immune effector functions, immune regulation, and immunological memory.

The mammalian immune system is divided into two branches: the innate immune system and the adaptive immune system. The innate immune response is mediated by a variety of cell types, including dendritic cells, macrophages, granulocytes and natural killer cells. This branch of the immune systems distinguishes "normal" vs. "abnormal; or microbial" and executes a rapid response involving secretion of inflammatory cytokines and, in the case of NK cells, killing of unhealthy cells. The innate immune system is also critical for shaping the adaptive immune response, which is executed by T and B cells. The adaptive response is more specific and in some contexts more effective, but takes longer to develop (days, vs. hours for innate immune responses). Antibodies produced by B cells persist after elimination of the pathogen and can prevent re-infection. Additionally, T and B memory cells can respond with sufficient rapidity to a re-infection to eliminate the pathogen before a full-blown infection is established.

Natural killer (NK) cells

NK cells develop in the bone marrow from the common lymphoid progenitor. Unlike T- and B-lymphocytes, however, they do not depend on the VDJ recombinase enzyme RAG for their development. Presumably, therefore, the receptors expressed by these cells are not highly diversified. Functionally, they have been defined as cells that kill target cells without a need for prior immunization, but subsequent studies have shown that they exhibit low killing activity unless pre-activated briefly with cytokines or inflammatory stimuli. NK cells kill by the same killing mechanisms as CD8 T cells do: they release cytotoxic granules onto the target cell that contain proteins that permeabilize the membrane, allowing entry of apoptosis-inducing effector proteins; and they express death receptor ligands such as TRAIL, which can engage TRAIL-ligands on the surface of target cells and initiate target cell apoptosis.

Natural Killer cell activation

NK cell activation is governed by the net balance between activating and inhibitory signals (Lanier 1998; Diefenbach and Raulet 2001; Moretta, Bottino et al. 2001). Most activating signals are provided though interactions of stimulatory receptors on NK cells with various ligands. In one documented case, the ligand is a virus-encoded protein (Arase, Mocarski et al. 2002). Some of the ligands are self-ligands, encoded in the animals own genome, that are upregulated due to a variety of "stresses" such as infection or DNA damage (Gasser, Orsulic et al. 2005). Inhibitory

signals arise primarily from the interaction of inhibitory NK receptors with MHC class I molecules on target cells.

NK cell receptors

Activating receptors: The best-characterized activating NK cell receptor is NKG2D. NKG2D recognizes "induced-self" ligands – molecules that are not expressed or are expressed at low levels on most normal cells and upregulated on unhealthy cells due to pathways activated in malignant, infected or abnormal cells (Raulet 2003; Lopez-Larrea, Suarez-Alvarez et al. 2008). NKG2D ligands include MICA/B and ULBP1-6 in humans, and MULT1, RAE-1a-e (five variants), and H60a-c (three variants) in mice (Raulet, Gasser et al. 2013). Another important NK cell stimulatory receptor is the Fc receptor FcγR, which triggers antibody-dependent cell cytotoxicity (ADCC) when it binds IgG bound to antigens on the surfaces of other cells. Some activating receptors are specific for MHC I molecules (though the interaction of MHC I molecules with activating receptors is usually of lower affinity than the interaction with the inhibitory counterparts (Vales-Gomez, Reyburn et al. 1998; Vales-Gomez, Reyburn et al. 1999). Numerous other activating receptors exist and have been implicated in host defense, but the corresponding ligands remain to be clearly defined. Among these are NKp46 and NKR-P1C. Activating receptors usually associate in the membrane of the killer cell with signaling adaptor molecules, including DAP10, DAP12, CD3ζ, and FcRγ, which transmit activation signals through a series of molecular recruitment and phosphorylation events (Wirthmueller, Kurosaki et al. 1992; Lanier 1998; Wu, Song et al. 1999).

Recently the SLAM family of activating receptors has been shown to be important in the recognition of hematopoietic cells by NK cells (Dong, Cruz-Munoz et al. 2009). The SLAM receptors are type I transmembrane proteins. The receptor family consists of SLAM, CD48, Ly9, 2B4, CD84, Ly108, and CRACC. They are broadly expressed on various hematopoietic cell types and are not found on most other cell types. All SLAM family receptors except SLAM are expressed by NK cells. Interestingly, most of these receptors make homotypic interactions with the same SLAM family member on other cells, with the exception being the heterotypic interaction between 2B4 and CD48. The SLAM receptors associate with signaling adapter molecules of the SAP family, which include three members: SAP, EAT-2, and ERT. SAP and EAT-2 are expressed in a variety of immune cells, including NK cells, while ERT is found exclusively in NK cells (at least in the mouse) (Veillette 2010). Mice deficient in all three SAP family adaptors are nearly devoid of the capacity to mediate cytotoxicity or produce IFN-γ in response to simulation with hematopoietic target cells, including hematopoietic tumor cells. In contrast, they continue to react against non-hematopoietic target cells, and this latter reaction is even amplified, for unknown reasons, compared to the reactivity of wild type NK cells (Dong, Cruz-Munoz et al. 2009).

NKp46 is an activating receptor on NK cells encoded by the *Ncr1* gene. It is conserved among mammals and expressed on all mature NK cells (Walzer, Blery et al. 2007). The only reported ligand for NKp46 is hemagglutinin from influenza virus (Mandelboim, Lieberman et al. 2001). It has been proposed that NKp46 plays a role in tuning the responsiveness of NK cells. Interestingly, NK cells carrying an ENU-induced mutation in the *Ncr1* gene were shown to be hyper-responsive to stimulation through other activating receptors, and mice with this mutation were more resistant than their wild-type littermates to MCMV infection (Narni-Mancinelli,

Jaeger et al. 2012). It was proposed that an endogenous ligand for NKp46 in wild type mice stimulates NK cells persistently and causes partial desensitization of the cells, which is relieved in NKp46-mutant mice. Surprisingly, however, a recent study showed that NK cells in NKp46 knockout mice are not hyper-responsive, suggesting that the hyper-responsive phenotype may reflect a dominant function of the ENU-mutant NKp46 (Sheppard, Triulzi et al. 2013).

Inhibitory receptors: NK cells are inhibited by MHC I molecules expressed by target cells. In mice, the Ly49 receptors recognize MHC class Ia molecules directly. Surprisingly, humans have only a single, nonfunctional, Ly49 gene; instead MHC class Ia recognition is performed by a distinct set of receptors called killer cell immunoglobulin-like receptors (KIRs), which are not present in mice. Both mice and humans express a functional CD94/NKG2A dimeric receptor that recognizes a complex of a peptide derived from the class Ia leader sequence presented by a specific nonclassical MHC I molecule (HLA-E in humans and Qa-1 in mice) (Lee, Llano et al. 1998; Vance, Kraft et al. 1998). Inhibitory receptors signal though immunoreceptor-tyrosine based inhibitory motifs (ITIMs) in their cytoplasmic tails. Upon receptor engagement, ITIMs are tyrosine-phosphorylated and recruit protein tyrosine phosphatases. The phosphatases are believed to inhibit activation by dephosphorylating one or more critical signaling molecules, including Vav1(Binstadt, Brumbaugh et al. 1996; Burshtyn, Scharenberg et al. 1996; Campbell, Dessing et al. 1996; Olcese, Lang et al. 1996).

In the B6 mouse strain there are 3 self-MHC I-specific inhibitory receptors: Ly49I and Ly49C both recognize H-2K^b, whereas the CD94/NKG2A dimer recognizes the leader peptide of H-2D^b presented on the non-classical MHC I molecule Qa1. The acquisition of the inhibitory receptors is stochastic. As a result, each NK cell can express many combinations of the available inhibitory receptors. Considering the self-MHC specific inhibitory receptors in B6 mice, it has been shown that each NK cell can express between 0 and 3 of the three self MHC-specific inhibitory receptors (Joncker, Fernandez et al. 2009). Approximately 85% of NK cells in B6 express at least one these receptors, but 15% express none of them (Fernandez, Treiner et al. 2005).

NK cell activating cytokines

In addition to stimulatory receptors, cytokines are major regulators of NK cells responses. IL-2 promotes proliferation of NK cells, cytotoxicity, and cytokine secretion (Trinchieri 1989). IL-15 is required for generation and survival of NK cells (Kennedy, Glaccum et al. 2000) and its production and presentation by DCs is important for priming NK cell effector functions (Koka, Burkett et al. 2004). IL-12 and IL-18 are NK activating cytokines, and they can reportedly provide a “co-stimulatory” signal that, when combined with stimulatory receptor engagement, can override inhibitory receptor signals and cause increased expression of perforin and enhanced cytokine production by NK cells (Hyodo, Matsui et al. 1999; Ortaldo and Young 2003; Ortaldo, Winkler-Pickett et al. 2006). IL-21 has been demonstrated to induce functional maturation of NK cells and stimulate cytotoxicity and cytokine secretion (Brady, Hayakawa et al. 2006). IFNα/β, cytokines secreted by DCs in early response to infections, enhance NK cytotoxicity and proliferation (Biron, Cousens et al. 1998), in part by inducing production of IL-15 and in part by acting directly on the NK cells (Martinez, Huang et al. 2008; Mack, Kallal et al. 2011).

Functions of NK cells

Natural killer cells are important in controlling infections and eliminating transformed cells. Upon activation NK cells produce cytokines, including IFN-γ and TNF-α, and can directly lyse infected or transformed cells in a perforin and granzyme – dependent manner (Smyth, Cretney et al. 2005).

NK cells in viral infections

Multiple studies suggest that NK cells can be directly activated during viral infections. In addition to the interaction between Ly49H on NK cells and the MCMV protein m157 on infected cells, which is discussed in more detail below, influenza hemagglutinin, according to one study, binds NKp46 on human cells and induces cytotoxicity (Mandelboim, Lieberman et al. 2001). Envelope proteins of West Nile virus and Dengue virus bind to NKp44 (Hershkovitz, Rosental et al. 2009). Engagement of Ly49P by H-2D^k on MCMV infected cells induces secretion of IFN-γ, which is dependent on the viral m04 gene (Kielczewska, Pyzik et al. 2009). Responses to and early control of dissemination of ectromelia virus infections is reportedly dependent on engagement of NKG2D and CD94/NKG2E on NK cells by NKG2D ligands and Qa-1^b, respectively, on the surface of infected cells (Fang, Lanier et al. 2008; Fang, Orr et al. 2011). Cytotoxicity of vaccinia virus infected cells by human NK cells is reportedly regulated by interactions with NKp30, NKp44, and NKp46 activating receptors (Chisholm and Reyburn 2006).

Indirect NK cell activation is described for many viruses, including MCMV and HSV-1. It involves secretion of type I interferons and IL-12 from plasmacytoid dendritic cells (Horowitz, Stegmann et al. 2011). Production of IFN-γ by NK cells during viral infection is dependent on IL-12 while IFN-α and IFN-β are critical for cytotoxicity (Steinberg, Eisenacher et al. 2009).

NK cells in MCMV infections

MCMV. Murine cytomegalovirus belongs to the β-Herpesvirinae subfamily of the Herpesviridae family. All CMVs are highly species-specific with tropism for hematopoietic cells, endothelial cells, macrophages, and secretory glands. After the resolution of initial infection, CMVs establish life-long latency.

Pathogenesis of MCMV infection in immunocompetent mice. Pathogenesis of MCMV infection is largely dependent on the virus preparation and the dose used for infection. Infection with tissue culture derived virus is mostly asymptomatic with doses up to 1×10^5 PFU. The same dose of salivary gland-derived virus results in high viral titers in spleen and liver, liver damage (Katzenstein, Yu et al. 1983; Shanley, Biczak et al. 1993) and immunosuppression (Campbell, Slater et al. 1989).

Immune response to MCMV infection. Dendritic cells are critical for initiating the antiviral immune response to MCMV. Early activation of DCs leads to production of the cytokines IFN-α, IL-12, and IL-18, all of which contribute to NK cell activation (Andrews, Andoniou et al. 2005). In B6 mice, CD8α$^+$ DCs are required for the proliferation of the Ly49H$^+$ NK subset that is responsible for restricting early viral replication (Andrews, Scalzo et al. 2003).

NK control of MCMV infection. Natural killer cells are important for controlling MCMV infection in some mouse strains, as demonstrated by an increased susceptibility when NK cells are depleted in B6 mice (Bukowski, Woda et al. 1984). In BALB/c or DBA/2 mice, in contrast, NK cells play no role in controlling the infection (Scalzo, Fitzgerald et al. 1990). NK cell-dependent control of MCMV in B6 mice has been mapped to a single gene, *Ly49h*, which encodes the activating receptor Ly49H. Ly49H recognizes the MCMV-encoded m157 protein on infected cells, and is expressed by ~50% of NK cells (Cheng, French et al. 2008). NK cells control MCMV infection by lysing infected cells as well as secreting cytokines (including TNF-α, IFN-γ, and GM-CSF) and chemokines (including RANTES and MIP1α). Cytokine secretion by NK cells is induced by IFNα/β, IL-12, IL-15, and IL-18 secreted by infected cells, DCs, and macrophages. Cytokines secreted by NK cells may help shape the adaptive immune response, in addition to mediating the noncytolytic control of infection (French and Yokoyama 2003).

NK cells in bacterial, protozoan, and fungal infections

In cases of bacterial infection the activation of NK cells is thought to be primarily indirect and involves accessory cells. This activation usually results from TLR-mediated activation of mDCs and subsequent secretion of IL-12, IL-18, and type I interferons (Newman and Riley 2007). Interestingly, NK cells can also play a regulatory role during bacterial infections. Studies of mice with acute systemic infections with *L. monocytogenes* and *Yersinia pestis* found that NK cells are a source of IL-10. This IL-10 secretion suppresses production of IL-12 by DCs and blocks NK cell activation (Perona-Wright, Mohrs et al. 2009).

Protozoan parasites induce a strong NK cell activation, and rapid secretion of IFN-γ by NK cells is necessary for effective clearance of many protozoan infections (Korbel, Finney et al. 2004).

The role of NK cells in fungal infections has not yet been investigated extensively. Studies of *Aspergillus fumigatus* infection have demonstrated NK cells to be the major producers of IFN-γ in the early stages of infection (Park and Mehrad 2009). NK cells can be activated by *A. fumigatus* hyphae (through a yet-to-be described mechanism) and can kill hyphae in a perforin-dependent manner (Schmidt, Tramsen et al. 2011). Additionally, resistance to *Candida albicans* is correlated with NK cell activation (Algarra, Ortega et al. 2002).

NK cells in tumor surveillance

The role of NK cells in the eradication of tumors is supported by numerous studies in both mice and humans. Multiple studies in the mouse system demonstrated increased growth of

implanted syngeneic tumors in animals that are genetically deficient in NK cells or depleted of these cells by antibody administration (Seaman, Sleisenger et al. 1987; Kim, Iizuka et al. 2000; Smyth, Hayakawa et al. 2002; Wu and Lanier 2003; Hayakawa and Smyth 2006). Treatment of mice with cytokines that have been shown to enhance NK cell activation, including IL-2, IL-12, IL-15, IL-21, and type I IFNs, results in enhanced elimination of implanted tumors, further supporting the role of NK cells in anti-tumor responses (Wu and Lanier 2003; Hayakawa and Smyth 2006). Studies addressing the control of spontaneously arising or carcinogen (such as methylcholanthrene) induced tumors showed that mice that are deficient for effector molecules that are expressed by NK cells exhibit a higher incidence of tumor development (Kaplan, Shankaran et al. 1998; Shankaran, Ikeda et al. 2001).

The strongest evidence for the contribution of NK cells to tumor elimination in humans comes from an 11-year follow-up study that found that low cytotoxic activity of peripheral blood NK cells correlates with an increased risk of cancer (Imai, Matsuyama et al. 2000).

NK memory

Recent studies of MCMV-infected mice point to the existence of immunological memory in the Ly49H+ NK cell population. In this study Ly49H+ NK cells were shown to respond more robustly to a second infection with MCMV compared to naïve NK cells (Sun, Beilke et al. 2009). Other studies have documented NK cells memory in cases of delayed hypersensitivity (O'Leary, Goodarzi et al. 2006) and following priming by TLR-activated DCs and IL-15 (Lucas, Schachterle et al. 2007) as well as of NK cells that were activated by a combination of IL-12, IL-15, and IL-18 (Cooper, Elliott et al. 2009).

Tolerance of NK cells that are not inhibited by self MHC I.

The stochastic nature of inhibitory receptor expression results in a population of mature NK cells that lack self MCH I-specific inhibitory receptors and raises the important question of how such NK cells become self tolerant. The first indication of a solution to this question came from analysis of NK cells that arise in MHC class I deficient mice. Unlike NK cells from wild-type mice, NK cells from MHC I-deficient (e.g. *β2m*-knockout) mice do not attack *β2m*–deficient bone marrow grafts *in vivo* (Bix, Liao et al. 1991) or *in vitro* (Liao, Bix et al. 1991). Interestingly, these NK cells exhibit a lower response to various other stimuli, including tumor cells, antibody-coated target cells (ADCC), activating receptor crosslinking, etc. (Hoglund, Ohlen et al. 1991; Liao, Bix et al. 1991; Dorfman, Zerrahn et al. 1997; Fernandez, Treiner et al. 2005; Kim, Poursine-Laurent et al. 2005).

Later, it became clear that a fraction of NK cells in normal MHC+ mice do not express receptors for self MHC (Fernandez, Treiner et al. 2005). In B6 mice 15% of NK cells lack Ly49I, Ly49C, and NKG2A which are the three receptors that are known to engage the MHC I molecules available in B6 mice. These so called "CIN-" NK cells do not attack normal WT or class I-deficient cells but rather are functionally hyporesponsive, akin to NK cells in class I-deficient mice (Fernandez, Treiner et al. 2005). Hyporesponsiveness was manifested by an inability of the cells to kill MHC I-deficient lymphoblasts *in vitro* or to reject MHC I – deficient bone marrow grafts *in vivo*. In addition, hyporesponsiveness was evidenced by a diminished

ability of these cells to produce IFN-γ or degranulate when the cells were stimulated *ex vivo* with tumor cells or with crosslinking antibodies specific for stimulatory receptors. Therefore, the 15% of NK cells that do not express any self MHC-specific inhibitory receptors in B6 mice, and all NK cells in class I-deficient mice, assume a hyporesponsive phenotype, and are incapable of attacking MHC I-deficient cells.

Various mechanisms have been proposed to account for the low responsiveness of CIN- NK cells and NK cells from *β2m*-deficient animals:

Regulatory cells: The role of T_{regs} in inhibition of autoreactive T cell responses is well established. A number of reports have provided evidence that they can inhibit NK cell responses as well (Ghiringhelli, Menard et al. 2005; Barao, Hanash et al. 2006; Smyth, Teng et al. 2006). However, analysis of natural killer cell responsiveness in $Rag^{-/-}$ mice that lack all B and T cells, including inhibitory T cells, revealed responsive and a hyporesponsive populations of NK cells, suggesting that T_{regs} are not necessary to impart NK cell hyporesponsiveness (Joncker and Raulet 2008). Furthermore, purified NK cells from WT vs. MHC I-deficient mice exhibit the expected difference in ERK phosphorylation following *in vitro* activation (W. Deng, unpublished data), arguing that hypo-responsiveness does not require any non-NK cell type. It has not been excluded that there exists a subset of regulatory NK cells that controls responsiveness of other NK cell populations.

MHC-I independent inhibitory receptors: Another proposal is that the NK cells lacking inhibitory receptors for self-MHC upregulate or express inhibitory receptors for non-MHC ligands. 2B4 is a SLAM-family receptor expressed by both mouse and human NK cells that can either activate or inhibit NK function based on the adaptor it associates with (Kumar and McNerney 2005; Clarkson, Simmonds et al. 2007). Activated 2B4-negative NK cells can kill CD48+ splenocytes, suggesting that 2B4 can mediate an inhibitory effect in NK cells (Lee, McNerney et al. 2004). This led to the hypothesis that 2B4 may be responsible for the lack of reactivity of CIN- and $\beta 2m^{-/-}$ NK cells towards MHC I – deficient targets (McNerney, Guzior et al. 2005). However, 2B4 expression is normal in these NK cells (Fernandez, Treiner et al. 2005) and there is no evidence for altered 2B4 function on these NK cells.

Other inhibitory receptors that have been proposed to play a role in suppressing the activity of CIN- NK cells include CEACAM1 (Markel, Achdout et al. 2004), KLRG1 (Butcher, Arney et al. 1998; Hanke, Corral et al. 1998), and NKR-P1D (Iizuka, Naidenko et al. 2003; Carlyle, Jamieson et al. 2004). However, none of these receptors show a pattern of expression or functionality that can account for the failure of CIN- NK cells to attack MHC-deficient cells.

Differences in expression of activating receptors: A distinct hypothesis to explain the hypo-responsiveness of NK cells that are not inhibited by class I MHC is that the cells display altered, presumably diminished, expression of relevant activating receptors. This is, however, not the case as these NK cells show normal surface levels of all activating receptors examined (Fernandez, Treiner et al. 2005).

Dampening of signaling pathways downstream of activating receptors. According to this hypothesis, the signaling pathways downstream of activating receptors are desensitized in hyporesponsive NK cells, resulting in an ineffective coupling of activating receptors with effector functions such as cytokine production and granule release. Adaptor proteins that are directly downstream of the activating receptors seem to be present in normal amounts in hyporesponsive NK cells (Joncker and Raulet 2008), but it remains possible that downstream steps in the relevant signaling pathways are desensitized or decoupled. Although not proven, this hypothesis is the most consistent with the available data.

Models for NK cell education

While there is no doubt that class I MHC plays a critical role in NK cell education, there is a debate regarding the type of interaction (or lack thereof) between NK cells and MHC I – expressing cells in this process. Since NK cells with receptors that bind MHC I are responsive and those that lack such receptor are hyporesponsive, one possibility is that the presence of MHC I – expressing cells induces responsiveness. The converse possibility is that interactions of NK cells with target cells in which MHC I is not engaged leads to hyporesponsiveness.

These possibilities have been described in different ways in the literature and conferred with different names. In one treatment, the two extremes were described as "disarming" and "arming" as detailed here:

Disarming: (Raulet and Vance 2006) In this concept, high responsiveness is the default state, but when NK cells cannot receive inhibitory signals though MHC I – specific receptors while encountering surrounding normal cells, the cells are driven into a hyporesponsive state. The simplest version of this proposal is that during such encounters the cells receive persistent stimulation by activating ligands on cells in the environment, without opposing inhibitory signals, and that this persistent stimulation drives the cells into a hypo-responsive state, akin to the anergy that occurs in self-reactive B and T cells.

Arming: (Raulet and Vance 2006) According to this model, NK cells require interactions with MHC class I to acquire high responsiveness. In its simplest form, NK cells fail to achieve full functional maturation unless they encounter MHC I expressing cells in their environment. When this interaction is impossible, NK cells persist in a hyporesponsive, possibly immature, state. The arming concept is essentially equivalent to the "licensing" concept of NK cell education (Jonsson and Yokoyama 2009), which suggests that MHC I encounters license the maturation of NK cells. It should be pointed out that NK cells from MHC I deficient mice are phenotypically mature, as evidenced by expression of maturation markers comparable to what is seen on NK cells from wild-type mice (Fernandez, Treiner et al. 2005).

The arming model predicts that when NK cells mature within a mixture of normal and MHC-deficient cells, MHC class I – expressing cells will dominantly induce NK responsiveness, whereas according to the disarming model the presence of MHC I – negative cells will dominantly induce hyporesponsiveness.

