THE ROLES OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I AND FOXK1 IN NATURAL KILLER CELL DEVELOPMENT

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DEDICATION

So many people have helped me throughout the ordeal that is graduate school. First I would like to thank my mentor, Michael Bennett, M.D., for his continued support of me, his confidence in my abilities and his infectious love of science. He is really responsible for making me become the independent scientist that I am today. He encouraged me to be independent in my studies very early on in my graduate career, which, although difficult at times, has turned out to be a blessing, in that I really feel well prepared for whatever challenges life will throw at me. So thank you, Mike, for all of your patience and guidance. John Schatzle, Ph.D. has always been a tremendous help during my years in the lab. Although not officially my co-mentor, he really played that role, giving me advice and even a good kick in the rear when I needed it most. I honestly don't think I'd be graduating now if not for his help. Since John was in the lab most of the time, I really credit the easy-going and laid-back atmosphere of the lab to him. It was the

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because we have always had such a great time together, whether we're watching the TV show du jour or playing any assortment of games. Life would have been boring and drab without Beth in it. Jamie, Jen and Beth were also all my roommates at one point, so all three of them had to put up with me and my little quirks and for that they all deserve a big thanks. I will always cherish the memories from the HOV house.

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Pathways leading to the development of functionally mature Natural Killer cells from bone marrow progenitors are incompletely characterized. Several reports have indicated the necessity of class I Major Histocompatibility Complex-Ly49 interactions to generate functionally mature Natural Killer cells. Natural Killer cells from mice deficient in Major Histocompatibility Complex class I exhibit impaired lytic ability against class I^+ and class I^- targets. It has been proposed that class I interactions with inhibitory Ly49s are required for generation of lytic

Natural Killer cells; cells that do not receive these signals fail to become activated. To investigate further the role of class I-Natural Killer cell interactions during development, we produced chimeric mice using class I^- mice, in which the hematopoietic system was derived from class I-expressing mice. We discovered that class I^+ Natural Killer cells that are developed in a class I^- environment are not functional, despite the presence of class I on hematopoietic cells. This indicates that the environment in which Natural Killer cells are developed determines their function and further supports the role of the bone marrow microenvironment in Natural Killer cell development.

A complete understanding of Natural Killer cell development would involve determining which transcription factors drive development of Natural Killer cells from stem cells to mature, functional