The rheostat concept: The rheostat model considers NK responsiveness from a quantitative point of view. The discussion until now has implied that NK responsiveness reflects two states (responsive vs. hyporesponsive). The rheostat concept, in contrast, is based on the observation that NK responsiveness varies quantitatively, rather than reflecting only two states (responsive vs. hyporesponsive). As stated previously, due to the stochastic nature of inhibitory receptor expression by NK cells, each cell can express between 0 and 3 self MHC I specific inhibitory receptors (in B6 mice). This fact, along with the fact that the affinity of the various inhibitory receptors for MHC ligands is known to vary, leads to the conclusion that NK cells encountering cells in the normal environment are exposed to varying degrees of inhibition, depending on their inhibitory receptor repertoire and the available MHC molecules that are expressed. Studies examining the responsiveness of NK cell as a function of the number of self-specific inhibitory receptors they express show that responsiveness is directly proportional to the number of these receptors. NK cells that receive the most inhibition (or the least stimulation) at the steady state comprise the most responsive subset and responsiveness diminishes as the number of inhibitory receptors decreases (Brodin, Lakshmikanth et al. 2009; Joncker, Fernandez et al. 2009).

Tuning model: (Raulet and Vance 2006) The tuning model incorporates the arming and disarming concepts as well as the rheostat concept. It proposes that each NK cell, depending on the inhibitory (and stimulatory) receptors expressed and the MHC genotype of the animal, receives a varying amount of stimulation and inhibition when encountering neighboring cells. The model proposes further that depending on the net stimulation received, the NK cell assumes a quantitatively appropriate responsiveness state. Strong net stimulation (e.g. steady-state stimulation without opposing inhibition) drives the cell to its lowest responsive state, whereas weak (or no) net stimulation drives the cell to its highest responsive state. Intermediate net stimulation results in intermediate responsiveness.

The roles of *cis* and *trans* interactions between MHC I and inhibitory receptors in NK cell education

Members of the Ly49 receptors family have the capacity to bind MHC I on neighboring cells (in *trans*) and on the NK cells themselves (in *cis*) (Doucey, Scarpellino et al. 2004; Scarpellino, Oeschger et al. 2007). While *trans* binding mediates NK cell inhibition there is no evidence that *cis* interactions dampen activation of NK cells (Doucey, Scarpellino et al. 2004). It has been proposed that binding of inhibitory receptors to MHC I in *cis* leads to sequestration of these receptors and makes them unavailable for *trans* binding resulting in enhanced activation of NK cells (Chalifour, Scarpellino et al. 2009). A study of NK cells that express an engineered variant of Ly49A which retains *trans* but not *cis* binding to its ligand H-2D^d showed that this receptor inhibited killing of H-2D^d expressing cells but did not contribute to the education of NK cells, suggesting a possible role for *cis* interactions between MHC I and Ly49 receptors in this process (Chalifour, Scarpellino et al. 2009). A reciprocal study involving NK cells that express a Ly49A variant that binds its ligand in *cis* but not in *trans* showed that *cis* interactions lead to alterations in the Ly49 repertoire, further supporting the possible role of such interactions in NK cells education. (Bessoles, Angelov et al. 2013).

Functionality of NK cells lacking self-specific inhibitory receptors

The previous discussion emphasizes NK function in steady state conditions, which is highly relevant to self-tolerance. An important issue, however, concerns how responsive vs. hyporesponsive NK cells function in the context of immune responses.

Interestingly, early studies showed that despite the low responsiveness of NK cells under steady state conditions in MHC-deficient *β2m*$^{-/-}$ mice, when such mice were infected with MCMV their NK cells limit the infection to a similar extent as occurs in wild-type mice (Tay, Welsh et al. 1995). These findings suggested that hyporesponsive NK cells can provide anti-viral activity under conditions of infection. Interestingly, analysis of CIN- NK cells provided a similar conclusion. First, it was shown that in wild-type mice infected with *Listeria monocytogenes*, CIN- NK cells produce as much IFN-γ as do CIN+ NK cells (Fernandez, Treiner et al. 2005). Subsequently, it was shown that NK cells lacking expression of the inhibitory receptors Ly49C and Ly49I (CI- NK cells) are the primary mediators of protection against MCMV infection in B6 mice (Orr, Murphy et al. 2010). Hence, under the conditions of infection,

hyporesponsive NK cells can exert functional activity, perhaps because such conditions provide inflammatory signals that increase the activity of those cells.

Dissertation Research

It is well established that tuning of NK cell responsiveness is dependent on the expression of class I MHC in the environment, and expression of inhibitory receptors that bind MHC I by NK cells themselves (Fernandez, Treiner et al. 2005; Brodin, Lakshmikanth et al. 2009; Joncker, Fernandez et al. 2009). However, many questions regarding the role of MHC I in the process of NK cell education remain unanswered.

One important question is whether the responsiveness of NK cells is set once in an NK cell, as it develops, or can be further adjusted once the NK cell is mature. If the latter is the case, it could help explain the failure of NK cells to clear certain tumors and viral infections.

Another question is whether the mechanism that accounts for NK hyporesponsiveness also accounts for self-tolerance. There is a close correlation between tolerance and functional hyporesponsiveness, but it is possible that the underlying mechanisms of the two phenomena are distinct or partially overlapping. If this were the case, it could help explain the ability of NK cells from *β2m*-deficient animals to mount immune responses without causing autoimmunity.

In addition to investigating the role of MHC I in setting NK cell tolerance and responsiveness, it is important to investigate other factors that may participate in or influence this process. Because changes in responsiveness/tolerance are of particular interest in situations of infections and malignancies, where inflammatory stimuli may vary, we chose to investigate the roles of pro-inflammatory cytokines in these phenomena.

Work presented in this thesis addresses the relationship between NK cell responsiveness and tolerance towards target cells (cells that either lack expression of class I or express high levels of stimulatory ligands), the plasticity of NK cell education as a function of their MHC environment, the roles that hematopoietic and non-hematopoietic cells play in this process, and finally the effect of inflammation on tolerance of NK cells towards MHC I deficient target cells.

Chapter 2: Materials and Methods

Mice

Mice were bred at the University of CA, Berkeley from breeders obtained from Jackson Laboratories (C57BL/6J and B6;129-Gt(ROSA)26Sortm1(DTA)Mrc/J (Wu, Wu et al. 2006)), Charles River (B6-Ly5.2/Cr, which express Ly5.1), W. Yokoyama (Washington University in St. Louis, St. Louis, MO; NKD mice (Kim, Iizuka et al. 2000)), E. Vivier (Centre d'Immunologie de Marseille-Luminy; *Nkp46*^{iCre} knock-in mice (Narni-Mancinelli, Chaix et al. 2011)), and L. Lanier (University of CA, San Francisco; *Rae-1e* transgenic mice (Ehrlich, Ogasawara et al. 2005)). *β2m*$^{-/-}$ mice on the B6 background (B6-*β2m*$^{-/-}$) were described previously (Zijlstra, Li et al. 1989). B6 *β2m*$^{-/-}$- Ly5.1 mice were bred in our facilities.

In-vitro NK stimulation and IFN-γ assay

Where indicated, mice were pre-treated with 200 μg of Poly(I:C) (Invivogen) IP 24-36 hours prior to assay. Stimulation assays were performed using flat-bottom, high-binding 96-well plates (Thermo Fisher) pre-coated with the relevant antibody. Plates were coated by applying 100 μl of the indicated antibody diluted in PBS and incubated overnight at 4^0C. Prior to stimulation the plates were washed 4 times with 200 μl of PBS/well. Wells coated with isotype control antibody or PBS (as indicated) served as controls. $1x10^6$ splenocytes/well were stimulated for 5 hours in the presence of 1 μg/ml of BrefeldinA and 1000 U/ml of recombinant human Il-2 (from NCI). In case of stimulation with the RMA-S cell line stimulations were performed in U-bottom TC-treated plates using $1x10^5$ RMA-S cells, or RMA cells as a negative control. In assays where, in addition to intracellular IFN-γ staining, CD107a was detected by staining the cell surface, 1 μg/ml of monensin was added to the samples.

CFSE-labeled engraftment assay

Spleen cells from *β2m*$^{-/-}$ or *Raet1ε*-transgenic mice were labeled with 10 μM CFSE. For use as an internal control, WT B6 splenocytes were labeled with 1 μM CFSE for 10 minutes at 37^0C. The two types of cells were mixed 1:1 and $1x10^7$ cells ($5x10^6$ of each genotype) were injected IV into recipient mice. Graft rejection was assessed 18-42 hours later (specified in figure legends) by harvesting recipient spleens and comparing the CFSE^{hi} and CFSE^{lo} populations among the CFSE$^+$ cells. Percent graft rejection was defined as: 100 x [1-(% *β2m*$^{-/-}$ / % WT)] after normalizing to the mean rejection obtained when *β2m*$^{-/-}$ mice were challenged with the same mixture. When rejection of *Rae1e*-transgenic splenocytes was measured, the data were normalized to the "input" percentages, determined by flow cytometry after mixing the cells.

Adoptive Transfers

WT or *β2m*$^{-/-}$ splenocytes (containing 2-2.5x10^6 NK cells) were injected intravenously into mice that had received 6 Gy of irradiation from a ^{137}Cs source 4-5 hours prior. For adoptive transfers into unirradiated mice, the hosts were treated with 200 μg of anti-CD4 and anti-CD8 antibody one day prior to adoptive transfer and on days 1, 4, and 7 post adoptive transfer, in order to minimize T cell mediated rejection of the grafts.

Radiation chimeras

Chimera hosts received Gy using a ^{137}Cs source (9.5 Gy) 4-5 hours before injecting a source of hematopoietic stem cells. For fetal-liver chimeras, 1 x 10^7 donor cells (from CD45-congenic, embryonic day 14-17 embryos of each genotype) were injected. For bone-marrow chimeras, 1 x 10^7 bone marrow cells (from CD45-transgenic mice of each genotype were injected. WT hosts that were to receive *β2m*$^{-/-}$ donor cells were pretreated with 200 μg of anti-NK1.1 antibody (PK136) i.p. 2 days prior to reconstitution, in order to deplete NK cells. When WT bone-marrow donors were used, the mice were depleted of NK cells (as described above) 1-2 days prior to bone-marrow harvest. Chimeras were analyzed 13-22 weeks post reconstitution.

Salivary gland preparations of MCMV

4-week-old BALB/c female mice were infected with 1 x 10^3 PFU of tissue-culture derived MCMV (obtained from ATCC). 14-17 days later, salivary glands were harvested, dissociated using the gentleMACS device (Miltenyi biotec), and sonicated 5 times, alternating between 30 seconds of sonication and 30 seconds of incubation at 4^0C. The resulting viral extract was filtered through a 0.4 μM filter and used to infect a second group of naïve BALB/c mice (1 x 10^3 PFU was used). After this amplification step, the salivary gland virus was prepared again after 14-17 days and used for another round of infection (for a total of 3 passages).

Plaque assays

One day prior to performing the plaque assay, approximately 3 x 10^4 NIH 3T3 fibroblasts (originally obtained from ATCC) were seeded in the wells of 24-well tissue culture plates. On the day of the assay, viral stocks were thawed at 37^0C. Ten-fold serial dilutions were prepared in complete DMEM, resulting in final dilutions of between 1:10^3 to 1:10^6. Each well received 0.5ml of each dilution. After a 2 hour incubation the viral supernatants were removed and replaced with 0.5 ml of DMEM diluted 1:1 with 1.5% low-melting point agarose. After 5 days of incubation, the agarose was removed and the cells were fixed with 10% PFA and stained with brilliant violet to visualize the plaques.

MCMV infection

Mice were infected intraperitoneally with 1 x 10^4 PFU of 3rd passage salivary gland virus in 100-200 μl of PBS.

In-vivo cytokine treatment

rmIL-12 (cat # 210-12) and rmIL-15 (cat # 210-15) were purchased from Peprotech. rmIL18 (cat # B004) and rmIL-15Rα (cat # 551-MR) were purchased from R&D systems. IL-15 and IL-15Rα were pre-complexed by co-incubation at 37^0C for 30 minutes. Mice were injected intraperitoneally every 2 to 3 days for a total of 2-3 injections.

Antibodies and flow cytometry analysis

Antibodies against NK1.1 (PK136), CD3-ε (145-2C11), NKG2A/C/E (20d5), Ly49I (YLI-90), CD45.1 (A20), CD45.2 (104), IFN-γ (XMG1.2), NKG2D (MI-6) (Jamieson, Diefenbach et al. 2002), NKp46 (29A1.4), KLRG1 (2F1), CD27 (LG.3A10), CD11b (M1/70), H-2Kb (AF6-88.5), CD107a (1D4B), Pan-Rae1 (199215) were purchased from eBioscience, BD, R&D systems, and Biolegend. The 4LO3311 hybridoma, which secretes Ly49C antibody (a gift from S. Lemieux, INRS–Institut Armand-Frappier , Laval, Québec, Canada;(Gosselin, Lusigan et al. 1997)) was purified according to the protocol outlined below and conjugated to biotin using the EZ-Link Sulfo-NHS-LC-Biotin Biotinylation Kit (Pierce, cat# PI 21435). Biotin-conjugated mAbs were detected with streptavidin Pacific blue (Invitrogen) or streptavidin Brilliant Violet 421 (BioLegend). Before staining, cells were preincubated for 20 min with 2.4G2 hybridoma supernatant to block FcγRII/III receptors. Flow cytometry was performed on a cytometer (LSR II or LSR Fortessa; BD), and data were analyzed with the FlowJo software (Tree Star, Inc.).

Anti-Ly49C antibody (4LO3311) purification protocol

The anti-Ly49C antibody was purified from CELLine classical 1000 supernatants (Integra Biosciences). Prior to purification the supernatant (~60mL) was combined with an equal volume of binding buffer (20 mM sodium phosphate pH 7.0, 0.001% Tween 80) and filtered through a 0.22 μM filter. The supernatant-binding buffer mixture was applied to a protein A HP column containing 5 mL resin (GE Healthcare, cat # 17-0403-01) and allowed to flow through with the aid of a peristaltic pump. The column was washed with 100 mL binding buffer before eluting with 20 mL of elution buffer (0.1 M citric acid, pH 3-6). As the antibody was eluted, the pH was neutralized by addition of aliquots of approximately 100μl 1M Tris pH 9.0 to the 20 ml of eluate until the pH reached neutrality (pH 7.0) as tested with litmus paper. The elution was performed on the lab bench with prewarmed 42^0C elution buffer, into a tube held in a 42^0C water bath. Immediately after elution, 0.001% Tween 80 was added to aid in avoiding precipitation.

Antibodies used for *in-vivo* depletion

NK1.1 antibody (PK136) used for depleting NK cells in vivo was purified from CELLine classical 1000 supernatants (Integra Biosciences). Depleting CD8a (2.43) and CD4 (GK1.5) antibodies were purchased from Bio X Cell. For in-vivo depletions 200 μg of each antibody was injected intraperitoneally.

Chapter 3: NK cell hyporesponsiveness and the acquisition of tolerance

An important role of NK cells is to eliminate cells that extinguish or diminish expression of self MHC class I molecules, which commonly occurs as a result of viral infection or cellular transformation (Karre, Ljunggren et al. 1986; Bix, Liao et al. 1991; Garrido, Cabrera et al. 1995; Raulet, Held et al. 1997; Karre 2002). This capacity arises because NK cells express stimulatory and inhibitory receptors that engage ligands on normal cells. The majority of inhibitory receptors belong to the KIR (in human), Ly49 (in mouse) and CD94/NKG2A (both in human and mouse) families and are specific for MHC I molecules (Raulet, Held et al. 1997; Moretta, Bottino et al. 2001; Lanier 2005). When an NK cell encounters a normal cell, engagement of the inhibitory receptors conveys signals that counteract stimulatory signaling. Lysis occurs when inhibition is lost because the target cell lacks one or more self-MHC molecule, or when target cells express high levels of stimulatory ligands that over-ride inhibition (Raulet 2006).

NK cells vary in the number and specificity of MHC-specific inhibitory receptors they express (Raulet, Held et al. 1997; Raulet, Vance et al. 2001). Recent studies demonstrate that NK cells vary in basal responsiveness to stimulatory receptor engagement depending on the number of expressed inhibitory receptors specific for self-MHC molecules (Brodin, Lakshmikanth et al. 2009; Joncker, Fernandez et al. 2009). Cells with several self-MHC specific receptors exhibit the greatest basal responsiveness, and hence mediate the greatest activity against target cells that lose MHC. Cells with no self MHC-specific receptors are the most hyporesponsive, to the extent that they fail to attack otherwise normal cells lacking MHC I molecules. These data suggest that the responsiveness set point of individual NK cells is tuned depending on the balance of inhibitory and stimulatory ligands that each NK cell encounters on neighboring cells in the normal environment (Joncker and Raulet 2008).

Chapter 3, Section 1. Responses of NK cells that develop in MHC-deficient hosts

Responsiveness of NK cells developing in *β2m*$^{-/-}$ chimera hosts to stimulation with antibodies specific for activating receptors

The processes of lymphocyte development, selection, and education require their interaction with multiple cells types to shape the optimal repertoire. During and after development in WT animals, a fraction of NK cells acquire the ability to respond to activating stimuli, including tumor cells, stimulatory ligands presented by otherwise normal cells that lack MHC I, and antibodies that crosslink activating receptors on the NK cells. In contrast, NK cells in *β2m*-deficient animals do not respond to self cells lacking MHC I, and also show lower responses to the various other stimuli just listed (Fernandez, Treiner et al. 2005). Furthermore, the NK cells in WT mice divide into a subset that expresses self MHC specific inhibitory receptors, which carries out rejection of MHC deficient cells and exhibits high responsiveness, and a subset that lacks such receptors, which is tolerant of MHC I-deficient cells and exhibits low responsiveness (Fernandez, Treiner et al. 2005). Thus, self-tolerance (no response to self cells lacking MHC I)

and hyporesponsiveness (low responses to antibody crosslinking of activating receptors) are correlated, and it has been assumed that hyporesponsiveness is a manifestation of self-tolerance. While hyporesponsiveness and the absence of missing-self recognition seem to go hand-in-hand it remains possible that the educational processes that lead to each of these outcomes are regulated at least partially independently, for example by interactions of NK cells with different cells.

To generate further understanding of these processes, we prepared irradiation chimeras, in which WT or $\beta 2m^{-/-}$ NK cells develop from hematopoietic precursor cells in $\beta 2m^{-/-}$ hosts. This protocol allowed us to determine the origin (hematopoietic or non-hematopoietic) of cells that regulate NK cell responsiveness and tolerance, and to determine how these processes are regulated during NK cell development.

To distinguish the roles of non-hematopoietic and hematopoietic cells in determining NK functional responsiveness and tolerance towards missing-self we generated the following fetal-liver chimeras:

Chimeras (Donor fetal liver→ irradiated host)	**Hematopoietic cells (including NK cells)**	**Non-hematopoietic cells**
WT→WT	WT	WT
$\beta 2m^{-/-}\rightarrow\beta 2m^{-/-}$	$\beta 2m^{-/-}$	$\beta 2m^{-/-}$
WT→$\beta 2m^{-/-}$	WT (+few $\beta 2m^{-/}$ cells)	$\beta 2m^{-/-}$
WT+$\beta 2m^{-/-}\rightarrow\beta 2m^{-/-}$	WT & $\beta 2m^{-/-}$	$\beta 2m^{-/-}$

NK cell responsiveness was analyzed 13-20 weeks following reconstitution with fetal liver cells, by stimulating splenic NK cells from the chimeras with plate-bound antibodies against different activating NK receptors followed by staining for CD107a and/or intracellular IFN-γ (Fig. 3-1).

The results indicated that hyporesponsiveness of NK cells was induced by *β2m*-deficient host cells and was independent of MHC I expression by donor hematopoietic cells. Thus, WT NK cells that developed in a $\beta 2m^{-/-}$ host exhibited low responsiveness similar to that of $\beta 2m^{-/-}$ NK cells that developed in a $\beta 2m^{-/-}$ host. In mixed chimeras, the outcome was determined by the *β2m*-genotype of the host, despite the presence of roughly equal numbers of WT and *β2m*-deficient cells among hematopoietic cells.

It is also noteworthy that the MHC-I expression on the NK cells had no effect on their responsiveness. It has been previously suggested that *cis* interaction between Ly49 receptors and MHC play a role in the education of NK cells. (Chalifour, Scarpellino et al. 2009). The results from the mixed fetal liver chimeras indicate that the factors that determine the responsiveness of the NK cells are cell-extrinsic with respect to MHC-I expression, and argue that cis-interactions alone cannot explain NK cell responsiveness.

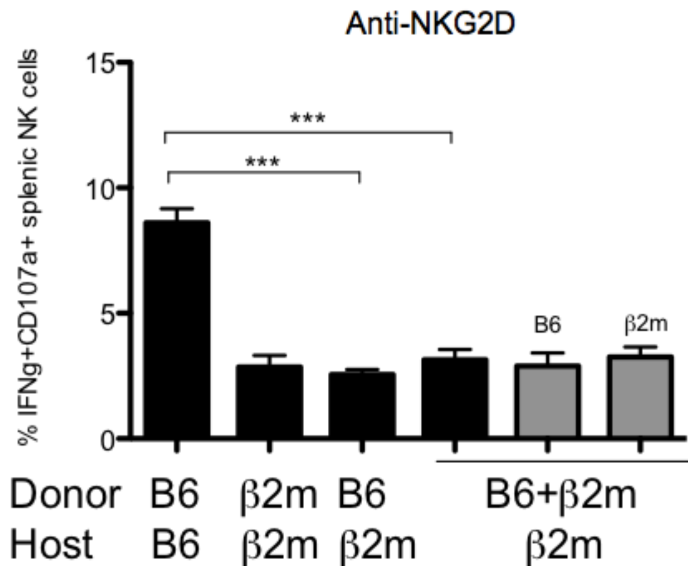


Figure 3-1. **NK cells developing in MHC I – deficient hosts are hyporesponsive.** Lethally irradiated hosts were reconstituted with WT, *β2m*$^{-/-}$, or 1:1 mix of WT and *β2m*$^{-/-}$ fetal liver cells. 13-20 weeks after reconstitution NK cells were stimulated for 5 h with 5µg/ml of plate-bound anti-NKG2D antibodies. The percentage of donor NK cells expressing IFN-γ and CD107a was determined by flow cytometry. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (***, P < 0.0005). Data represent means ± SEM.

NK cells developing in MHC I – deficient hosts are tolerant of *β2m*$^{-/-}$ cells

In order to address the roles of different cells types that play a role in ensuring the self-tolerance of NK cells we have utilized the fetal liver chimeras described above.

We used two approaches to assess the tolerance of NK cells to MHC-I deficient targets. First we evaluated the maintenance of chimerism in the mixed fetal liver chimeras. Fig. 3-2 shows the percent of *β2m*-deficient cells among the donor-derived blood lymphocytes. Chimerism was stably maintained following reconstitution in *β2m*-deficient hosts, for at least 125 days.

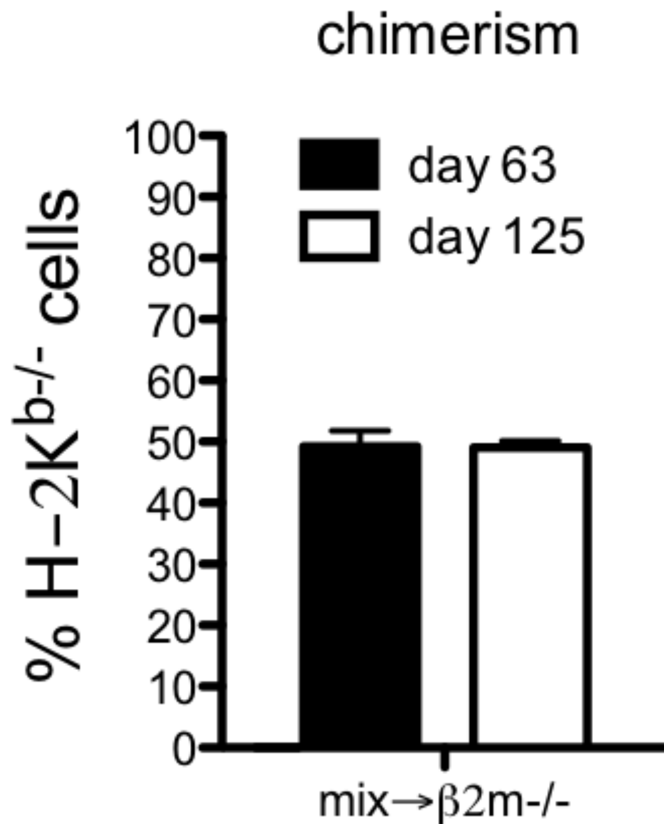


Figure 3-2. Long-term maintenance of chimerism in mixed fetal liver chimeras. Lethally irradiated *β2m*$^{-/-}$ hosts were reconstituted with a 1:1 mixture of WT and *β2m*$^{-/-}$ fetal liver cells (n=5 mice in each group). Chimerism was determined by flow cytometry of PBLs stained for H-2K^{b} (in this experiment) or the congenic CD45 marker (in experiments not shown) 63 and 125 days following reconstitution. Data represent means ± SEM.

As a second way to measure tolerance to *β2m*-deficient cells we challenged the mice with a 1:1 mixture of differentially CFSE-labeled wild-type and *β2m*$^{-/-}$ splenocytes or bone marrow cells and assessed their rejection 18-40 hours later (18 hours in the experiment shown in Fig. 3-3 and up to 40 hours in replicates not shown). Figure 3-3 shows that WT→ WT chimeras rejected the grafts, but NK cells that developed in the presence of MHC-I deficient host cells, most of which are non-hematopoietic cells, were tolerant of MHC I deficient grafts. This was true whether the donor hematopoietic cells were purely MHC+ (as in WT→ *β2m*$^{-/-}$ chimeras) or were a mixture of WT and *β2m*$^{-/-}$ cells (as in Mix→ *β2m*$^{-/-}$ chimeras)

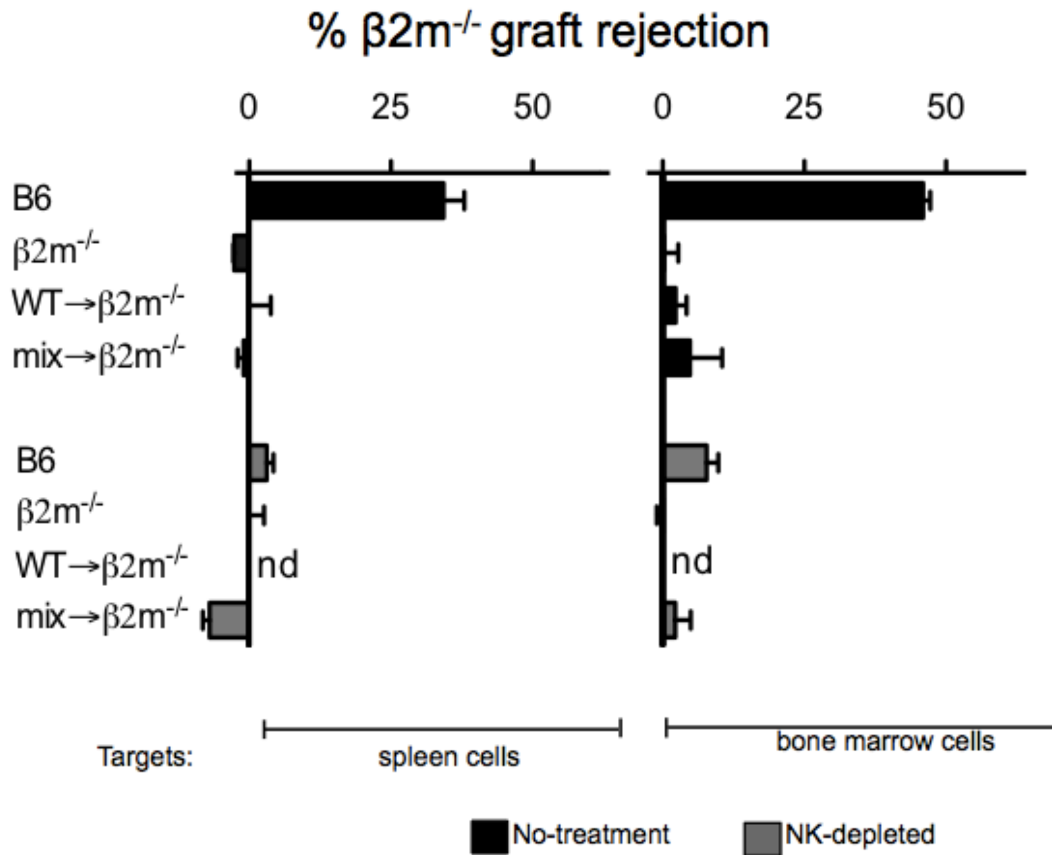


Figure 3-3. The tolerance of developing NK cells is set depending on the MHC environment in which they mature. After 9–17 wk of reconstitution, chimeras were tested for rejection of $\beta 2m^{-/-}$ spleen cells or bone marrow cells, as indicated. Unmanipulated B6 and $\beta 2m^{-/-}$ mice were tested in parallel as controls for rejection and tolerance, respectively. The rejection assay was performed by injecting $CFSE^{high}$-labeled $\beta 2m^{-/-}$ cells (spleen or bone marrow) mixed with an equal number of $CFSE^{low}$-labeled WT spleen cells as an internal control. Some groups of chimeras were pretreated i.p. twice (on days -2 and -1) with 200 µg PK136 (NK1.1) antibody (NK depleted), as indicated. Rejection of $\beta 2m^{-/-}$ spleen cells was determined by flow cytometry of spleen cells 18 h later. Data represent means ± SEM (n = 2–5 mice). The experiment was performed three times with spleen cell targets and once with bone marrow cell targets. nd, not done.

NK cells developing in MHC I – deficient hosts do not respond to the NK-sensitive cell line RMA-S

RMA-S is a widely used NK-sensitive lymphoma cell line. It is sensitive to NK cells because of its lack of MHC I expression, due to a mutation in the *Tap2* gene (Ljunggren, Ohlen et al. 1991). We have tested the ability of NK cells developing in fetal liver chimeras to respond to *ex-vivo* stimulation with either this cell line or its NK resistant counterpart RMA (the parent cell line, which expresses WT *Tap2*) following *in-vivo* pre-activation of the NK cells with the TLR3 ligand poly(I:C). Poly(I:C) preactivation is typically necessary in order to observe in vitro responses to tumor cells. Consistent with our results for rejection of MHC I-deficient grafts, NK cells that developed in the $\beta 2m^{-/-}$ host, where the cells are exposed to MHC-deficient non-hematopoietic cells, showed a strongly reduced response to RMA-S cells, measured as production of IFN-γ and display of the degranulation marker CD107a. In comparison, control chimeric WT NK cells that developed in WT hosts responded to RMA-S cells. The responses to RMA tumor cells (MHC+) were much lower, showing the specificity of the response.

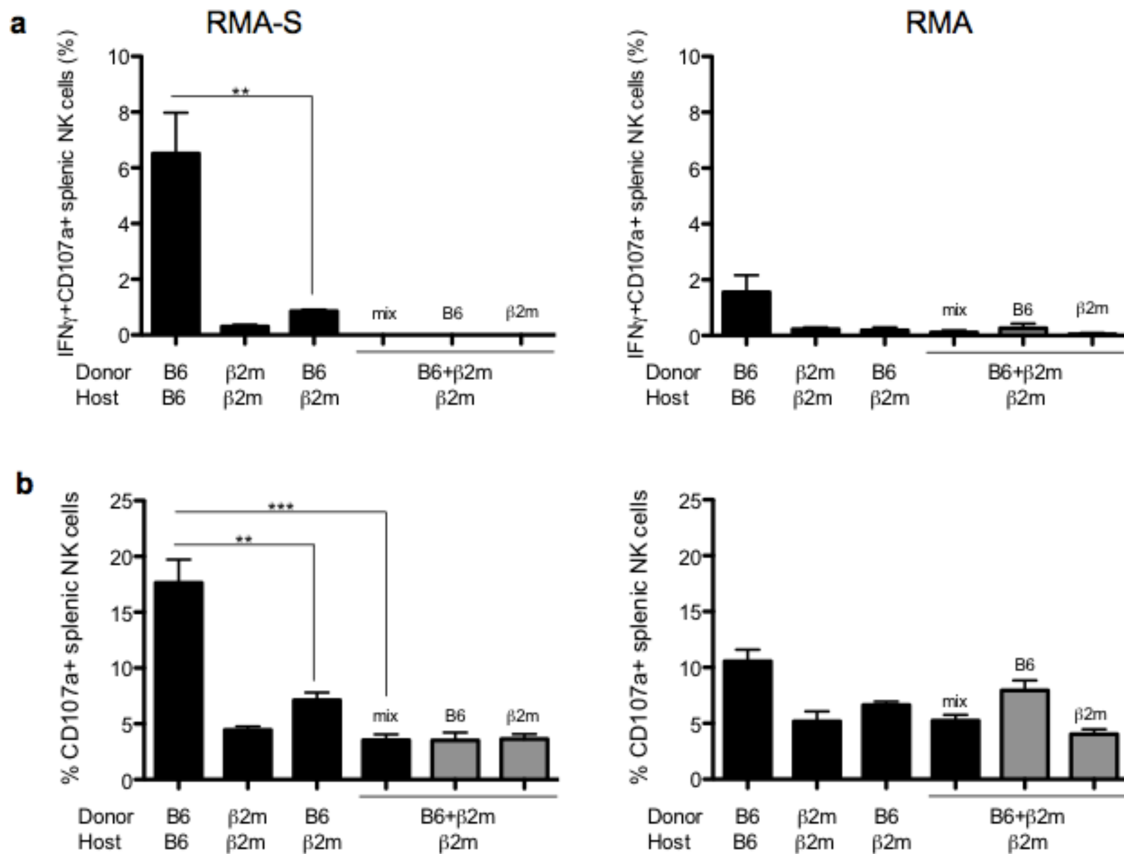


Figure 3-4. NK cells developing in the presence of MHC I-deficient non-hematopoietic cells do not respond to the NK-sensitive cell line RMA-S. (a, b) 16 weeks post reconstitution, groups of chimeras (n=5) were pre-treated with 200 μg poly(I:C) intraperitoneally. 24 hours later splenocytes were harvested and co-incubated with 1×10^5 RMA-S or RMA cells for 5 hours. The percentages of (a) IFN-γ+CD107a+ or (b) CD107a+ donor NK cells were determined by flow cytometry. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (**, P < 0.005; ***, P < 0.0005). Data represent means ± SEM. Results are preliminary.

NK cells developing in MHC I – deficient hosts are tolerant of RAE1 expressing grafts

Having determined that NK cells developing in the presence of MHC I – deficient non-hematopoietic cells do not reject MHC I-negative grafts, we asked whether lack of rejection would extend to distinct NK sensitive target cells, such as MHC I+ target cells that express a ligand for NKG2D. To test this we used splenocytes from *Raet1e* transgenic mice (Ehrlich, Ogasawara et al. 2005) as targets in a CFSE rejection assay *in vivo*. These cells display normal amounts of MHC I, but are NK sensitive because they display cell surface RAE-1 ligands, which engage the activating receptor NKG2D on NK cells.

Surprisingly, the *Raet1e*-transgenic spleen cells were not rejected by any of the mice unless the mice were pretreated with poly(I:C). This was unexpected because of published data with the same transgenic mice indicating that rejection occurred without pre-treatment (Ehrlich, Ogasawara et al. 2005; Oppenheim, Roberts et al. 2005). The discrepancy may be due to differences in microbiota or other factors in different animal colonies.

The first notable finding from this analysis (Fig. 3-5) was that when mice were pretreated with poly(I:C), $\beta 2m^{-/-} \rightarrow \beta 2m^{-/-}$ chimeras showed a defect, compared to WT→WT chimeras, in rejection of spleen cells from *Raet1e* transgenic mice. Therefore, the MHC-deficient environment resulted in hyporesponsiveness of NK cells to RAE-1-expressing cells that are otherwise normal with respect to MHC expression and other properties. Most significantly, WT→$\beta 2m^{-/-}$ chimeras and mix→ $\beta 2m^{-/-}$ chimeras also showed impaired rejection of the transgenic cells. This rejection pattern closely resembled that observed for the rejection of MHC I-deficient cells. In conclusion, NK cells exposed to MHC-deficient non-hematopoietic cells in fetal liver chimeras are impaired in their capacity to reject RAE-1-expressing, MHC+ spleen cells. Thus, tolerance to MHC-deficient grafts is correlated with hyporesponsiveness to otherwise healthy, MHC+ cells displaying activating ligands.

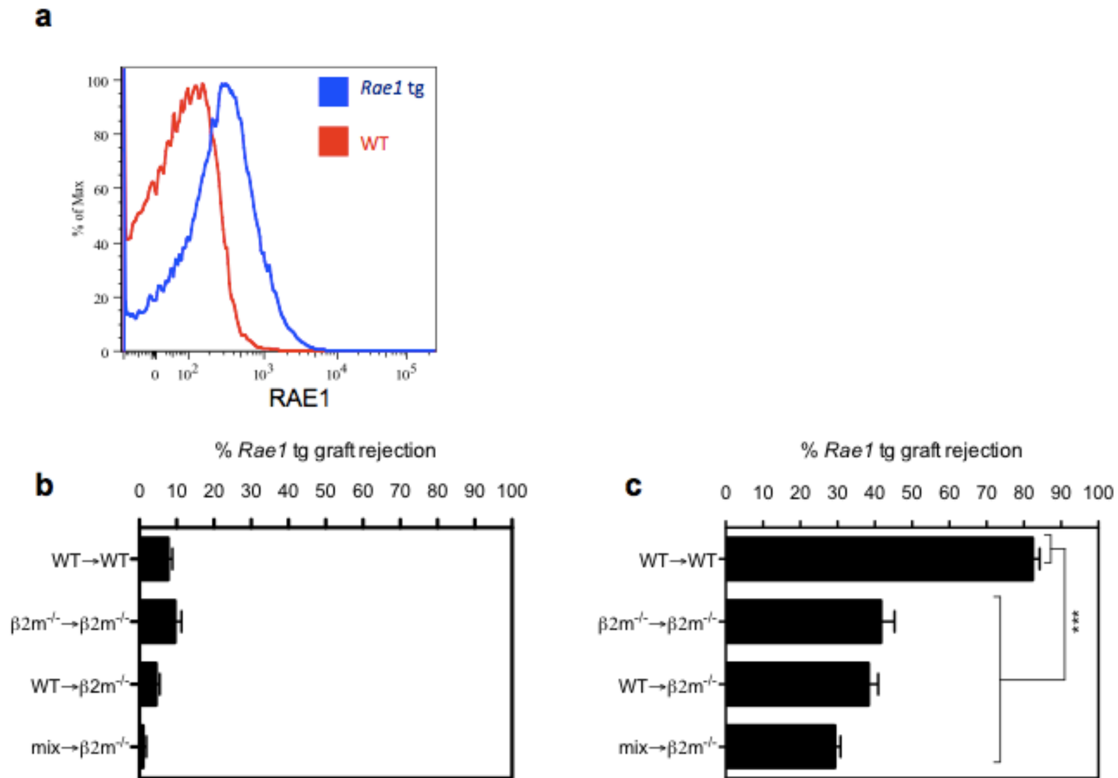


Figure 3-5. NK cells developing in the presence of *β2m*-deficient non-hematopoietic cells show reduced capacity to reject *Raet1e* transgenic splenocytes grafts. (a) Representative plot of RAE1 expression on transgenic splenocytes and splenocytes from WT littermate controls. (b and c) 13-20 weeks post reconstitution, groups of chimeras (n = 3-5) received grafts of CFSE^{high}-labeled *Rae1e* tg spleen cells mixed with an equal number of CFSE^{low}-labeled WT spleen cells. Rejection of *Raet1e* tg target cells was determined by flow cytometry of spleen cells 48 h later. Mice were either left untreated (b) or pretreated i.p. (on day −1 relative to the time of engraftment) with 200 µg poly(I:C) (c). The experiment was done once for no pretreatment (b) and three times with poly(I:C) pretreatment (c) with n = 3-5 mice for each. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (***, $P < 0.0005$). Data represent means ± SEM.

NK subset composition in chimeric mice

We determined whether the acquisition of phenotypic markers on NK cells, or the composition of NK cell subsets, were altered in chimeras depending on the MHC expression of host cells. In WT mice CIN+ and CIN- NK cells correspond to responsive and hyporesponsive subsets, respectively. In *β2m*-deficient mice, in contrast, both subsets are hyporesponsive (Fernandez, Treiner et al. 2005). Analysis of Ly49I, Ly49C, and NKG2A/C/E receptor expression on NK cells from fetal liver chimeras showed a similar percentage of CIN+ NK cells in all groups (Fig. 3-6a). Analysis of responsiveness of the CIN+ subset in each group of chimeras, after stimulation with NKG2D antibody, showed that the CIN+ NK cells were responsive in WT→WT chimeras, but were much less responsive in any of the chimeras prepared with MHC-deficient hosts, suggesting that the CIN+ subset had been rendered hyporesponsive in the MHC-deficient hosts (Fig. 3-6b).

The percentage of cells that express KLRG1 is known to be higher on NK cells from WT mice compared to *β2m*$^{-/-}$ mice and in WT mice, KLRG1^{high} cells are more frequent among CIN+ NK cells than among CIN- NK cells (Corral, Hanke et al. 2000; Fernandez, Treiner et al. 2005). This pattern was reproduced in the WT→WT chimeras (Fig. 3-6c). In contrast, relatively lower percentages of NK cells expressed KLRG1 in all the chimeras prepared with *β2m*$^{-/-}$ hosts (Fig. 3-6c), consistent with published data (Joncker, Shifrin et al. 2010). Hence, chimeric NK cells prepared in MHC-deficient hosts resemble NK cells in fully MHC-deficient hosts with respect to functional responsiveness, tolerance to MHC-deficient cells, and KLRG1 phenotype.

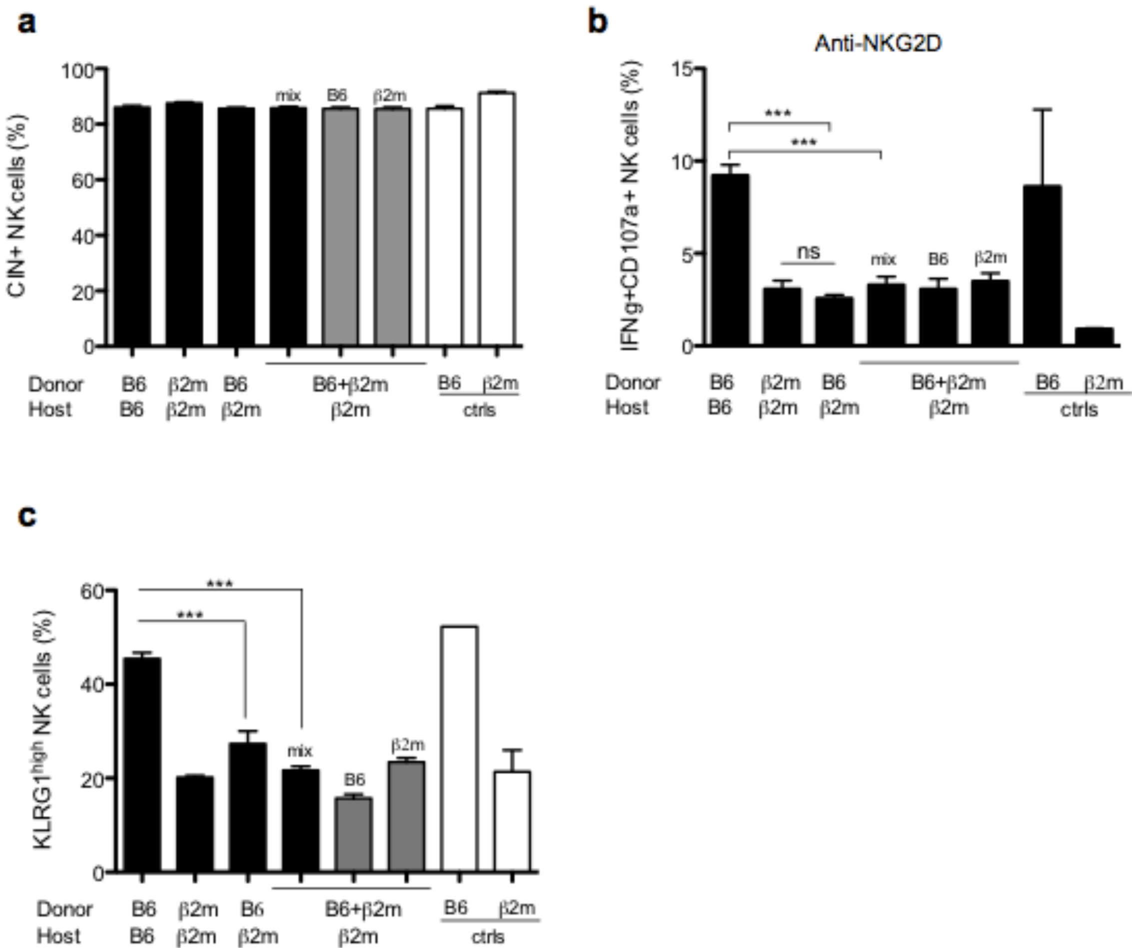


Figure 3-6. Phenotype of NK cells in fetal liver chimeras. 13-20 weeks after reconstitution, donor-derived splenic NK cells were stained for expression of (a) Ly49I, Ly49C, and NKG2A/C/E or (c) KLRG1. (b) analysis of IFN-γ and CD107a expression on CIN+ NK cells following a 5hr stimulation with 5 μg/ml of plate-bound anti-NKG2D antibody (MI-6). For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (***, $P < 0.0005$). Data represent means ± SEM. Results are preliminary.

Chapter 3, Section 2. Responses of mature NK cells after adoptive transfer into MHC-deficient hosts.

Downward resetting of mature NK cells after adoptive transfer

An important unanswered question is whether the basal responsiveness of NK cells is set only once during NK cell development, or alternatively can be readjusted when the fully mature NK cell is exposed to a changing MHC environment. Readjustments of NK cell responsiveness, if they occur, may account for instances where NK cells fail to eliminate tumors or pathogen-infected cells, and will be important to address when testing therapies designed to augment or suppress NK cell activity in the context of disease.

Splenic NK cells re-set their functional potential downwards when transferred to MHC I-deficient mice.

We employed an adoptive transfer system to test whether the responsiveness of NK cells can be reset after the NK cells reach maturity. Splenocytes from C57BL/6 mice, containing a majority of responsive NK cells (Fernandez, Treiner et al. 2005; Joncker, Fernandez et al. 2009), were transferred to irradiated congenic MHC I-deficient or control wild type mice, and responsiveness was tested 10 days later. To test responsiveness, splenocytes from the recipients were stimulated *in vitro* with plate-bound antibodies specific for the stimulatory receptors NKG2D or NKR-P1C. Intracellular IFN-γ production by donor NK cells was determined by gating on the congenic CD45 marker. The results demonstrated a striking hyporesponsiveness of WT NK cells to NKG2D and NKR-P1C stimulation after transfer to MHC I-deficient hosts, comparable to that of MHC I-deficient NK cells after transfer to MHC I-deficient hosts (Fig. 3-7a). Hyporesponsiveness resulted from exposure to the MHC-deficient environment, because the same NK cells transferred to WT mice exhibited strong responsiveness (Fig. 3-7a and data not shown). Time course studies demonstrated that some re-setting occurred as little as 4 days after transfer but required at least 7-10 days to be fully established (data not shown).

The alterations in NK cell responsiveness after transfer occurred for the most part in the CIN+ NK cell population, as expected (Fig. 3-7b). The CIN- population, was relatively hyporesponsive after transfer in all combinations tested and the differences in CIN- cells responsiveness were neither statistically significant, nor reproducible (data not shown).

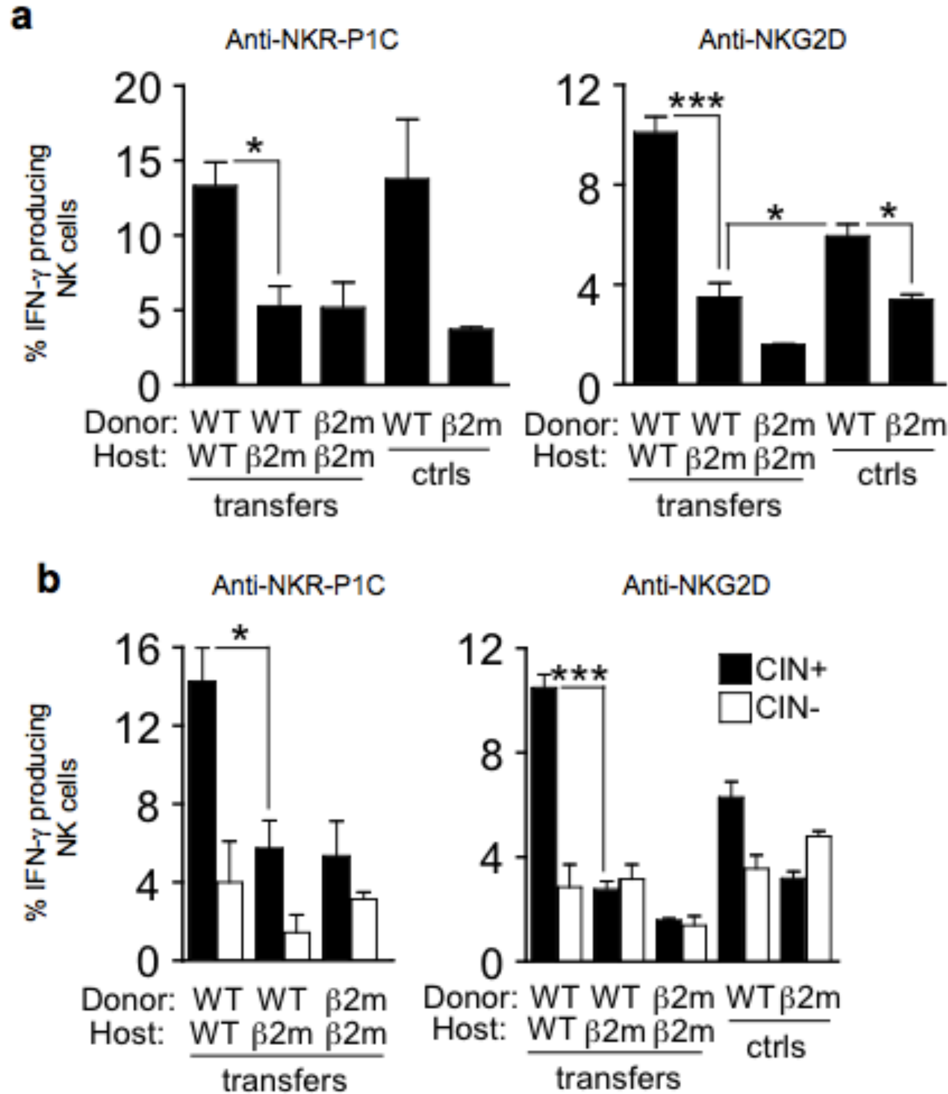


Figure 3-7. Responsive NK cells reset their functional potential downward in the absence of MHC I contact. Responses of WT NK cells after transfer to WT or *β2m*-deficient hosts. (a and b) 10 d after transfer, splenic NK cells were stimulated for 5 h with 5 µg/ml of plate-bound NKG2D or 50 µg/ml NKR-P1C antibody as indicated. The percentage of IFN-γ–producing NK cells among gated donor NK cells (a) or gated donor NK cells expressing one or more of Ly49C, Ly49I, and/or CD94/NKG2A (CIN+) or lacking those three receptors (CIN−; b) was determined by flow cytometry. Responses of NK cells from unmanipulated WT and $\beta 2m^{-/-}$ mice are shown for comparison. Experiments were repeated five to six times with n = 3–5 each for transfer groups. For relevant comparisons, statistical significance was determined with a two-tailed unpaired (a) or paired (b) Student's t test (*, P < 0.05; ***, P < 0.0005). ctrls, controls. The experiment was repeated three times with n = 3–6 mice for each (***, P = 0.0002). Data represent means ± SEM.

Downward resetting occurs in mature NK cells

A shorter transfer time of 4 days revealed similar trends in terms of downward resetting of NK activity, but the differences were not statistically significant, suggesting that >4 days are required for maximal resetting (data not shown). Although we would not expect extensive NK differentiation from stem cells to occur in ≤10 days, it remained possible that the changes in activity we observed after transfer of spleen cells applied only to new NK cells that differentiated in the adoptive host from donor hematopoietic precursor cells. If this occurred, the new results would not speak to whether the MHC environment influences the activity of mature (as opposed to differentiating) NK cells. In order to distinguish NK cells that were already mature at the time of transfer from NK cells that differentiated after transfer, we took advantage of the knowledge that NK cell differentiation involves extensive cellular proliferation (Kim, Iizuka et al. 2002). For this analysis, donor spleen cells were labeled with CFSE before transfer, and the extent of CFSE dilution was assessed in NK cells harvested 10 days after transfer. A significant fraction of NK cells (between 10% and 30% in different experiments) had not divided 10 days after transfer (data not shown). By gating on NK cells that had not divided after transfer, we observed the same patterns of responsiveness that we had observed in the bulk populations: WT NK cells transferred to MHC I-deficient hosts exhibited relatively low responsiveness (Fig. 3-8a). These data argue that the changes in NK responsiveness after transfer occur in non dividing mature NK cells, as opposed to representing the activity of newly differentiated NK cells that arose after transfer.

The experiments to this point were performed in irradiated hosts, so an additional analysis was performed to determine whether downward NK cell resetting occurs after transfer to unirradiated hosts. Previous studies have shown that unirradiated MHC I-deficient hosts reject WT hematopoietic cells. Rejection was due in part to the action of rare CD8+ T cells in such hosts which are amplified after challenge (Glas, Ohlen et al. 1994), but a CD4 T cell-dependent mechanism also caused rejection independent of CD8 T cells (data not shown). To prevent rejection of transferred WT cells, the hosts were depleted of both CD8+ and CD4+ T cells before transfer, and the cells were parked for a slightly shorter time period before analysis. WT cells transferred to unirradiated MHC I-deficient mice were rendered hyporesponsive to NKG2D stimulation, similar to our findings in irradiated mice (Fig. 3-8c). In unirradiated hosts, the donor NK cells underwent little or no homeostatic proliferation, as expected (data not shown), and therefore the cells analyzed are all mature, non-proliferating NK cells. Thus, downward resetting does not depend on the use of irradiated hosts.

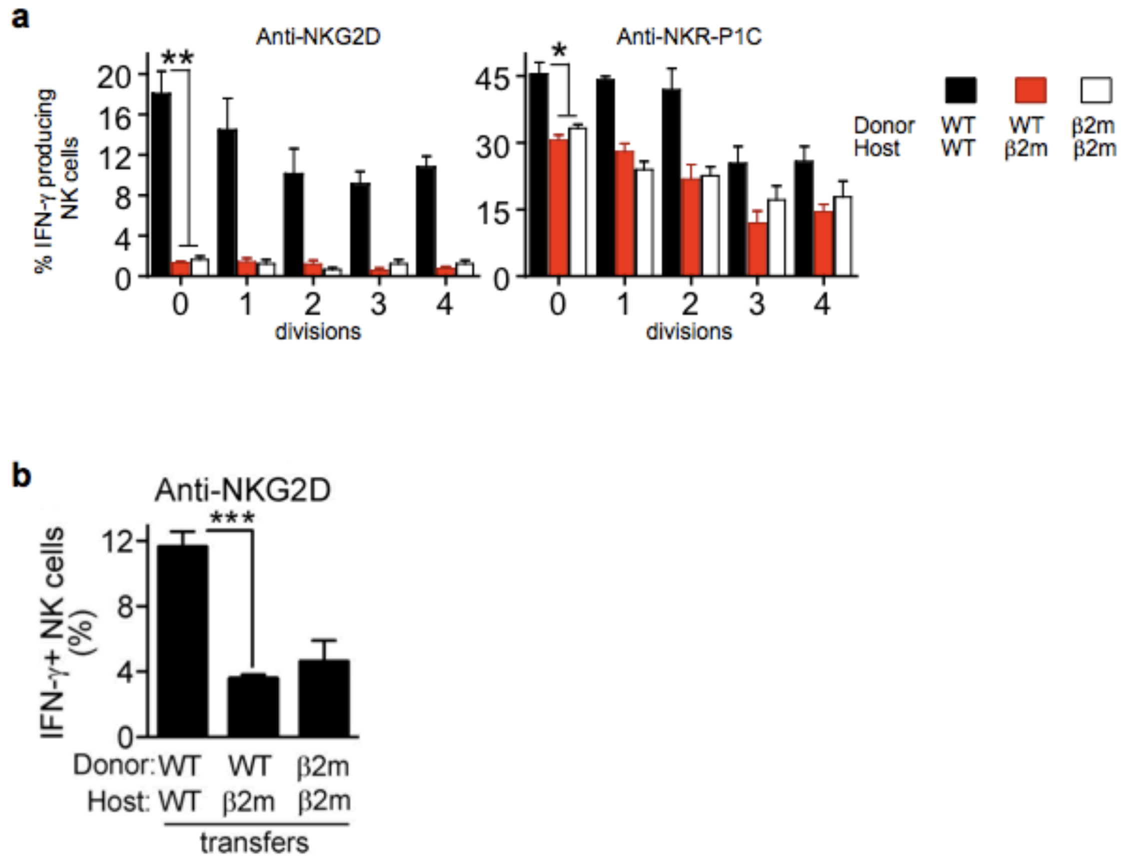


Figure 3-8. Downward functional re-setting occurs even in NK cells that do not divide after transfer. (a) CFSE-labeled WT splenocytes were transferred to irradiated WT or *β2m*-deficient hosts. 10 d after transfer, NK cells were stimulated with NKG2D antibody (left) or NKR-P1c antibody (right) and assayed as in Fig. 3-7, except that the donor cells were gated based on CFSE level, distinguishing cells that had divided 0, 1, 2, 3, or 4 times after initial labeling. Experiments were repeated two to four times with n = 3–6 mice for each. (b) WT splenocytes were transferred to unirradiated, CD8a- and CD4-depleted WT, or *β2m*-deficient hosts. 9 d after transfer, NK cells were stimulated and assayed as in Fig. 3-7. The experiment was performed twice with n = 3–5 mice. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$). Data represent means ± SEM.

Tolerance of NK cells to MHC I – deficient grafts after adoptive transfer

The hyporesponsive phenotype of natural killer cells from $\beta 2m^{-/-}$ animals and CIN- NK cells goes hand-in-hand with their inability to reject MHC I – deficient hematopoietic grafts (Fernandez, Treiner et al. 2005). We therefore asked whether changes in responsiveness that result from downward resetting are correlated with a decreased capacity to reject grafts of MHC-deficient spleen cells.

Re-setting of NK cell function after transfer to MHC I-deficient mice was indeed evident in an *in vivo* assay of NK cell tolerance. Twelve days after transfer of WT splenocytes to irradiated WT or MHC I-deficient mice, we tested the capacity of the mice to reject spleen cell grafts from MHC I-deficient mice (Fig. 3-9). Only minimal rejection occurred in irradiated WT control mice that did not receive a WT cells transfer (data not shown). When WT recipients were restored with WT spleen cells, strong rejection occurred. The rejection was mediated by NK cells, as shown by depleting NK cells before engraftment. In contrast, MHC I-deficient recipients that were restored with WT NK cells failed to reject MHC I-deficient grafts. Absence of NK cells could not account for the failure to reject the grafts since these recipients contained as many donor NK cells as did WT recipients of WT NK cells (data not shown).

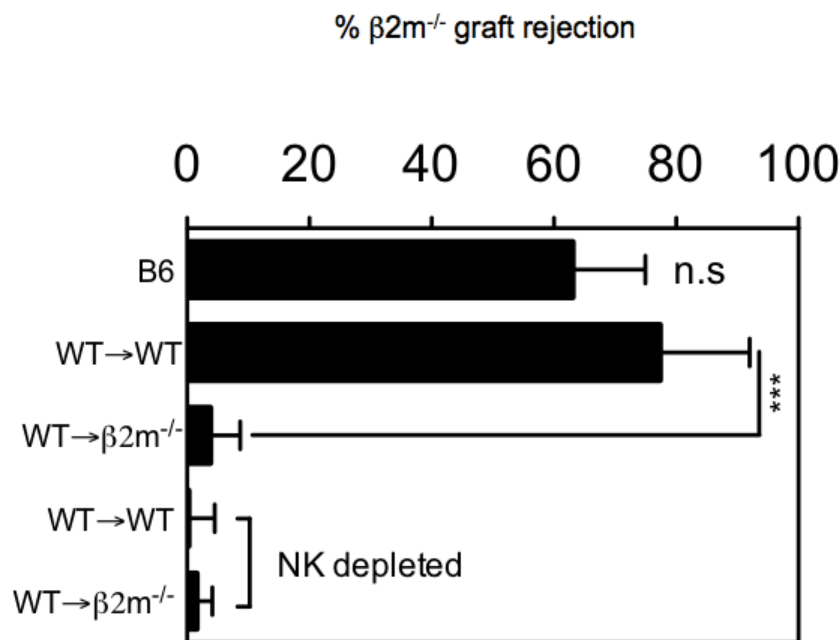


Figure 3-9. Tolerance of NK cells to MHC I – deficient grafts after adoptive transfer
At day 10-12 after transfer, groups of adoptive transfer recipients (n = 3–4) received grafts of CFSE^{high}-labeled $\beta 2m^{-/-}$ spleen cells mixed with an equal number of CFSE^{low}-labeled WT spleen cells. Rejection of $\beta 2m^{-/-}$ target cells was determined by flow cytometric analysis of spleen cells 18 h later. Some groups of transfer recipients in panel a were pretreated i.p. twice (on days −2 and −1 relative to the time of engraftment) with 200 μg PK136 (NK1.1) antibody to deplete NK cells, as indicated ("NK-depleted"). For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (***, $P < 0.0005$). Data represent means ± SEM.

NK subset composition after transfer

To determine whether functional re-setting correlated with changes in subset composition after transfer, we determined the phenotypes of gated CFSE^{high} NK cells, which we infer correspond to transferred NK cells rather than NK cells that differentiated from precursor cells after transfer. Because CIN+ and CIN- NK cells correspond to responsive and hyporesponsive NK cells in normal B6 mice (Fernandez, Treiner et al. 2005) we determined whether the proportions of these subsets changed after transfer. Analysis showed that the proportions of CIN+ and CIN- NK cells among total NK cells or among those that did not divide after transfer did not change significantly in the various transfer combinations (Fig. 3-10a). As already emphasized, the changes in responsiveness after transfer can be accounted for by changes in the responsiveness of CIN+ NK cells after transfer (Fig. 3-7 and 3-8).

As previously discussed, high responsiveness correlated with a high percentage of KLRG1-hi cells in previous studies (Corral, Hanke et al. 2000; Fernandez, Treiner et al. 2005; Joncker, Fernandez et al. 2009). Direct assays confirmed that in normal B6 mice, KLRG1^{high} NK cells reproducibly responded better to anti-receptor antibodies than KLRG1^{low} NK cells (Fig. 3-10b). Interestingly, WT NK cells that had been transferred to MHC-I-deficient hosts exhibited a modestly reduced percentage of KLRG1^{high} NK cells, compared to WT NK cells that had been transferred to WT hosts (Fig. 3-10c). These differences were small, however, compared to the functional differences we observed (Fig. 3-7 and 3-8), suggesting that resetting results in changes in both the composition of NK subsets and their functional activity.

Expression of the CD11b and CD27 markers define 4 NK subsets, labeled R0 (CD27-CD11b-), R1 (CD27+ CD11b-), R2 (CD27+ CD11b+) and R3 (CD27- CD11b+). The R3 population includes somewhat higher percentages of Ly49+ and KLRG1^{high} NK cells, and greater expression of genes involved in NK effector functions ((Hayakawa and Smyth 2006; Chiossone, Chaix et al. 2009), data not shown). Consistent with these features, but in contrast to the findings of others (Hayakawa and Smyth 2006), we consistently observed that R3 NK cells were more responsive than R2 NK cells, or in some cases roughly equal in responsiveness (Fig. 3-10d, and data not shown). R1 and R0 cells show substantially less functional activity. Although there were modest changes in the percentages of R2 and R3 cells when comparing the various transfer combinations, the changes did not provide an adequate explanation for the functional differences we observed (Fig. 3-10d).

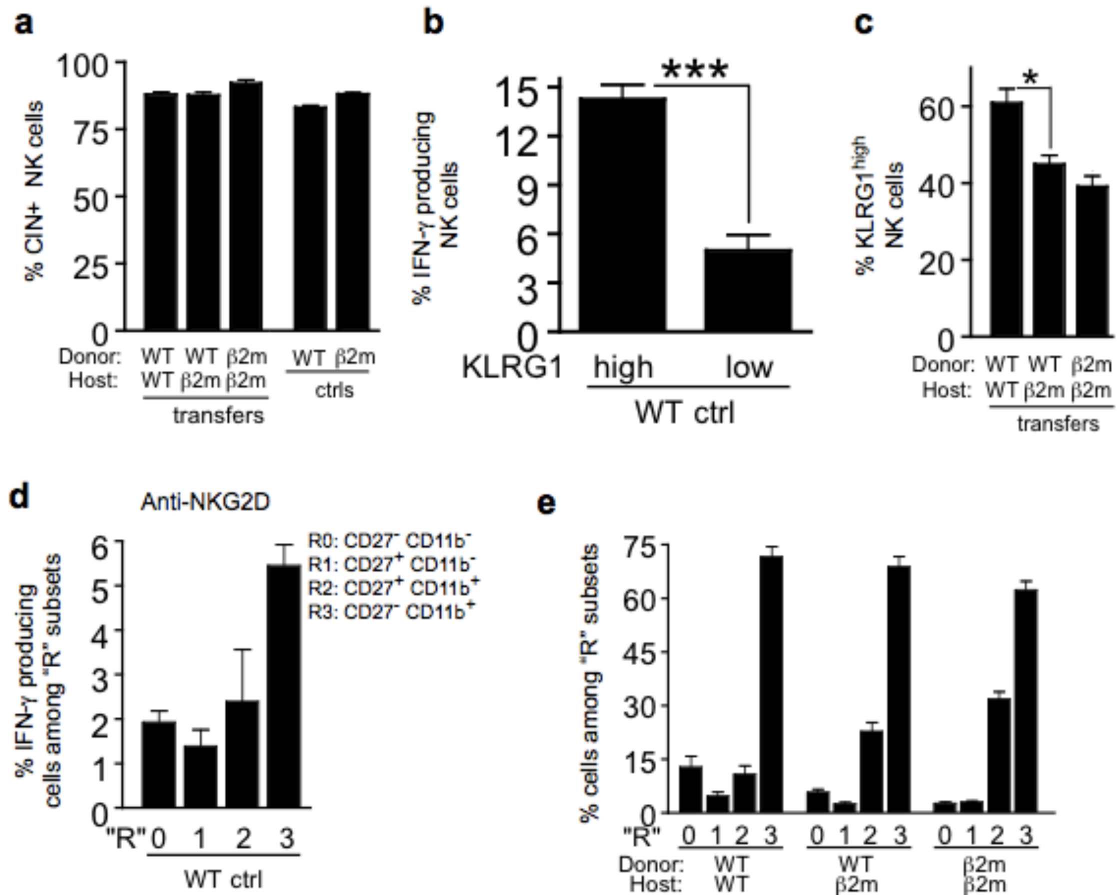


Figure 3-10. Phenotypic changes associated with resetting (a) 10 d after transfer of CFSE-labeled splenocytes, the frequencies of CIN+ and CIN− NK cells were determined among gated donor NK cells that remained undivided after transfer by staining with Ly49C, Ly49I, and NKG2A antibodies. (b) IFN-γ response of KLRG1high and KLRG1low WT NK cells to stimulation with 5 μg/ml plate-bound NKG2D antibody. (c) Percentages of KLRG1high cells among undivided donor NK cells at day 10 after transfer. (d) IFN-γ response of CD27− CD11b−, CD27+ CD11b−, CD27+ CD11b+, and CD27− CD11b+ NK cell subsets from WT mice upon stimulation with 5 μg/ml anti-NKG2D. (e) Distribution of undivided donor NK cells among the CD27/CD11b-defined subsets at day 10 after transfer. Asterisks indicate a statistically significant difference as calculated by two-tailed paired (b) or unpaired (c) Student's t test (*, $P < 0.05$; ***, $P < 0.0005$). Experiments were performed two to four times with n = 3–6 mice, except one repeat of panel d where n = 2 mice. Data represent means ± SEM.

Concluding remarks

Data presented in this chapter demonstrate that WT NK cells exposed to *β2m*-deficient hosts assume a hyporesponsive phenotype, similar to that of NK cells in *β2m*-deficient mice. This was true not only when the NK cells differentiated from hematopoietic precursor cells in an MHC-deficient environment, as in the fetal liver chimeras, but also when mature WT NK cells were transferred to MHC-deficient mice. Furthermore, it also occurred when the WT NK cells differentiated in a mixture with MHC-deficient hematopoietic cells, in MHC-deficient hosts. The NK cells that developed in these hosts were hyporesponsive to both stimulation with anti-receptor antibodies, MHC-deficient tumor cells, and RAE-1-expressing MHC+ spleen cells, similar to NK cells in unmanipulated MHC-deficient mice, or CIN- NK cells in WT mice.

As demonstrated by the CFSE-labeling experiment, downward resetting occurred in mature NK cells (that have not undergone cell divisions after adoptive transfer) and does not require irradiation, or any inflammatory signals associated with irradiation, as shown by adoptive transfer into unirradiated hosts.

Taken together these findings suggest several important conclusions. One is that MHC-deficient non-hematopoietic cells dominantly induce hyporesponsiveness. Whether the induction of hyporesponsiveness is imposed by a specific MHC-deficient non-hematopoietic cell type, or many, remains to be determined. The second conclusion is that NK cells exposed to MHC I-deficient non-hematopoietic cells also exhibit tolerance to MHC-deficient cells, as they fail to reject these cells *in vivo*. Hence, low responsiveness and tolerance to MHC-deficient grafts were correlated in these studies. Third, NK cells exhibit plasticity with respect to the basal level of NK cell responsiveness, a conclusion suggested by the similar results obtained with NK cells that differentiated in MHC-deficient hosts (i.e. in the chimeras) and mature NK cells that were transferred to MHC-deficient hosts for a few days. The fourth conclusion, which follows from the third, is that the mechanisms that set responsiveness must be of a type that can be adjusted quite rapidly. Finally, the results suggest that the induction of hyporesponsiveness by MHC-deficient cells dampens NK activation through multiple activating receptors, including NKG2D, NKR-P1c, and, presumably, the activating receptor responsible for killing of MHC-deficient normal cells as well as of the MHC-deficient tumor cell line RMA-S. The latter receptor may be SLAM family receptors, given the evidence that they play a role in lysis of both MHC-deficient normal cells and RMA-S cells (see Chapter 4 for analysis of responses to SLAM receptors).

Chapter 4: Upward Resetting of NK cell responsiveness and maintenance of tolerance

Having established that NK cells that develop in or are transferred into MHC I-deficient hosts acquire a hyporesponsive phenotype as well as tolerance to *β2m*$^{-/-}$ hematopoietic grafts we proceeded to analyze the responsiveness and tolerance of NK cells that develop in MHC I – expressing hosts or following adoptive transfer into such animals. Some of the experiments to address this issue were carried out in parallel with the studies in the previous chapter in order to provide direct comparisons, and hence some of the figures include the corresponding data from Chapter 3.

Chapter 4, Section 1. Responsiveness of NK cells developing in WT chimera hosts

To study the role that *β2m*$^{+/+}$ non-hematopoietic cells play in NK cell development in terms of functional responsiveness and tolerance towards *β2m*$^{-/-}$ grafts we generated the following fetal liver chimeras:

Chimeras (Donor fetal liver→ irradiated host)	**Hematopoietic cells (including NK cells)**	**Non-hematopoietic cells**
WT→WT	WT	WT
β2m$^{-/-}$→*β2m*$^{-/-}$	*β2m*$^{-/-}$	*β2m*$^{-/-}$
β2m$^{-/-}$→WT	*β2m*$^{-/-}$ (+few WT cells)	*β2m*$^{-/-}$
WT+*β2m*$^{-/-}$→WT	WT & *β2m*$^{-/-}$	*β2m*$^{-/-}$

As in the previous chapter, NK cell responsiveness was analyzed 13-20 weeks following reconstitution with fetal liver cells, by stimulating splenic NK cells from the chimeras with plate-bound antibodies against different activating NK receptors followed by staining for CD107a and/or intracellular IFN-γ (Fig. 4-1).

Interestingly, and somewhat unexpectedly, MHC-deficient NK cells that developed in WT hosts exhibited a responsive phenotype. This occurred whether or not the *β2m*$^{-/-}$ NK cells were mixed with WT hematopoietic cells. In such mixed chimeras, the *β2m*$^{-/-}$ NK cells in the mixture were as responsive as WT NK cells in the mixture, when they were analyzed separately. Each of these populations were responsive to a similar extent as WT NK cells that had developed in control WT→ WT chimeras. Since the large majority of NK cells and other hematopoietic NK cells were MHC-deficient in e.g. *β2m*$^{-/-}$ → WT chimeras, yet the NK cells exhibited high responsiveness, these data suggested that in chimeras, responsiveness, using this assay, is largely determined by the non-hematopoietic cells of the host.

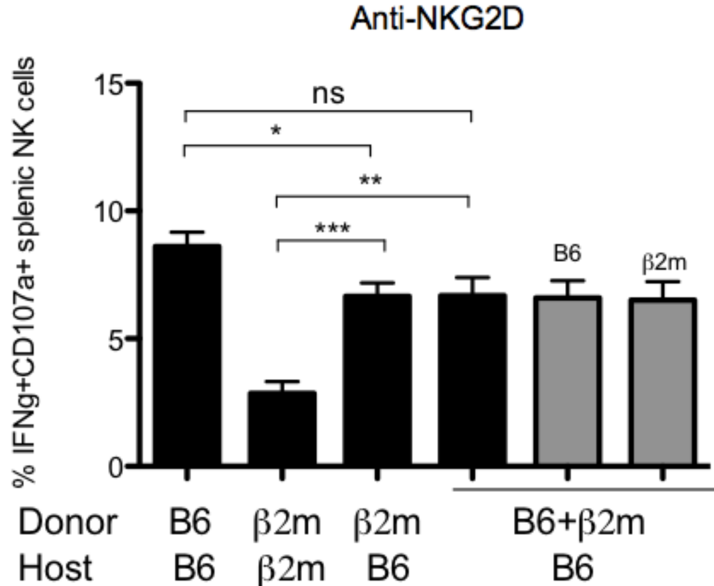


Figure 4-1. NK cells developing in MHC I – expressing hosts are responsive. Lethally irradiated and NK depleted hosts were reconstituted with WT, $\beta 2m^{-/-}$, or a 1:1 mixture of WT and $\beta 2m^{-/-}$ fetal liver cells. 13-20 weeks after reconstitution NK cells were stimulated for 5 h with 5μg/ml of plate-bound anti-NKG2D antibodies. The percentage of donor NK cells expressing IFN-γ and CD107a was determined by flow cytometry. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$). Data represent means ± SEM.

Responsiveness of NK cells to antibody-mediated stimulation of SLAM family activating receptors is dependent primarily on MHC-expression by non-hematopoietic cells

NKG2D is not involved in recognition of $\beta 2m^{-/-}$ hematopoietic cell grafts (Guerra, Tan et al. 2008) and may therefore not be representative of the relevant activating receptor for such responses. Recently the SLAM family receptors have been shown to play a critical role in immune surveillance of hematopoietic cells by NK cells, and were specifically shown to be essential for rejection of MHC-deficient hematopoietic cell grafts (Dong, Cruz-Munoz et al. 2009). When we performed the *ex vivo* stimulation assay using antibodies against two different SLAM-family receptors expressed by NK cells, 2B4 and Ly108, we observed the same pattern as we had before for NKG2D stimulation: the responsiveness of NK cells was determined primarily by MHC expressed by non-hematopoietic cells (Figure 4-6). High responses were observed with NK cells from WT→WT, $\beta 2m^{-/-}$ →WT, and mix →WT chimeras, but low responses were observed with NK cells from $\beta 2m^{-/-}$→$\beta 2m^{-/-}$, WT→$\beta 2m^{-/-}$, and mix→$\beta 2m^{-/-}$chimeras.

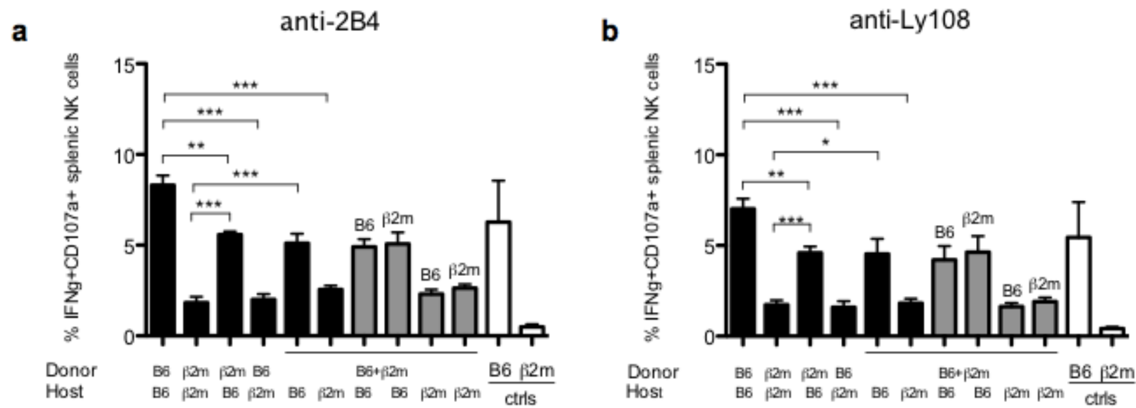


Figure 4-2. Responsiveness of NK cells to antibody-mediated stimulation of SLAM family activating receptors. Lethally irradiated and NK depleted hosts were reconstituted with WT, *β2m*$^{-/-}$, or 1:1 mix of WT and *β2m*$^{-/-}$ fetal liver cells. 15-21 weeks after reconstitution NK cells were stimulated for 5 h with 5μg/ml of plate-bound (a) anti-2B4 or (b) anti-Ly108 antibodies. The percentage of donor NK cells expressing IFN-γ and CD107a was determined by flow cytometry. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (**, $P < 0.005$; ***, $P < 0.0005$). Data represent means ± SEM.

Tolerance of NK cells developing in WT chimera hosts

As in the previous chapter, we analyzed tolerance of NK cells developing in WT chimeras by examining chimerism as well as rejection of *β2m*-deficient grafts. Figure 4-3 shows that chimerism of the hematopoietic compartment was maintained long-term in the mix→WT chimeras, a clear indication that tolerance was sustained in these animals.

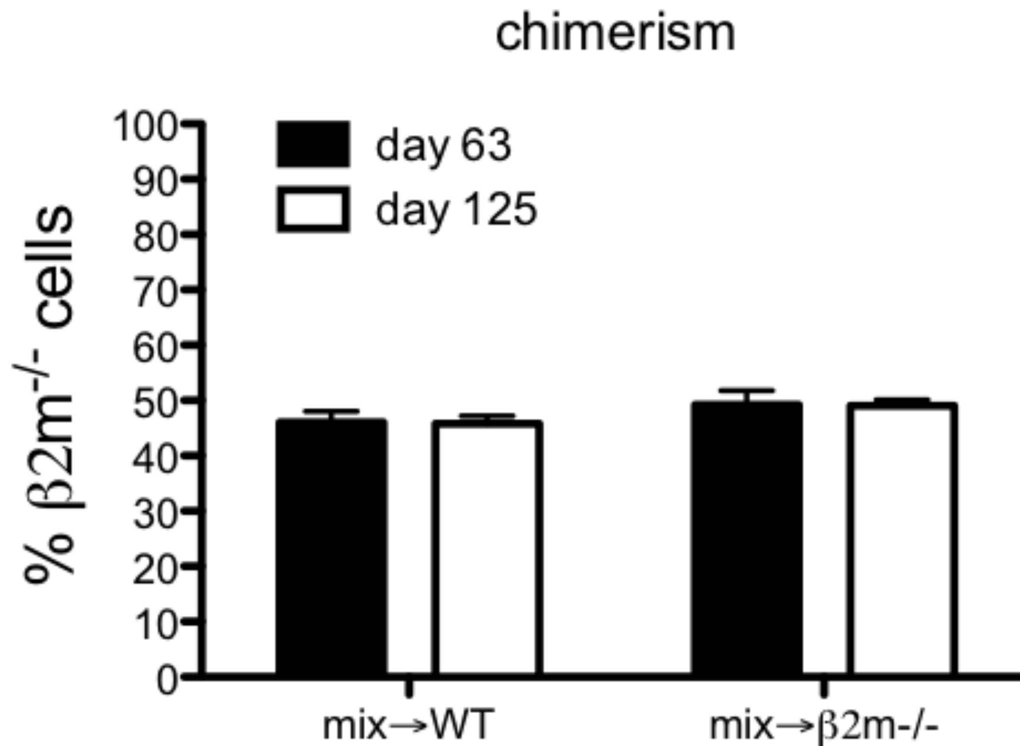


Figure 4-3. Long-term maintenance of chimerism in mixed fetal liver chimeras. Lethally irradiated WT or $\beta 2m^{-/-}$ hosts were reconstituted with a 1:1 mixture of WT and $\beta 2m^{-/-}$ fetal liver cells (n=5 mice in each group). Chimerism was determined by flow cytometry of PBLs stained for H-2K^b (in this experiment) or the congenic CD45 marker (in experiments not shown) 63 and 125 days following reconstitution. Data represent means ± SEM.

As a second way to measure tolerance to *β2m*-deficient cells we challenged the mice with MHC-deficient grafts, because it was possible that engraftment provides extra signals compared to the steady state situation. The WT hosts received a 1:1 mixture of differentially CFSE-labeled wild-type and $\beta 2m^{-/-}$ splenocytes or bone-marrow cells and we assessed the depletion of MHC-deficient cells relative to WT cells 18-40 hours later by flow cytometry. Figure 4-4 shows that NK cells that developed in WT→WT chimeras rejected the grafts. Furthermore, as already mentioned in the previous chapter, NK cells that developed in the presence of MHC-I deficient cells of non-hematopoietic origin, such as those in WT→$\beta 2m^{-/-}$ and mix→$\beta 2m^{-/-}$chimeras, were

tolerant of MHC-deficient grafts. Notably, NK cells that developed in the presence of MHC-I deficient cells of hematopoietic origin, such as those in *β2m*$^{-/-}$ →WT or mix →WT chimeras, were also tolerant of MHC-I deficient grafts. Therefore although NK cells in these latter chimeras showed higher responsiveness to antibody-mediated stimulation through activating receptors, they were tolerant of MHC-deficient grafts *in vivo*.

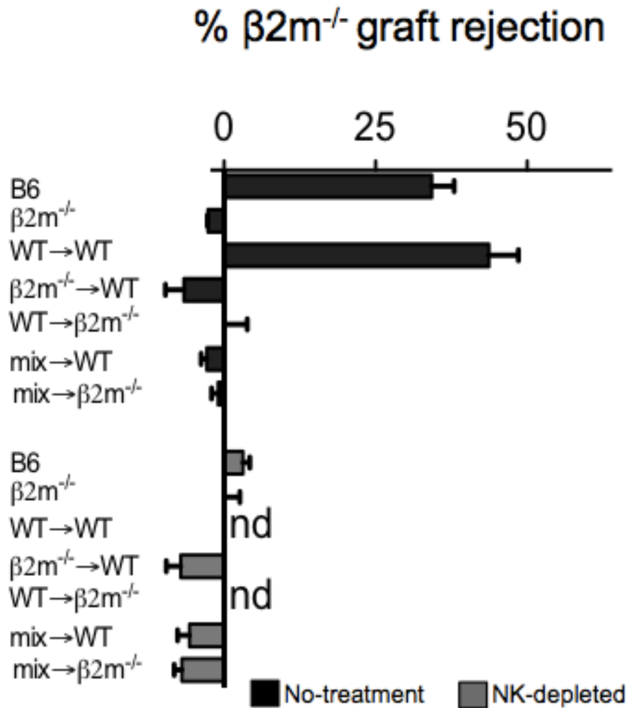


Figure 4-4. The tolerance of developing NK cells is set depending on the MHC environment in which they mature. After 9–17 wk of reconstitution, chimeras were tested for rejection of *β2m*$^{-/-}$ spleen cells. Unmanipulated B6 and *β2m*$^{-/-}$ mice were tested in parallel as controls for rejection and tolerance, respectively. The rejection assay was performed by injecting CFSE^{high}-labeled *β2m*$^{-/-}$ spleen cells mixed with an equal number of CFSE^{low}-labeled WT spleen cells as an internal control. Some groups of chimeras were pretreated i.p. twice (on days -2 and -1) with 200 µg PK136 (NK1.1) antibody (NK depleted), as indicated. Rejection of *β2m*$^{-/-}$ spleen cells was determined by flow cytometry of spleen cells 18 h later. Data represent means ± SEM (n = 2–5 mice). The experiment was performed three times. nd, not done.

NK cells developing in the presence of MHC I – deficient cells do not respond to the NK-sensitive cell line RMA-S

Because MHC-deficient tumor cells may stimulate NK cells in a manner different from MHC-deficient bone marrow and spleen cells, we next tested the ability of NK cells developing in WT fetal liver chimera hosts to respond to *ex-vivo* stimulation with the MHC-deficient RMA-S cell line as well as its NK resistant MHC+ counterpart RMA, following *in-vivo* pre-activation of NK cells in the chimeras with the TLR3 ligand poly(I:C).

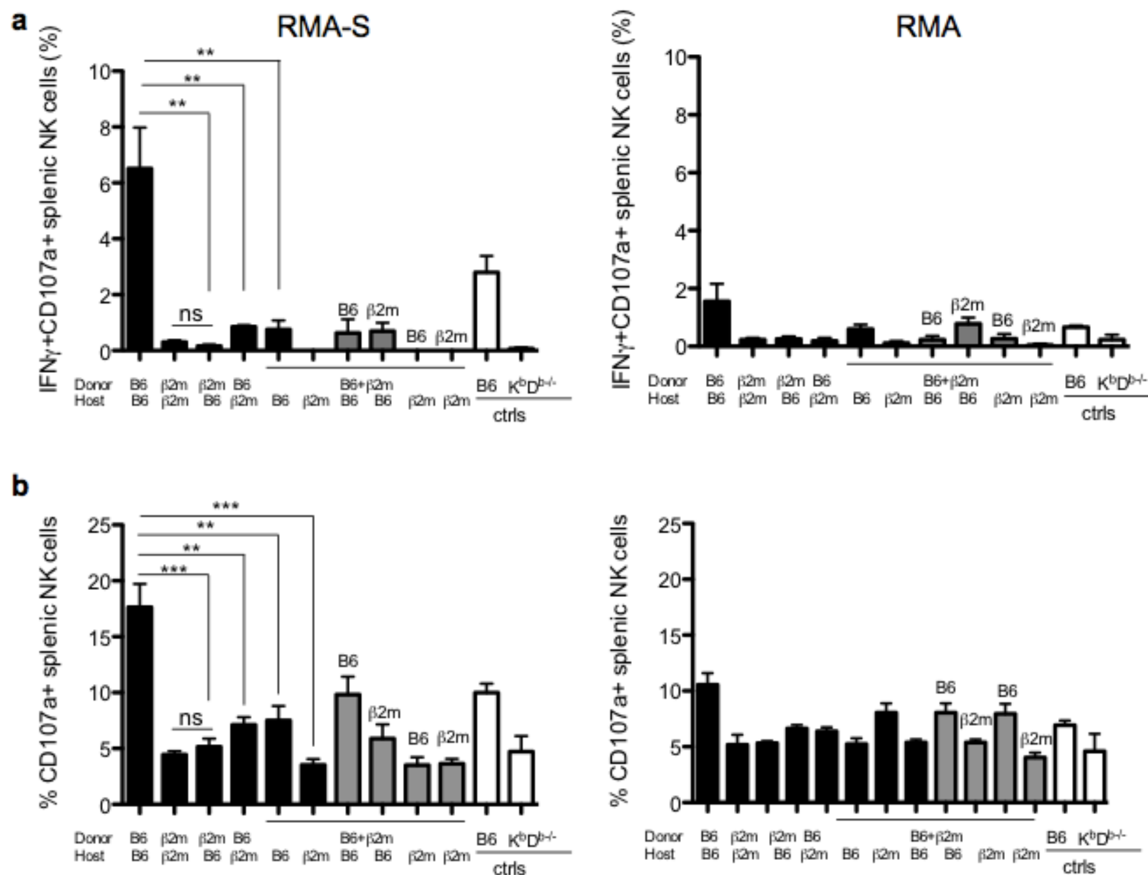


Figure 4-5. NK cells developing in the presence of MHC I-deficient nonhematopoietic cells do not respond to the NK-sensitive cell line RMA-S. (a,b) 16 weeks post reconstitution, groups of chimeras (n=5) were pre-treated with 200 μg poly(I:C) intraperitoneally. 24 hours later splenocytes were harvested and co-incubated with $1x10^5$ RMA-S or RMA cells for 5 hours. The percentages of (a) IFN-γ+CD107a+ or (b) CD107a+ donor NK cells were determined by flow cytometry. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (**, P < 0.005; ***, P < 0.0005). Data represent means ± SEM. Results are preliminary.

Compared to stimulation with RMA cells, stimulation with RMA-S tumor cells caused a significant increase in IFN-γ production and CD107a expression in NK cells from WT→WT chimeras (Fig. 4-5). In contrast, NK cells from all the other chimeras tested, all of which contained MHC I-deficient cells, showed little or no NK cell activation in response to RMA-S cells. These included chimeras in which the host was MHC-deficient (e.g. WT→ *β2m*$^{-/-}$*,* or mix→ *β2m*$^{-/-}$*),* as well as chimeras that contained MHC-deficient cells among the donor cells (e.g. *β2m*$^{-/-}$→WT, or mix→ WT). These results are preliminary, but they suggest that the presence of either *β2m*$^{-/-}$ hematopoietic cells or non-hematopoietic cells in the chimeras resulted in impaired responses to RMA-S cells. This pattern was similar to the pattern of tolerance to MHC-deficient spleen or bone marrow grafts, but was distinct from the pattern of "responsiveness" defined as stimulation with antibodies specific for NK-activating receptors, where NK cells from *β2m*$^{-/-}$→WT and mix→ WT chimeras were both responsive (see discussion).

NK cells developing in the presence of MHC I – deficient hematopoietic cells are tolerant of RAE1 expressing grafts

Having studied responses of the various chimeras to MHC-deficient tumor cells, spleen cell grafts and bone marrow cell grafts, we examined rejection responses to spleen cells from *Raet1e* transgenic mice (Ehrlich, Ogasawara et al. 2005). As already discussed in Chapter 3, the WT→*β2m*$^{-/-}$ and mix→*β2m*$^{-/-}$ chimeras showed impaired rejection of *Raet1e* tg cells, indicating that *β2m*$^{-/-}$ non-hematopoietic cells in the chimeras induce hyporesponsiveness to *Raet1e* tg cells. Interestingly, impaired rejection was also observed in *β2m*$^{-/-}$→ WT or mix→WT chimeras, indicating that *β2m*$^{-/-}$ hematopoietic cells in the chimeras also induce hyporesponsiveness to *Raet1e* tg cells. Therefore, *β2m*$^{-/-}$ hematopoietic cells induce hyporesponsiveness to *Raet1e* tg cells, but not to antibody crosslinking of the NKG2D or SLAM family activating receptors.

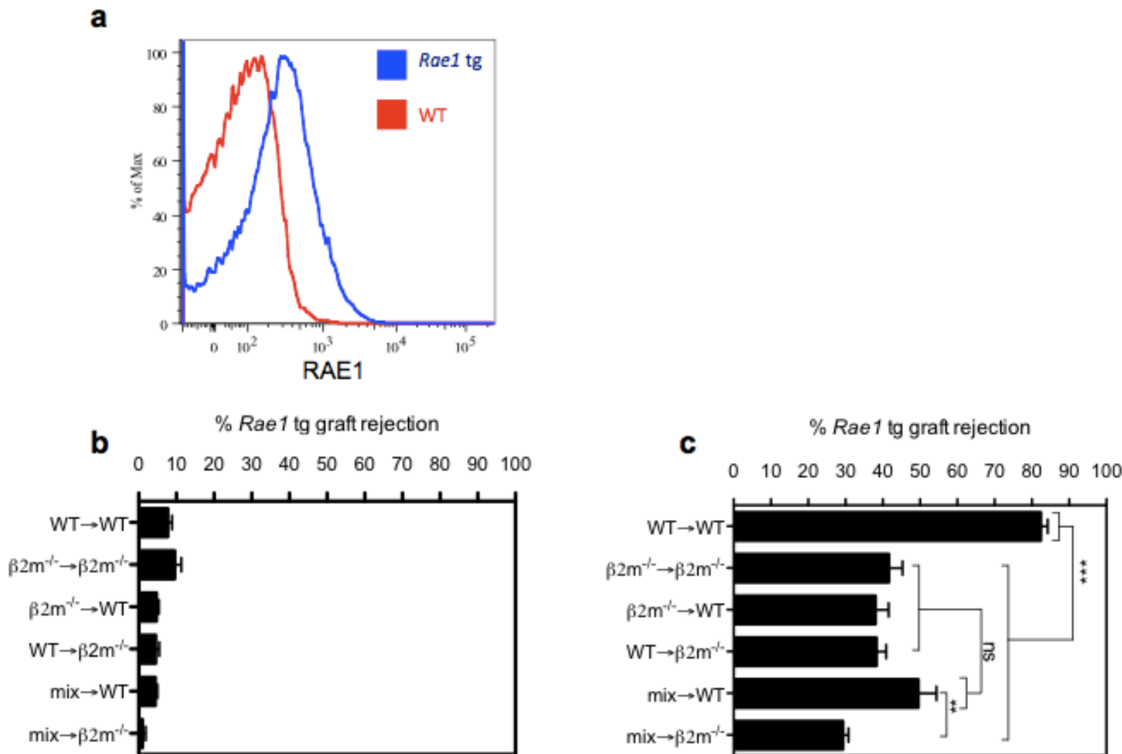
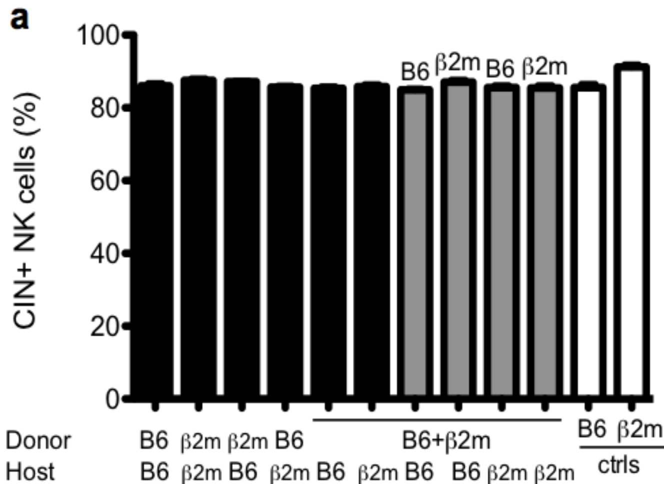


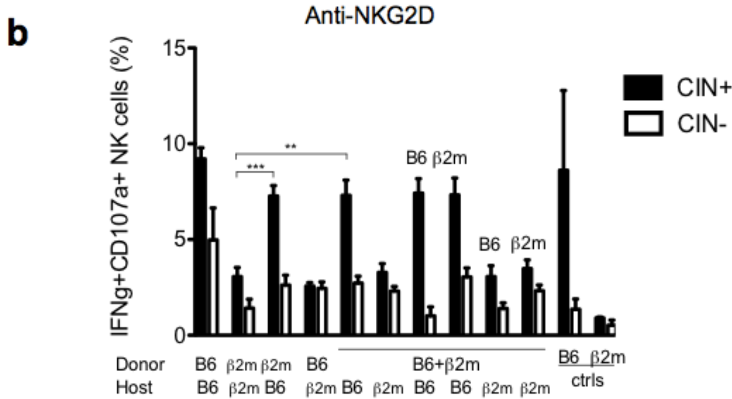
Figure 4-6. MHC I-deficient hematopoietic or non-hematopoietic cells in chimeras result in defective rejection of *Raet1e*-transgenic spleen cells (a) Representative plot of RAE1 expression on transgenic splenocytes and splenocytes from WT littermate controls. (b and c) 13-20 weeks post reconstitution, groups of chimeras (n = 3-5) received grafts of CFSE^{high}-labeled *Raet1* tg spleen cells mixed with an equal number of CFSEl^{ow}-labeled WT spleen cells. Rejection of *Raet1* tg target cells was determined by flow cytometry of spleen cells 48 h later. Mice were either left untreated (b) or pretreated i.p. (on day −1 relative to the time of engraftment) with 200 µg poly(I:C) (c). The experiment was done once for no pretreatment (b) and three times with poly(I:C) pretreatment (c) with n = 3-5 mice for each. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (**, $P < 0.005$; ***, $P < 0.0005$). Data represent means ± SEM.

Phenotype of NK cells developing in WT chimera hosts

Analysis of Ly49I, Ly49C, and NKG2A/C/E receptor expression on NK cells from fetal liver chimeras showed a similar percentage of CIN+ NK cells in all groups (Fig. 4-7a) with the CIN+ NK cells being the responsive subset in WT hosts and both subsets being hyporesponsive in *β2m*-deficient host regardless of the NK cell genotype (Fig. 4-7b).

Analysis of KLRG1 expression in chimeras revealed a higher percent of KLRG1^{high} NK cells in WT→WT chimeras compared to NK cells that developed in *β2m*$^{-/-}$ hosts. NK cells that developed in *β2m*$^{-/-}$→ WT or mix→WT chimeras tended to show intermediate percentages of KLRG1^{high} NK cells (Fig. 4-7c).





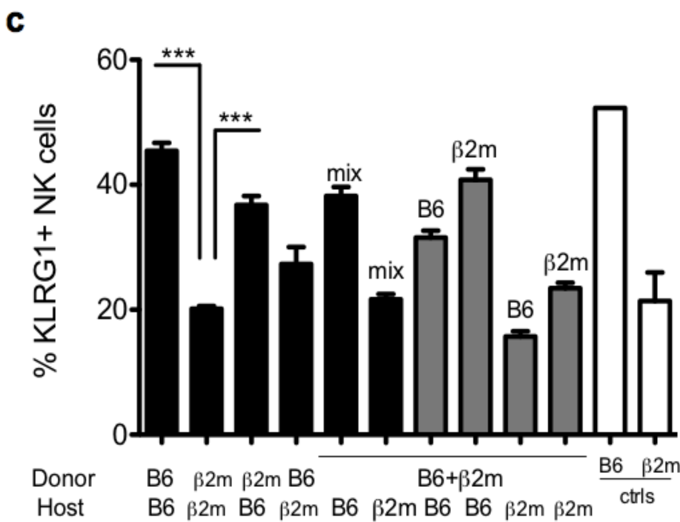


Figure 4-7. Phenotype of NK cells in fetal liver chimeras. Donor-derived NK cells (gated on the congenic CD45 marker) were examined 13-20 weeks after reconstitution. (a) Splenic NK cells were stained with antibodies for Ly49I, Ly49C, and NKG2A/C/E and the percentages of cells expressing one or more of these self MHC-specific inhibitory receptors is shown. (b) analysis of IFNg and CD107a expression on CIN+ and CIN- NK cells following a 5hr stimulation with 5 μg/ml of plate-bound anti-NKG2D antibody (MI-6). (c) Splenic NK cells were stained with antibody for KLRG1, and the percentages of positive cells are depicted. n=5 for chimeric mice and 2-3 for ctrls; For some comparisons, statistical significance was determined with a two-tailed unpaired (c) or paired (b) Student's t test (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$). Data represent means ± SEM.

Chapter 4, Section 2. Altered responses of mature MHC-deficient NK cells, after transfer to MHC+ hosts.

Hyporesponsive NK cells acquire responsiveness to stimulation with antibodies against activating receptors after transfer to MHC I+ mice: upward "re-setting".

Having collected data on the function of NK cells that differentiated in the presence of MHC+ or MHC- cells, we tested whether the hyporesponsiveness of already mature NK cells from MHC-deficient mice could be restored after adoptive transfer to MHC+ mice. A caveat is that MHC I-deficient splenocytes could not be studied after transfer into normal WT hosts because the host's NK cells rejected the donor cells. Therefore we used an NK cell-deficient strain (NKD mice (Kim, Iizuka et al. 2000)) as recipients.

When MHC I-deficient splenocytes were transferred to irradiated NKD (MHC I+) mice for ten days, the responsiveness of the NK cells was significantly enhanced compared to the responsiveness of the same NK cells after transfer to MHC I-deficient hosts (Fig. 4-8). In some repetitions, the response approached the level observed in WT mice (Fig. 4-8a), and in others, it was intermediate between WT and MHC I-deficient (Fig. 4-8b). Similar results were obtained whether the adoptive transfer hosts were pre-treated with poly(I:C) for 36 hours to enhance NK activity (Fig. 4-8 b and c) or were tested without pre-treatment (Fig. 4-8a). The increase in responsiveness after transfer to NKD mice was most pronounced in the CIN+ population, whereas the CIN- population was by comparison relatively hyporesponsive (Fig. 4-8c). Taken together, these data and data from Chapter 3 indicate that the responsiveness of peripheral NK cells can be re-set both downwards or upwards depending on the steady state MHC environment to which the NK cells are exposed.

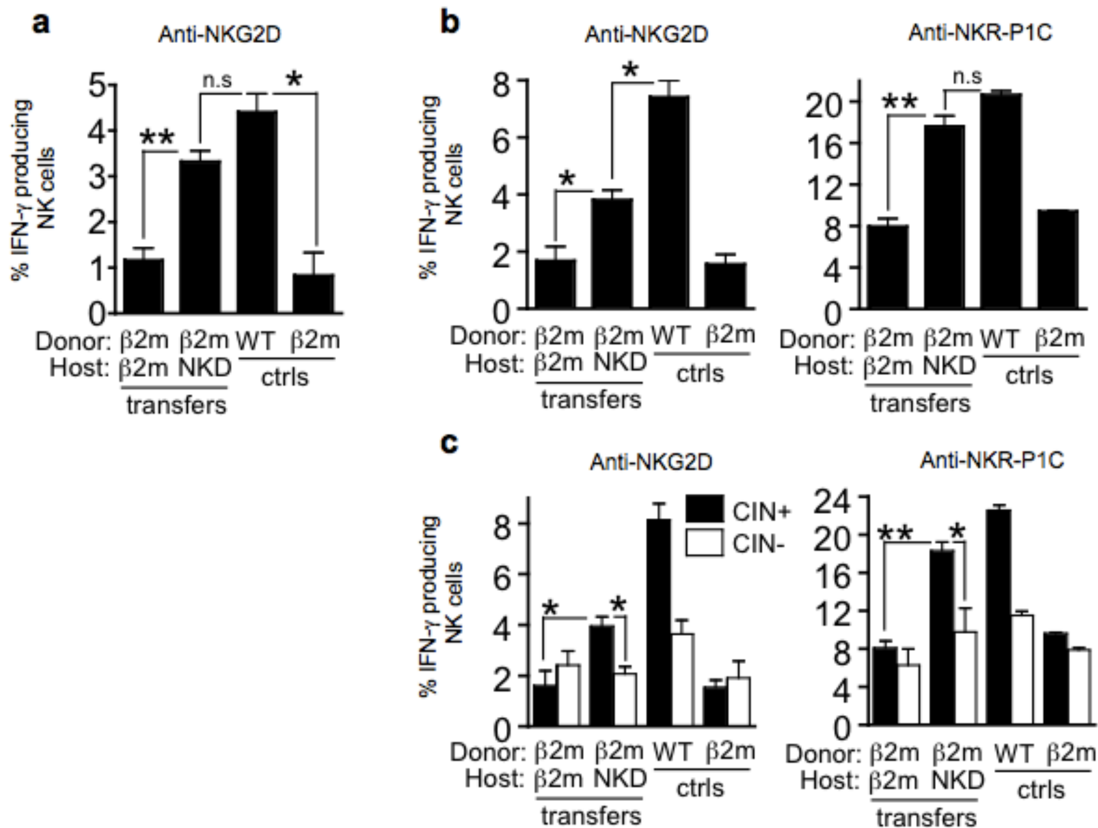


Figure 4-8. Hyporesponsive NK cells re-set their functional potential upwards when exposed to MHC I-expressing cells. Responses of $\beta 2m^{-/-}$ splenocytes after transfer to MHC-I-expressing NK cell-deficient (NKD) or *β2m*-deficient hosts. (a, b) Stimulations and assays were carried out as in Figure 3-7. Recipients were either left untreated (a) or treated with poly I:C (b) 36 hours prior to *in vitro* stimulation, in order to augment the responses (b, c). Experiments were repeated 4 times with n = 3-6 mice for each transfer group. For relevant comparisons, statistical significance was determined with a two-tailed unpaired (a-c) or paired (comparisons of CIN+ vs CIN- in c) Student's t test (* p<0.05; ** p<0.005; *** p<0.0005; n.s: non-significant). Data represent means ± SEM.

Upward resetting occurs in mature NK cells

To distinguish between upward resetting that occurs in mature NK cells after adoptive transfer and the possibility that the NK cells studied were newly differentiated after transfer, we labeled donor cells with CFSE prior to transfer and 10 days later analyzed the responsiveness of NK cells with various levels of CFSE dilution.

By gating on NK cells that had not divided after transfer, we observed the same patterns of responsiveness that we had observed in the bulk populations: MHC I-deficient NK cells transferred to NKD hosts exhibited relatively high responsiveness (Fig. 4-9). These data argue that upward resetting occurs in non-dividing mature NK cells, as opposed to representing the activity of newly differentiated NK cells that arose after transfer.

We were unable to test upward resetting following adoptive transfer into un-irradiated hosts because of rejection of donor cells by residual NK cells in unirradiated NKD mice.

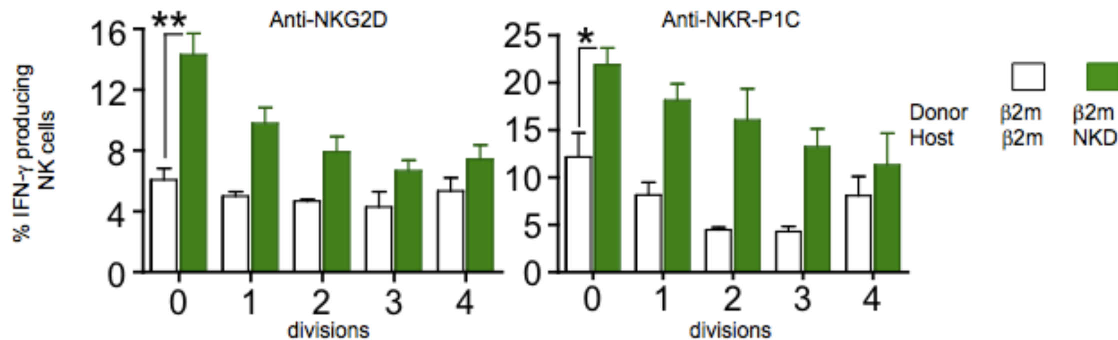


Figure 4-9. Upward functional re-setting occurs even in NK cells that do not divide after transfer. CFSE-labeled *β2m*-deficient splenocytes were transferred to irradiated NKD or *β2m*-deficient hosts. 10 d after transfer, NK cells were stimulated and assayed as in Fig. 4-8, except that the donor cells were gated based on CFSE level, distinguishing cells that had divided 0, 1, 2, 3, or 4 times after initial labeling. Experiments were repeated two to four times with n = 3–6 mice for each. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$). Data represent means ± SEM.

Maintenance of tolerance of NK cells following adoptive transfer

We next determined whether the increased responsiveness of *β2m*$^{-/-}$ NK cells that occurred after transfer to WT hosts was accompanied by a loss of tolerance of the cells to *β2m*$^{-/-}$ cells. To test this possibility, 10 days after transfer we challenged the adoptive transfer recipients with MHC-deficient grafts. Despite the increased responsiveness of the NK cells from the adoptive transfer recipients, they failed to reject *β2m*$^{-/-}$ spleen cell grafts (Fig. 4-10). As already emphasized, these mice contained normal numbers of NK cells, which exhibited high responsiveness in the *in vitro* assay. Therefore, the high responsiveness of NK cells in *β2m*$^{-/-}$→NKD adoptive transfer scenario to anti-receptor crosslinking was not correlated with the capacity to reject MHC-deficient grafts.

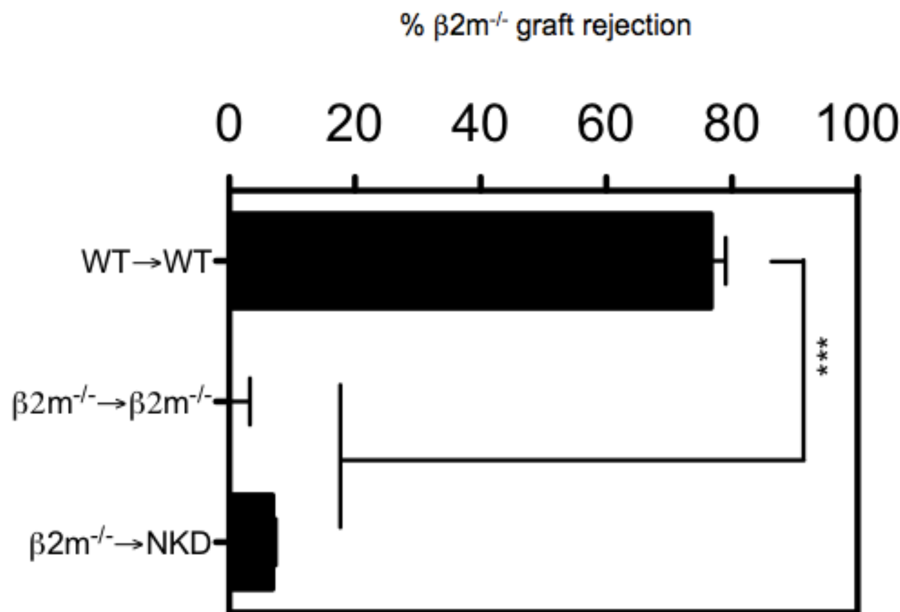


Figure 4-10. Tolerance of NK cells to MHC I-deficient grafts after adoptive transfer. At day 10 after transfer, groups of adoptive transfer recipients (n = 3–4) received grafts of CFSE^{high}-labeled *β2m*$^{-/-}$ spleen cells mixed with an equal number of CFSE^{low}-labeled WT spleen cells. Rejection of *β2m*$^{-/-}$ target cells was determined by flow cytometric analysis of spleen cells 18 h later. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test ($P < 0.0005$). Data represent means ± SEM.

Phenotypic changes associated with upward resetting

To determine whether upward functional re-setting correlated with changes in subset composition after transfer, we determined the phenotypes of gated CFSE^{high} NK cells, which correspond to transferred NK cells as opposed to NK cells that differentiated from precursor cells after transfer. Analysis showed that the proportions of CIN+ and CIN- NK cells among total NK cells or among those that did not divide after transfer did not change significantly in the various transfer combinations (Fig. 4-11a). As already emphasized, the changes in responsiveness after transfer can be accounted for by changes in the responsiveness of CIN+ NK cells after transfer (Fig. 3-7 and 4-8).

MHC I-deficient NK cells that had been transferred to MHC I+ hosts exhibited a modestly increased percentage of KLRG1^{high} NK cells compared to MHC I-deficient NK cells recovered from MHC I-deficient hosts.

Although there were modest changes in the percentages of R2 and R3 cells when comparing the various transfer combinations, the changes did not provide an adequate explanation for the functional differences we observed (Fig. 3-10d).

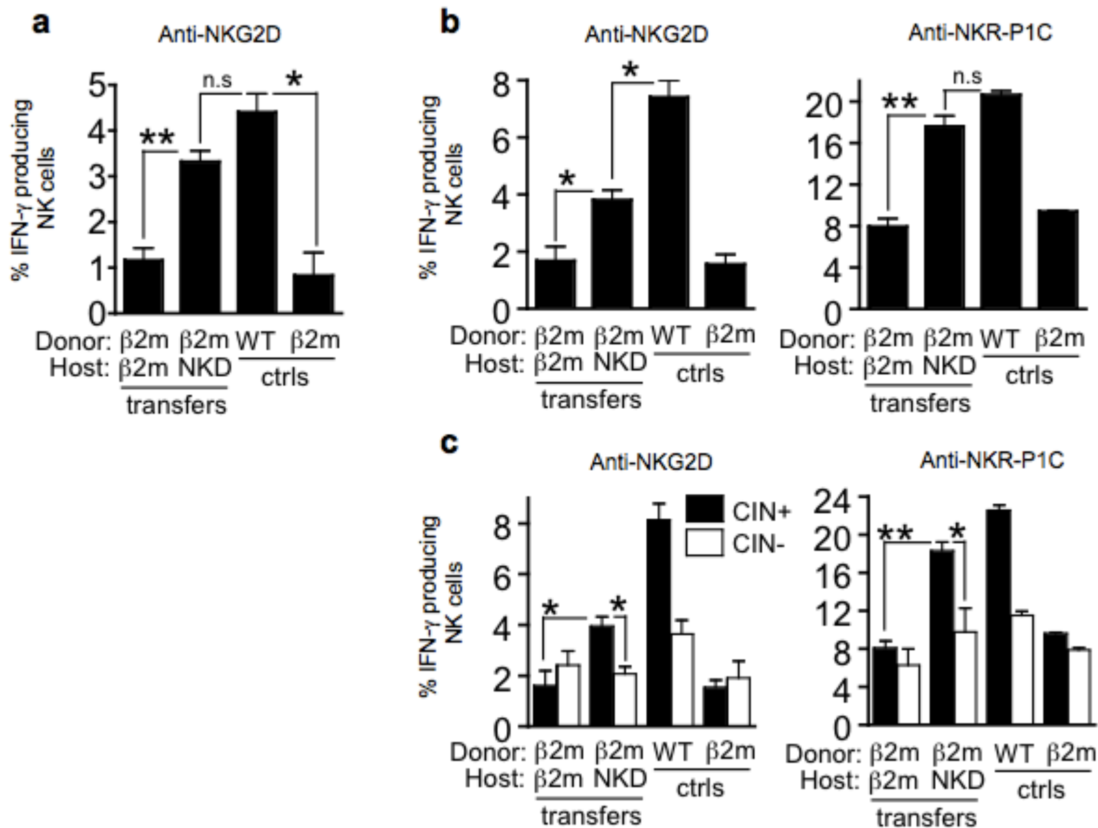


Figure 4-11. Phenotypic changes associated with resetting. (a) 10 d after transfer of CFSE-labeled splenocytes, the frequencies of CIN+ and CIN− NK cells were determined among gated donor NK cells that remained undivided after transfer by staining with Ly49C, Ly49I, and NKG2A antibodies. (b) Percentages of $KLRG1^{high}$ cells among undivided donor NK cells at day 10 after transfer. (c) Distribution of undivided donor NK cells among the CD27/CD11b-defined subsets at day 10 after transfer. Asterisks indicate a statistically significant difference as calculated by unpaired Student's t test (*, $P < 0.05$; ***, $P < 0.0005$). Experiments were performed two to four times with n = 3–6 mice. Data represent means ± SEM.

Concluding remarks

Data presented in this chapter demonstrate that NK cells, including MHC-deficient NK cells, exposed to the environment of an MHC I+ host exhibit high responsiveness to stimulation with antibodies that engage all activating receptors tested. The high responsiveness was observed in situations where the host was MHC+ ($\beta 2m^{+/+}$), even when the hematopoietic cells (including NK cells) were themselves $\beta 2m^{-/-}$, or a mixture of $\beta 2m^{-/-}$ and $\beta 2m^{+/+}$ cells. Despite the high responsiveness of NK cells in $\beta 2m^{-/-}$→WT and mix→WT chimeras, the mice were tolerant of $\beta 2m^{-/-}$ hematopoietic cells, providing the first evidence of circumstances where hyporesponsiveness and self tolerance can be dissociated. The results do not rule out a role of hyporesponsiveness in self tolerance, but they do indicate that hyporesponsiveness is not the sole mechanism of self tolerance.

The fact that self tolerance occurs in mix→$\beta 2m^{+/+}$ chimeras provides strong evidence against a simple model in which encounters of developing NK cells with $\beta 2m^{+/+}$ cells, either non-hematopoietic or hematopoietic, "licenses" the NK cells to gain the capacity to reject $\beta 2m^{-/-}$ cells. Instead, the results suggest that encounters of NK cells with $\beta 2m^{-/-}$ hematopoietic cells induces tolerance. However, in this instance, tolerance prevails despite the high responsiveness of the NK cells to crosslinking of activating receptors.

The data also suggest that responsiveness of NK cells to crosslinking of activating receptors with antibodies is not always correlated with responsiveness to NK-sensitive target cells. In the case of $\beta 2m^{-/-}$→WT and mix→WT chimeras, NK cells exhibited high responses to immobilized anti-receptor antibodies but low rejection responses against *Raet1e* transgenic spleen cells expressing RAE-1 *in vivo*, and low responses to RMA-S tumor cells in vitro. The low response to *Raet1* transgenic cells is relevant, because the NK cells in these chimeras gave high responses to immobilized antibodies binding NKG2D, the receptor for RAE-1. The low response to RMA-S tumor cells is relevant, because the NK cells in these chimeras gave high responses to antibodies binding SLAM family receptors, which are critical for NK responses to RMA-S cells. These results suggest that despite the high sensitivity of the relevant activating receptors in these chimeras, other events or receptors that participate in the interactions of target cells with NK cells are dysregulated.

One of the most important implications of the findings is that interactions of NK cells with hematopoietic cells and non-hematopoietic cells have different roles in regulating NK cell function. Interactions of NK cells with $\beta 2m^{-/-}$ non-hematopoietic cells causes the desensitization of activating receptors on the cells (see concluding chapter for more discussion of this point). In contrast, interactions of NK cells with $\beta 2m^{-/-}$ hematopoietic cells causes tolerance without having a substantial effect on activating receptor function. On this basis, we propose that there are two mechanisms of tolerance, which affect different aspects of NK cell physiology. One mechanism, controlled by interactions with non-hematopoietic cells, controls the sensitivity of NK cell activating receptors. The other, controlled by interactions with hematopoietic cells, has little direct impact on NK activating receptor signaling, but instead desensitizes other pathways that are critical for NK cell-target cell interactions. One possibility is that this mechanism diminishes signals downstream of adhesion molecules such as LFA1 and/or DNAM (which are involved in interactions with target cells) (see concluding chapter for more discussion).

The high responsiveness of NK cells in both mix→WT chimeras and $\beta 2m^{-/-}$→WT has another important implication. These results show that $\beta 2m^{-/-}$ NK cells can exhibit high responsiveness.

Indeed, WT and *β2m*-deficient NK cells in the same mix→$\beta2m^{+/+}$ chimeras exhibited identical, high responsiveness. These data demonstrate that responsiveness is not determined by *cis*-interactions of inhibitory receptors and MHC in the same NK cells, contrary to previous proposals (Kase, Johansson et al. 1998; Scarpellino, Oeschger et al. 2007; Bessoles, Angelov et al. 2013).

The results also extend the results in Chapter 3 showing that the responsiveness state is plastic, based on the finding that $\beta2m^{-/-}$ NK cells undergo re-setting when transferred to MHC I+ hosts. As demonstrated by the CFSE-labeling experiment, upward resetting occurs in transferred mature NK cells (that have not undergone cell divisions after adoptive transfer). This suggests that responsiveness is an adaption to the NK cell environment rather than a developmental process, and that an individual NK cell may vary in responsiveness throughout its lifetime, depending on environmental influences.

One caveat to the study is that we noticed that the responsiveness of NK cells from $\beta2m^{-/-}$→WT and mix→WT chimeras, though highly elevated compared to NK cells from $\beta2m^{-/-}$→$\beta2m^{-/-}$ chimeras, was typically slightly lower than the responsiveness of NK cells from WT→WT mice. The differences were not always statistically significant, but often they were. We believe it is unlikely that the slightly diminished responsiveness of these NK cells can account for the low responses to various target cells and tolerance to MHC-deficient grafts, and that the cells must exhibit other functional defects to account for these differences.

Finally, we showed that the changes in function and tolerance were not accompanied by relevant changes in the composition of CIN+ and CIN- NK cells, suggesting that the alterations represented adaptations of the activity of the cells as opposed to selection of cells with different activities. We also noted minor changes in the composition of CD27/CD11b defined NK subsets after adoptive transfer. The pattern of these changes did not obviously account for the increased responsiveness of NK cells exposed to MHC+ hosts. We also noted an increase in KLRG1 expression upon transfer of $\beta2m^{-/-}$ NK cells into MHC+ mice. These changes were relatively small, but they accord with our finding that KLRG1 expression generally correlates with high responsiveness to stimulation with antibodies against activating receptors.

Chapter 5: Role of inflammation in breaking NK cell tolerance

Despite their poor ability to respond to stimulation through activating receptors and mediate missing-self responses, NK cells from *β2m*-deficient animals (as well as CIN- NK cells) can nevertheless be activated in response to infection and mediate viral clearance (Tay, Welsh et al. 1995; Fernandez, Treiner et al. 2005). It is likely that the pro-inflammatory stimuli present at the time of infection are sufficient to overcome hyporesponsiveness in these cells. Therefore, we explored the effects of these stimuli on tolerance towards "missing-self" targets.

Data in chapters 3 and 4 show that there are at least two forms of tolerance to missing self:
(i) Tolerance that is accompanied by functional hyporesponsiveness to antibodies against activating receptors;
(ii) Tolerance that is accompanied by near-normal responsiveness to antibodies against activating receptors.

Sun and Lanier showed that inflammation caused by mouse cytomegalovirus (MCMV) infection can break tolerance to missing self in mixed bone marrow chimeras in wild-type hosts (Sun and Lanier 2008). That led us to hypothesize that tolerance that is accompanied by normal responsiveness is more easily broken/less stable than tolerance that is accompanied by low responsiveness. This chapter addresses the circumstances in which infection and inflammation can break tolerance of NK cells.

MCMV infection breaks tolerance of NK cells to *β2m*$^{-/-}$ cells in mix→WT chimeras but not in mix→*β2m*$^{-/-}$ chimeras.

We generated mixed fetal liver chimeras in wild-type and *β2m*$^{-/-}$ hosts, by reconstituting WT or *β2m*$^{-/-}$ mice with a 50:50 mixture of WT and *β2m*$^{-/-}$ fetal liver cells. As already shown in Chapters 3 and 4, hematopoietic cell chimerism is maintained long term in these animals if they are not further manipulated. We then infected the chimeras with MCMV and monitored the chimerism of the animals to gauge whether tolerance was broken. A specific loss of *β2m*$^{-/-}$ cells relative to WT hematopoietic cells represents a loss of tolerance.

In agreement with published data, the fraction of *β2m*$^{-/-}$ cells was diminished in the wild-type hosts shortly after infection. In contrast, however, there was no loss of chimerism in mix→*β2m*$^{-/-}$ chimeras after infection, suggesting that the mechanism(s) of tolerance in the latter scenario may be distinct (Fig. 5-1). These data support the hypothesis that tolerance that is accompanied by low responsiveness is more stable than tolerance accompanied by high responsiveness.

Interestingly, in mix→WT chimeras, loss of the *β2m*$^{-/-}$ cells was confined to the first two weeks following infection and then stabilized. This roughly correlates with viral clearance, suggesting that the loss of tolerance is transient, and self-tolerance is reestablished once the inflammatory stimulus is removed.

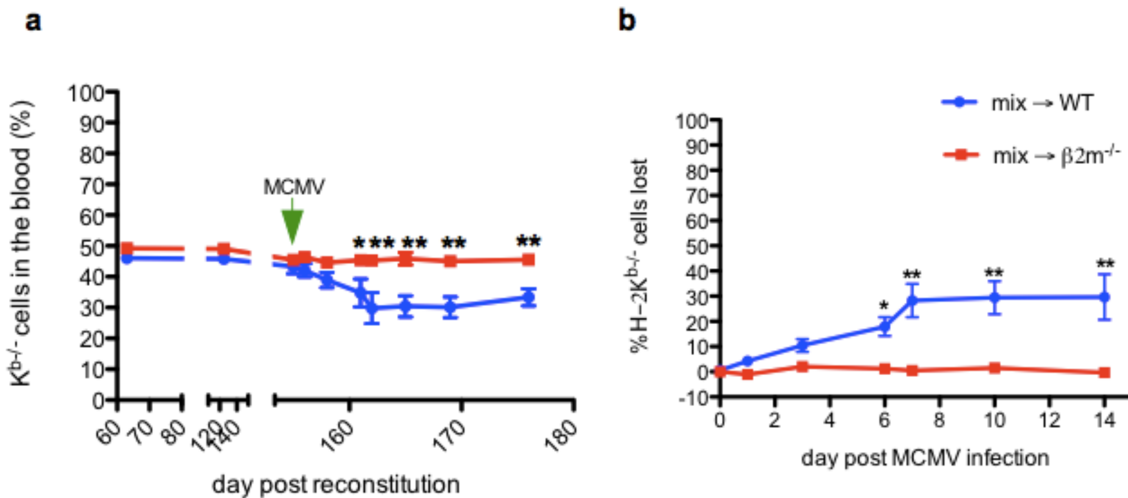


Figure 5-1. Loss of *β2m*-deficient cells from fetal liver chimeras in WT hosts. Lethally irradiated and NK depleted hosts were reconstituted with a 1:1 mixture of WT and $\beta 2m^{-/-}$ fetal liver cells. 15-21 weeks after reconstitution, the chimeras were infected intraperitoneally with 1×10^4 PFU MCMV. Chimerism was monitored by flow cytometry of PBLs with antibodies specific for H2-K^b to distinguish WT and $\beta 2m^{-/-}$ cells. (a) % H-2$K^{b-/-}$ cells in the blood. (b) % H-2$K^{b-/-}$ cells lost from the blood following MCMV infection. The percentage loss of H-2$K^{b-/-}$ cells was calculated as 100 x[1- (%H-2$K^{b-/-}{}_{[timepoint]}$/%H-2$K^{b-/-}{}_{[initial]}$)]. n=4. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (*, $P < 0.05$; **, $P < 0.005$). Data represent means ± SEM.

Self tolerance of genetically $\beta 2m^{-/-}$ NK cells in mix→WT hosts can be broken by MCMV infection.

The mix→WT chimeras contain both WT and $\beta 2m^{-/-}$ NK cells, and the previous experiment does not address whether tolerance of only one or both of these populations is broken by MCMV infection. Some published reports suggest that self tolerance is imposed by *cis*-interactions between MHC and Ly49 receptors on the same NK cells (Chalifour, Scarpellino et al. 2009; Bessoles, Angelov et al. 2013), which could imply that the $\beta 2m^{-/-}$ NK cells are self tolerant regardless of the MHC environment in which they develop. Hence, this issue is a substantive one.

In order to address this issue, we took advantage of the *NKp46*-Cre mice generated by the Vivier group (Narni-Mancinelli, Chaix et al. 2011). We crossed these mice to the ROSA26 DTA mice (Wu, Wu et al. 2006) to generate mice selectively deficient in NK cells. A 1:1 mixture of bone marrow cells from $\beta 2m^{-/-}$ mice and the NK-deficient mice, which were $\beta 2m^{+/+}$, was used to reconstitute lethally irradiated WT animals. In these chimeras, all the NK cells were MHC I-deficient, as expected (data not shown). Control chimeras were prepared with a mixture of bone marrow cells from WT mice and $\beta 2m^{-/-}$ mice, which contained both $\beta 2m^{+/+}$ and $\beta 2m^{-/-}$ NK cells. We infected these chimeras with MCMV and monitored the maintenance of $\beta 2m^{-/-}$ cells in the

blood. Interestingly, we observed a loss of *β2m*$^{-/-}$ cells in the experimental chimeras that was indistinguishable from the controls (Fig. 5-2). Since the experimental chimeras contained only *β2m*$^{-/-}$ NK cells, these data suggest that self tolerance of *β2m*$^{-/-}$ NK cells in the mix→WT chimeras can be broken by MCMV infection, and hence that the *β2m* genotype of the NK cells themselves is not decisive in determining NK cell tolerance nor in the mechanism resulting in broken tolerance in MCMV infected mice.

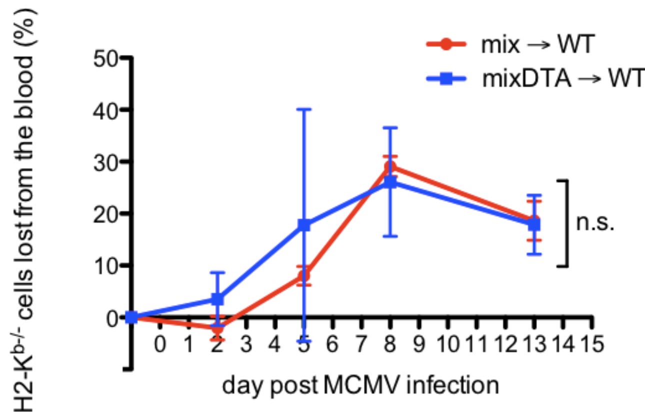


Figure 5-2. MHC I expression on NK cells is dispensable for establishing "missing-self" tolerance. Lethally irradiated and NK depleted hosts were reconstituted with a 1:1 mixture of NKp46Cre x ROSA26DTA and *β2m*$^{-/-}$ bone marrow cells, or, as a control, a 1:1 mixture of WT and *β2m*$^{-/-}$ bone marrow cells. 13-20 weeks after reconstitution mice were infected with $1x10^4$ PFU MCMV and the loss of *β2m*-deficient cells was monitored in the blood. Experiment was repeated twice with n=2-5 in each group. See Fig. 5-1 legend for details. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test. Data represent means ± SEM. Results are preliminary.

NK cell tolerance is broken following treatment with pro-inflammatory cytokines

MCMV infection results in systemic increases in numerous inflammatory cytokines, as well as direct activation of NK cells due to interactions of the virally encoded M157 protein with the Ly49H NK receptor. In order to address the roles of the specific inflammatory stimuli that are present during a viral infection in breaking tolerance in the chimera model, we treated the mixed fetal liver chimeras with different combinations of IL-12, IL-18, and IL-15 (the latter pre-complexed with IL-15Rα protein in vitro, which results in efficient trans-presentation of IL-15). Treatment with a combination of IL-12 and IL-18, or with a low dose of IL-15+IL-15Rα did not break self-tolerance (Fig. 5-3a) in the chimeras. In contrast, combining IL-12, IL-18, and IL-15+IL-15Rα, or treatment with a high dose of IL-15+IL-15Rα, resulted in a loss of *β2m*-deficient cells from the mix→WT chimeras (Fig. 5-3). The kinetics of loss of *β2m*-deficient cells from the blood following treatment with cytokines were similar to the kinetics observed following infection: tolerance was re-established once cytokines were withdrawn and no further

loss of $\beta 2m^{-/-}$ cells was observed. As in the viral infection experiments, the effect of the cytokines was largely restricted to mix→WT chimeras, and we observed little or no loss of chimerism in the mix→$\beta 2m^{-/-}$ chimeras (Fig. 5-3a, right panel).

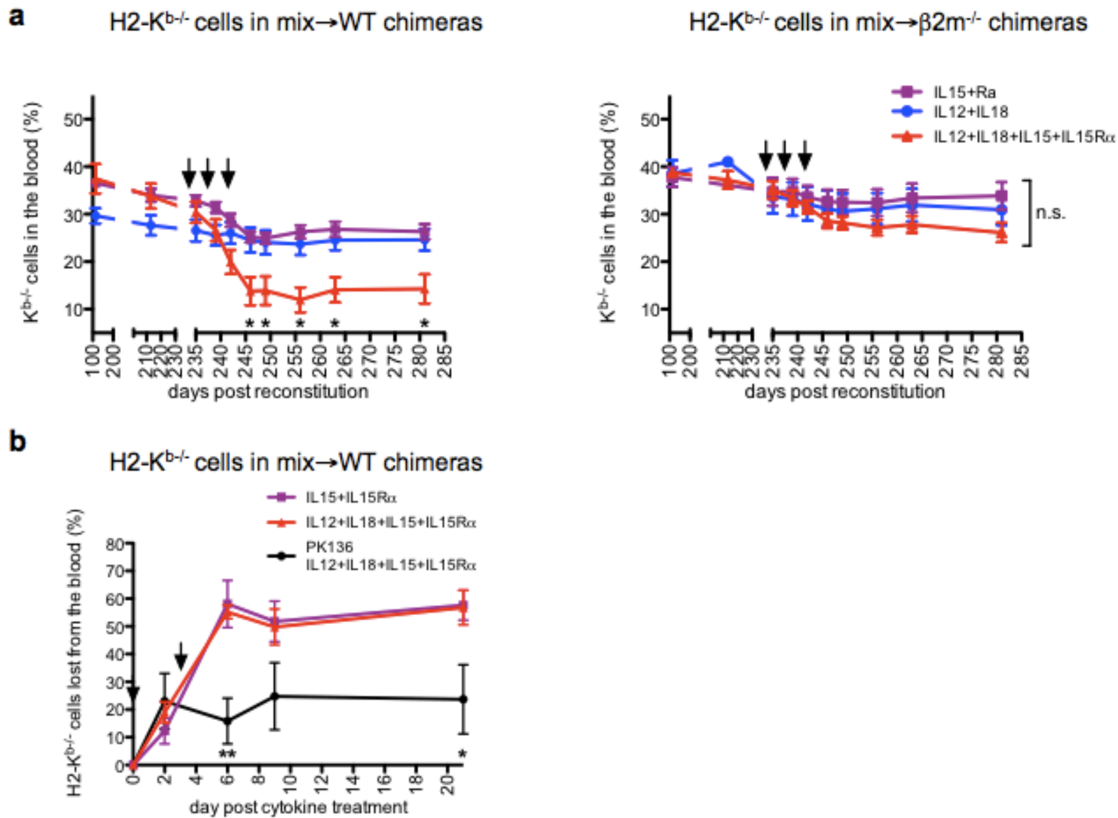


Figure 5-3. Treatments with pro-inflammatory cytokines break tolerance to missing self in mix→WT chimeras. Chimerism was monitored after treating mix→WT (a left panel and b) and mix→β2m$^{-/-}$ (a right panel) fetal liver chimeras intraperitoneally with cytokines 2-3 times at the time points indicated with vertical arrows.
(a) Mice were treated with IL-12 + IL-18 (200 ng each/mouse), or pre-complexed IL-15 + IL15Rα (2μg + 0.6 μg each/mouse, respectively), or with a mixture of all of these cytokines. (b) Mice were treated with pre-complexed IL-15 + IL15Rα (1 μg + 3 μg each/mouse, respectively), or IL-12 + IL-18 (200 ng each/mouse) mixed with IL-15 + IL15Rα (2μg + 0.6 μg); one group treated with the latter cytokine mix was depleted of NK cells by injecting 200 μg PK136 antibody one day prior to and one day after the first cytokine treatment. N=5 mice in each group. Experiment was performed 32 weeks following reconstitution. For some comparisons, statistical significance was determined with a two-tailed unpaired Student's t test (*, $P < 0.05$; **, $P < 0.005$). Data represent means ± SEM.

Concluding remarks

The data presented in Chapter 5 address the role of viral infection and inflammation in self tolerance of NK cells in the context of missing self recognition. In combination with the other findings in the thesis, the data make several important points. The most important result is that although we confirmed the findings of Sun and Lanier (Sun and Lanier 2008) that MCMV infection (transiently) breaks tolerance of NK cells in mix→WT chimeras, resulting in a partial loss of chimerism, we observed no loss of tolerance in mix→*β2m*$^{-/-}$ chimeras. In line with the results reported in the previous chapters, we propose that the different outcomes reflect that fact that the mechanisms of tolerance differ at least partially in the two situations. The steady state exposure of the NK cells to MHC-deficient non-hematopoietic cell types in the mix→*β2m*$^{-/-}$ chimeras may impose a type of tolerance that cannot be readily broken by viral infection, whereas the tolerance resulting from steady state exposure to MHC-deficient hematopoietic cells may differ and be readily broken by MCMV infection. Consistent with this possibility, the tolerance in mix→WT chimeras is correlated with a relatively high responsiveness of the NK cells to stimulation with antibodies specific for activating receptors, whereas tolerance in the mix→*β2m*$^{-/-}$ chimeras is correlated with low responsiveness of the NK cells to stimulation with antibodies specific for activating receptors. We propose that MCMV infection can readily break tolerance when responsiveness is high, but not when it is low as in mix→ *β2m*$^{-/-}$ chimeras, or in normal *β2m*$^{-/-}$ mice. If this interpretation is correct, there may be two mechanisms of tolerance in *β2m*$^{-/-}$ mice, one that results in low responsiveness and another that operates by a distinct mechanism. By extension, these two mechanisms may also operate in normal self tolerance of NK cells, for example in the CIN- population of NK cells in normal B6 mice, which lack inhibitory receptors for self MHC.

The confirmatory findings in this chapter that MCMV infection breaks tolerance of NK cells in mix→WT chimeras is also important. It probably has clinical significance, as it is possible that bone marrow transplant recipients subjected to major infections may also be induced to undergo significant losses of chimerism. Although the present experiments involved tolerance to fully MHC-deficient grafts, a scenario unlikely to be seen in the clinic, the rules and mechanisms governing missing self rejection of MHC-deficient grafts are similar to the rules governing rejection of allogeneic (partially or completely MHC-mismatched bone marrow grafts). It remains an open question whether such events could account for some instances of graft failure in the clinic, especially in light of the common reactivation of latent human cytomegalovirus infections that sometimes accompanies bone marrow transplantation in human patients.

Our results in mix→WT chimeras differ in some respects from the published data (Sun and Lanier 2008). First, Sun and Lanier observed a gradual reduction in chimerism in uninfected mix→WT chimeras (though the reduction was much more rapid after MCMV infection). In most of our chimeras the chimerism in uninfected chimeras was stable over many months. However, we did observe some “spontaneous” loss of chimerism in some experiments. A possible reason for the discrepancy is a difference in the commensal and/or pathogen populations present in mice that are maintained in different facilities or in different groups of mice. These commensals and/or pathogens may create the type of inflammatory signals that breaks tolerance, though less intensely than MCMV does. Another difference from the published data was that we observed only a partial loss of chimerism after MCMV infection, whereas Sun and Lanier observed a complete loss of chimerism. In fact, we observed a similar % loss of chimerism as in the Sun and

Lanier study (≥20%), but their chimeras were only about 20% *β2m*$^{-/-}$ at the time of infection, due to the gradual pre-infection loss mentioned above, whereas ours were typically composed of ~50% *β2m*$^{-/-}$ cells at the time of infection. The loss of chimerism ceases just around the time when that acute infection is expected to resolve, suggesting that the break in tolerance requires sustained exposure to inflammatory stimuli associated with acute infection.

Another important finding in our study of mix→WT chimeras is that tolerance of even *β2m*$^{-/-}$ NK cells in the mixture can be broken. A few researchers had previously proposed that tolerance was to a considerable extent imposed in *cis*, by the interaction, or not, of Ly49 receptors on NK cells with MHC molecules displayed on the same cell surface. Our results suggest that *β2m*$^{-/-}$ NK cells can be induced to reject *β2m*$^{-/-}$ cells in vivo under the appropriate circumstances, and therefore that *cis* interactions do not play an overriding role in causing tolerance to MHC-deficient cells. Furthermore, the mixed chimera data indicate that *trans*-interactions between NK cells and either *β2m*$^{-/-}$ hematopoietic cells or *β2m*$^{-/-}$ non-hematopoietic cells can induce tolerance, though the type of tolerance may be different in those two situations (see previous paragraphs and conclusions as well as Chapter 6).

A final finding in this study was that loss of tolerance to missing-self as a result of MCMV infection can be mimicked by *in-vivo* treatment of the mix→WT chimeras with pro-inflammatory cytokines that are known to be produced during the infection (Pien, Satoskar et al. 2000; Nguyen, Salazar-Mather et al. 2002). Specifically, treatment of mice with a high dose of IL-15 complexed with IL-15Rα resulted in loss of tolerance to *β2m*-deficient hematopoietic cells in the chimeras. A lower dose IL-15/IL-15Rα failed to break tolerance, nor did treatments with doses of IL-12 and IL-18 that have been used in other studies to activate NK cells (Hafner, Falk et al. 1999; Arai, Akamatsu et al. 2000; Cai, Kastelein et al. 2000). However, combining the same doses of IL-12, IL-18, and IL-15/IL-15Rα also broke tolerance, resulting in loss of chimerism. The additive or synergistic effects of these cytokines in breaking tolerance are notable because they are all produced in numerous infections.

Chapter 6: Concluding Discussion

The results in this thesis have addressed several important issues concerning tolerance and responsiveness of NK cells. They will be discussed sequentially below.

Relationship of responsiveness and self-tolerance

Previous studies examining the tolerance and responsiveness of natural killer cells have concluded that NK cell tolerance to MHC I – deficient grafts and hyporesponsiveness to *ex-vivo* activating receptor stimulation go hand in hand (Fernandez, Treiner et al. 2005; Kim, Poursine-Laurent et al. 2005; Brodin, Lakshmikanth et al. 2009; Joncker, Fernandez et al. 2009). Based on the previous findings, it was assumed that self-tolerance is accounted for by the reduced responsiveness of the NK cells (Johansson and Hoglund 2006; Raulet and Vance 2006; Yokoyama and Kim 2006). The work described in this thesis demonstrates for the first time that the two can be separated. NK cells in mix→WT or *β2m*$^{-/-}$→WT chimeras exhibited high responsiveness, but were tolerant of MHC-deficient cells. Hence tolerance to MHC-deficient cells can occur despite high responsiveness to stimulation with immobilized antibodies against activating receptors. On the other hand, no converse example has yet been reported of NK cells that exhibit low responsiveness to anti-receptor stimulation but are reactive against MHC-deficient target cells. We therefore propose that low responsiveness is one mechanism or one component of tolerance to MHC-deficient cells, but another mechanism(s) must exist as well, which can be in effect in NK cells that exhibit high responsiveness.

Hypothesis: At least two mechanisms cooperate in the self-tolerance of NK cells

Based on the data in this thesis we propose that there are two mechanisms of self tolerance, though they may have partially overlapping features. One mechanism, which is operative in mix→WT or *β2m*$^{-/-}$→WT chimeras or adoptive transfers, is not correlated with desensitization of activating receptors. It is, however, correlated with low responses to *Raet1e*-transgenic spleen cells and MHC-deficient tumor cells. The second mechanism is operative in mix→ *β2m*$^{-/-}$ or WT→ *β2m*$^{-/-}$ chimeras or adoptive transfers, and is accompanied by global desensitization of activating receptors (denoted "hyporesponsiveness"). It is possible or likely that both mechanisms are operative in the mix→ *β2m*$^{-/-}$ or WT→ *β2m*$^{-/-}$ chimeras, but proving this will require better molecular markers for the different mechanisms. We similarly suspect that both mechanisms are operative in the case of NK cells in *β2m*$^{-/-}$ mice, or in CIN- NK cells in WT mice. The existence of two or more mechanisms of self tolerance in NK cells is in one sense no surprise, given the evidence that multiple mechanisms account for self tolerance of T cells and B cells.

The self tolerance that prevails in mix→WT or *β2m*$^{-/-}$→WT chimeras is not associated with de-sensitization of activating receptors. It is, however, associated with low responses to *Raet1*-transgenic spleen cells and MHC-deficient tumor cells. Previously, responsiveness to activating

receptor engagement and target cells went hand-in-hand. Because we have dissociated responsiveness to immobilized activating receptor antibodies and responsiveness to NK sensitive target cells, the term “hyporesponsive” needs to be better specified in future publications.

Mechanisms of self tolerance and hyporesponsiveness

As just discussed, NK cells in mix→ *β2m*$^{-/-}$ or WT→ *β2m*$^{-/-}$ chimeras or adoptive transfers exhibit low responsiveness to immobilized antibodies that engage various activating receptors. Because the amounts of the various activating receptors are not altered, the underlying mechanism presumably involves a desensitization of activating receptor signaling pathways. The specific molecular defects remain to be defined, but they are accompanied by low phosphorylation of ERK and AKT (W. Deng, M. Ardolino, unpublished data).

The mechanism of the second form of tolerance, operative in mix→WT or *β2m*$^{-/-}$→WT chimeras, is even less well defined. It is notable, however, that the NK cells in these chimeras, despite being responsive to stimulation with antibodies specific for NKG2D, SLAM family receptors and NKR-P1c (albeit less dramatically), were hyporesponsive to stimulation with MHC-deficient spleen or bone marrow cells (*in vivo*) and tumor cells (*in vitro*), and to RAE-1 expressing spleen cells. NK responses to RAE-1 are mediated by the NKG2D receptor, and responses to MHC-deficient spleen/bone marrow cells and to RMA-S tumor cells are dependent on SLAM family receptors. Therefore, the same NK cells that exhibited high responsiveness to NKG2D or SLAM receptor antibodies were unable to be stimulated by cells that presented ligands for these very receptors. As one possibility to explain this discrepancy, we note that productive interactions of NK cells with these various target cells are also dependent on ligand interactions by other NK receptors, such as the adhesion receptors LFA-1 and DNAM-1, and may in some cases be dependent on costimulatory interactions as well. Therefore, it is possible that the low responses of NK cells from mix→WT and *β2m*$^{-/-}$→WT chimeras to various stimulatory cells is due to defects in signaling via those other receptors.

In contrast to a number of published reports suggesting a role for cis interactions between Ly49 receptors and MHC I molecules in NK cell education (Doucey, Scarpellino et al. 2004; Scarpellino, Oeschger et al. 2007; Schweitzer, Mitmaker et al. 2010; Bessoles, Angelov et al. 2013), we have found no role for the importance for such interactions in our experiments. In mixed chimeras, WT and *β2m*$^{-/-}$ NK cells always behaved similarly with respect to both responsiveness and self tolerance regardless of whether they were present in WT or *β2m*$^{-/-}$ hosts. We therefore propose that *trans*-interactions between inhibitory and activating receptors on NK cells, and MHC and other ligands on other cells in the host, play the dominant role in regulating NK cell self tolerance and responsiveness.

Plasticity of NK cell responsiveness

Data presented in chapters 3 and 4 of this thesis show that the responsiveness of NK cells remains plastic even after the cells reach maturity. NK cell responsiveness to activating receptor antibodies can be re-set upwards after transfer to WT hosts, and downward, after transfer to MHC-deficient hosts. The alterations occurred in fully mature (non-dividing) NK cells as well as

in NK populations that divided after transfer. We propose that the capacity of NK cells to reset their responsiveness represents a mechanism to set the triggering threshold of NK cells to an appropriate level under steady state conditions, in order to prevent autoreactivity, while preserving optimal reactivity to MHC I-deficient or other stimulatory target cells.

Downward resetting: The downward resetting in NK activity that is observed after adoptive transfer of NK cells into an MHC I – deficient environment might help explain how NK cells become inactivated in certain tumors and in cases of persistent viral infections. Tumors often exhibit partial or complete loss or downregulation of MHC I, probably as a result of immunoselection as a result of killing by CD8 T cells (Garrido, Cabrera et al. 1995; Seliger 2008). Loss of MHC I should, in principle, leave these tumors susceptible to killing by NK cells. While this may occur in some cases before tumors are diagnosed, numerous studies have shown that NK cells within tumors often become "anergic" and insensitive to stimulation by tumor cells (Costello, Sivori et al. 2002; Epling-Burnette, Bai et al. 2007). It is possible that upon initial encounter with an MHC I-deficient tumor mass, NK cells become activated and lyse the tumor cells, but if they fail to eliminate the tumor the NK cells eventually adopt a hyporesponsive state as a result of persistent stimulation. Other aspects of the tumor microenvironment, which is often immunosuppressive (Alderton and Bordon 2012), might also be contributing to this phenomenon.

Downregulation of MHC I is an immune-escape strategy that is not unique to tumors. Viruses have evolved mechanisms that target almost every step in the MHC I antigen presentation pathway to avoid detection by CD8 T cells (Hewitt 2003). Downward resetting of NK cells might be one phenomenon that allows certain viruses to persist in the host and escape surveillance by NK cells.

While downward resetting seems to counter the ability of NK cells to respond to infections and malignancies, it is important to note that it is a time-dependent process. We propose that upon initial encounters with target cells NK cells become activated to kill those targets and secrete pro-inflammatory cytokines. Following prolonged, persistent stimulation, however, they lose the ability to respond to activation. This might represent a mechanism to terminate the inflammation and immunopathology that would otherwise result from persistent NK activation.

Upward resetting. The finding that NK cells can gain responsiveness under some circumstances may have relevance to autoimmunity. Multiple studies have implicated NK cells in various autoimmune disorders. In some cases NK cells seem to protect against autoimmunity (Lunemann, Lunemann et al. 2009), while in others they contribute to it (Poirot, Benoist et al. 2004; Feuerer, Shen et al. 2009; Lunemann, Lunemann et al. 2009). Conceivably, the potential of mature NK cells to reset their responsiveness upward may contribute to their ability to promote autoimmunity.

Resetting in other contexts. While this work exclusively addressed the role of MHC class I – dependent inhibition on the activation threshold and responsiveness setting of mature NK cells, work by Tripathy et. al demonstrated that this phenomenon can be extended to other settings. In that study, the authors showed that WT Ly49H+ NK cells acquire a hyporesponsive phenotype following adoptive transfer into hosts ubiquitously expressing the activating ligand for this receptors, m157 (Tripathy, Keyel et al. 2008). This finding suggests that our model of resetting of NK responsiveness is a general adaptation mechanism and is not restricted to signaling

through specific receptors. In the case of NK cells in NKG2D ligand transgenic mice, however, the results are more contradictory. In all cases reported, the NK cells in such mice show defective responses to target cells expressing NKG2D ligands. In some of the studies, the NK cells showed more global defects, such as the inability to reject MHC-deficient grafts (Oppenheim, Roberts et al. 2005; Coudert, Scarpellino et al. 2008). In other studies, however, the effects seemed to be restricted to NKG2D-dependent responses (Champsaur, Beilke et al. 2010). The discrepancies may reflect differences in ligand expression levels in the different transgenic animals or other unknown variables. It seems likely that the specific signaling properties of some receptors can come into play in causing receptor-specific desensitization of NK cells, whereas in other cases more global mechanisms of desensitization must be mobilized.

Plasticity of NK cell self tolerance

As noted above, tolerance and hyporesponsiveness of NK cells to activating receptor stimuli are not always correlated. The question arises whether self tolerance of NK cells is also plastic. In the case of WT→ $\beta 2m^{-/-}$ adoptive transfers, we did observe a loss of the capacity to reject MHC-deficient spleen cell grafts, suggesting that tolerance can be acquired by mature NK cells exposed to an MHC-deficient non-hematopoietic environment. In that scenario, the NK cells also acquire hyporesponsiveness to activating receptor stimuli, and this may account for the self tolerance of the NK cells.

In contrast, we did not observe a gain in the capacity of NK cells to reject MHC deficient grafts $\beta 2m^{-/-}$ →WT adoptive transfers. In that scenario, we suspect that the second mechanism of tolerance remains active, preventing rejection of $\beta 2m^{-/-}$ cells. Therefore, under steady state conditions (no infection or inflammation), it appears that although mature NK cells may be induced to become tolerant after transfer to MHC-deficient mice, they cannot be induced to lose self-tolerance after transfer to WT mice.

The situation was different in MCMV-infected mice or in mice injected with inflammatory cytokines, where self tolerance was reversed in specific circumstances, specifically in mix→WT chimeras. During the infection or after exposure to cytokines, there was a sharp decrease in chimerism, which persisted for a short time until the infection was resolved or the cytokine treatments ended. Notably, an experiment showed that chimerism was lost even when the WT fetal liver donor was genetically NK-deficient, meaning that the NK cells were solely of $\beta 2m^{-/-}$ origin. That data suggested that in such chimeras, when infected, even genetically $\beta 2m^{-/-}$ NK cells can destroy $\beta 2m^{-/-}$ cells. These data demonstrated that the state of tolerance was reversible, at least temporarily, under conditions of infection.

Notably, although tolerance was reversed in mix→WT chimeras, little or no loss of chimerism occurred in mix→$\beta 2m^{-/-}$ chimeras. This finding and our results suggesting that multiple mechanisms are likely to be responsible for tolerance and responsiveness lead us to hypothesize that tolerance that is also accompanied by low responsiveness is more stable, probably because multiple dampening mechanisms must be overcome. One possibility is that the constant presence of MHC I-deficient non-hematopoietic cells in those chimeras may persistently drive NK cells into a hyporesponsive state. In contrast, the second mechanism of self tolerance, which is imposed by interactions with hematopoietic cells and which we have speculated may involve alterations in adhesion receptor function (see above), may be readily

overridden by inflammatory signals. The inflammatory signals could restore the signaling downstream of adhesion receptors or override the requirement for them.

Cytokine treatment of mixed chimeras resulted in some combinations of cytokines mimicking the effect of MCMV infection on tolerance towards *β2m* deficient cells and loss of chimerism. Namely, a combination of IL-12, IL-18, and a low dose of IL-15 complexed with its soluble receptor IL-15Rα resulted in loss of tolerance. IL-15 trans-presentation by dendritic cells has been shown to be crucial for priming the responses of NK cells (Mortier, Woo et al. 2008) whereas IL-12 and IL-18 can enhance both elimination of target cells and cytokine production by NK cells (Hyodo, Matsui et al. 1999; Ortaldo and Young 2003; Ortaldo, Winkler-Pickett et al. 2006). The most likely scenario therefore is that during an infection NK cells are primed through IL-15 transpresented by DCs, but this priming alone is not sufficient to break tolerance. This is followed by production of IL-12 and/or IL-18, which can overcome the activation defects in NK cells in WT, but not *β2m* deficient hosts, and lead to elimination *of β2m*$^{-/-}$ cells from the chimeras.

Interestingly, administration of a higher dose of IL-15 and its receptor can lead to loss of tolerance in WT hosts even in the absence of IL-12 and IL-18. Under physiological conditions only the membrane bound form of IL-15 is biologically active (Mortier, Woo et al. 2008), it is therefore difficult to determine which dose of IL-15/IL-15Rα used in our experiments better mimics the effect IL-15 has *in-vivo*. Another possible interpretation of our data is that IL-15 alone can result in loss of tolerance, but only in concentrations that are higher than what is present at physiological levels during an infection. To distinguish between the two possibilities antibody blockade can be used to block IL-12 and/or IL-18 during MCMV infection to determine the degree to which these cytokines contribute to loss to self tolerance. As a second approach, loss of chimerism can be assessed in chimeras that were prepared from mice with genetic deletions of the cytokines or their receptors.

The contribution of IL-15 and IL-15Rα are difficult to assess directly since in addition to activating NK cells, IL-15/IL15Rα are crucial for their survival (Kennedy, Glaccum et al. 2000; Koka, Burkett et al. 2003). Because IL-15 expression is induced by type I IFN (Mattei, Schiavoni et al. 2001) it is possible to test the effect of IL-15 upregulation on NK tolerance during infection by blocking type I IFN or performing the experiment in mice that are deficient for the type I IFN receptor. Studies that involve blocking the actions of various cytokines during an acute viral infection carry the potential caveat of making the mice more susceptible to the pathogen. To avoid lethality the pathogen dose will likely have to be lowered. It remains to be seen whether a dose of pathogen that is sufficient to result in loss of chimerism, but is not lethal in the absence of inflammatory cytokines, can be found.

While we have only addressed the roles of pro-inflammatory cytokines in breaking NK tolerance towards "missing-self" other factors may play a role. One such factor could be m157, a viral protein that is expressed on infected cells that directly binds to the activating Ly49H receptor on NK cells. To address the requirement of m157-Ly49H interactions for breaking tolerance an m157-deficient virus can be tested.

To our knowledge MCMV is the only pathogens whose role in breaking NK tolerance has been examined but it is likely that other infections will have a similar result. Breaking of tolerance as a result of infection is a potential mechanism that can account for autoimmunity that is sometimes seen following the clearance of a pathogen.

Although mix→$\beta 2m^{-/-}$ chimeras generally did not suffer much loss of chimerism in infected mice or mice treated with cytokines, we did observe small effects in some experiments. If that can occur, it raises the possibility that even in normal WT animals, NK cells subjected to major inflammatory conditions may in some cases develop autoreactivity, and destroy autologous cells. Such autoreactivity, even if transient, could account for some of the pathology that occurs in major viral infections. In this regard, it is notable that pathology in severe influenza infections, such as with the reconstructed 1918 strain, has been correlated to the actions of NK cells (Kash, Tumpey et al. 2006).

Consequences of the plasticity of NK cell responsiveness and self tolerance

The ability of NK cells to adjust their responsiveness depending on their MHC environment can potentially be a double edge sword. On one hand downward resetting can prevent autoimmunity by protecting cells that have downregulated MHC expression. On the other hand, it could account for failure to clear malignant or virally infected cells that have low MHC I. We propose that lowering the responsiveness of NK cells following infection can serve as a negative feedback mechanism to curtail the immune response once the infection is cleared and/or the adaptive immune response takes over. Additionally, downregulation of class I MHC by tumors as well as virally infected cells can serve as an immune escape mechanism. Although it is traditionally thought that MHC I downregulation serves to avoid the CD8 T cell response, in light of data presented in this work, we propose that it can also serve to escape elimination by NK cells.

Cell types that educate NK cells

In the education of T cells, it is well established that different cell types, such as hematopoietic versus non-hematopoietic cells, play distinct roles in the process. Studies using radiation chimeras and other methods have demonstrated that MHC:protein expression on host-derived cortical thymic epithelial cells allows for positive selection. cTECs express both MHC I and MHC II molecules and have been implicated in both positive (Anderson, Lane et al. 2007) and negative (Goldman, Park et al. 2005) thymocyte selection. In contrast, MHC expression on bone-marrow derived cells or medullary epithelial cells play important roles in induction of tolerance (vanMeerwijk, Marguerat et al. 1997), but no role in positive selection.

The work presented in this thesis provides the first evidence that interactions of NK cells with different cell types regulate distinct aspects of NK cell functional activity. Specifically, the results indicate that cells of non-hematopoietic origin are critical for setting the responsiveness of natural killer cells, whereas cells of hematopoietic origin are responsible for inducing a less stable form of tolerance by a distinct mechanism.

Hematopoietic cell types that determine self tolerance. The hematopoietic cell type(s) that imposes this second form of tolerance is unknown. Because mice deficient in lymphocytes ($RAG^{-/-}$) have grossly normal NK cells and are tolerant of MHC-deficient cells (Kim, Poursine-Laurent et al. 2005; Raulet and Vance 2006), it is unlikely that the responsible cells are T_{regs} or other T cell or B cell subtypes. Instead it seems likely the relevant cell type is of myeloid origin, based on multiple

studies showing the importance to NK-DC crosstalk for NK cell functions (Moretta, Ferlazzo et al. 2006). Furthermore, both macrophages and dendritic cells appear to be important for the final stages of NK cell maturation (Mortier, Advincula et al. 2009), and neutrophils have also been shown to influence NK cell activity (Narni-Mancinelli, Jaeger et al. 2012)).

Non-hematopoieitic cell types that determine hyporesponsiveness. The results indicate that cells of non-hematopoietic origin are critical for setting the responsiveness of natural killer cells. NK cells that developed in a host where MHC I –expression is restricted to non-hematopoietic cells acquired a responsive phenotype, regardless of MHC expression on hematopoietic cells, including the NK cells themselves. In contrast, NK cells that developed in *β2m* – deficient host became hyporesponsive.

Although little in detail is known about the requirement for stromal cells types in the support of NK cell development and maintenance, it has been shown that the cytokine IL-15 and its high affinity receptor IL-15Rα are critical for this process (Kennedy, Glaccum et al. 2000; Koka, Burkett et al. 2003). It has also been demonstrated that IL-15Rα expression on non-hematopoietic cells alone is sufficient to support differentiation and survival of NK cells (Koka, Burkett et al. 2003). IL-15 in trans-presented on the cell surface by IL-15Rα (Lodolce, Burkett et al. 2001; Burkett, Koka et al. 2003; Koka, Burkett et al. 2003; Burkett, Koka et al. 2004; Mortier, Woo et al. 2008), making direct cell-cell contact critical for signaling through the IL-15 receptor on NK cells (Mortier, Woo et al. 2008). It is therefore reasonable to hypothesize that the non-hematopoietic cells that are involved in setting NK cell responsiveness are capable of trans-presenting IL-15. Both IL-15 and IL-15Rα are broadly expressed to multiple tissue and cell types (Giri, Kumaki et al. 1995), therefore precluding a specific prediction as to the relevant cell type.

Another clue as to the origin of the critical non-hematopoeitic cell type is the finding that a set of protein tyrosine kinase receptors, called the TAM family, plays a critical intrinsic role in the development of NK cell functional activity (Caraux, Lu et al. 2006; Roth, Rothlin et al. 2007). The three TAM family kinases (TYRO 3, AXL and MER) bind to two cell surface ligands, GAS6 and protein S, which are expressed by stromal cells and not by hematopoietic cells. The results suggest that interactions of NK cells with non-hematopoietic cells expressing GAS6 and protein S play a critical role in NK cell development and it is attractive to hypothesize that MHC expression by these critical stromal cells may also play a role in educating NK cells by determining NK cell responsiveness.

Arming or disarming? Previously, the question was posed whether NK cell responsiveness and self tolerance are imposed by interactions with MHC-deficient cells, which DE-sensitize the NK cells (a process called "disarming"), or by dominant interactions with MHC+ cells, that "license" the NK cells to acquire full functional activity (a process called "arming") (Raulet and Vance 2006; Yokoyama and Kim 2006). Given the separation of responsiveness and self-tolerance described in this thesis, it must now be asked whether one or both mechanism of tolerance involves an "arming" as opposed to "disarming" type process.

The fact that tolerance of NK cells to MHC-deficient cells occurs in mixed fetal liver chimeras (WT+$\beta 2m^{-/-}$→WT) demonstrates that tolerance is dominantly induced by MHC I - deficient hematopoietic cells, supporting the "disarming" model of NK cell education for establishing self-tolerance (Raulet and Vance 2006).

Whether an "arming" or "disarming" type process occurs during interactions with non-hematopoietic cells remains unclear. In the chimeras, the non-hematopoietic cells were homogenously WT or $\beta 2m^{-/-}$, and therefore it has not been determined whether interactions with $\beta 2m^{-/-}$ non-hematopoieitic cells dominantly induce hyporesponsiveness, as opposed to the possibility that WT hematopoietic cells dominant induce responsiveness. Mice with chimeric non-hematopoietic compartments will be necessary to address that question.

One thing that does seem clear is that the original notion of "licensing", as a developmental process that determines NK cell responsiveness, must be incorrect. When WT mature NK cells were transferred to $\beta 2m^{-/-}$ hosts, the NK cells rapidly acquired a hyporesponsive phenotype, ruling out the notion that responsiveness is determined developmentally. Furthermore, the adoptive transfer results tend to suggest that encounters with $\beta 2m^{-/-}$ non-hematopoietic cells rapidly induce hyporesponsiveness, as suggested by the "disarming" concept, and inconsistent with the spirit of the "licensing" concept. It remains possible, however, that constant interactions of NK cells with MHC I+ non-hematopoietic cells are necessary to sustain a high level of responsiveness in NK cells, whereas interactions with MHC-deficient non-hematopoietic cells has little effect. If that proved to be the case, it would fit in some respects with the "arming" concept of NK cell education but less so with the licensing concept, which implies a one-time only event, or at least a relatively infrequent renewal process.

Concluding Remarks

The results presented here represent a substantial revision of current thinking regarding NK cell self tolerance and responsiveness. The next phase of research on this topic must involve molecular and cellular definitions of the critical events described here. These include: (1) the definition of the molecular circuits that regulate responsiveness; (2) the locus of action and molecular mechanisms of the presumed second mode of tolerance, imposed by hematopoietic cells; (3) the definition of the specific non-hematopoietic and hematopoietic cell types that impose hyporesponsiveness and self-tolerance; (4) mechanistic understanding of how tolerance and hyporesponsiveness are reversed by inflammatory signals and exposures to an MHC I+ environment, respectively; (5) an understanding of how such events impart or prevent pathology, impact autoimmunity, and regulate NK responses against tumors and infections.

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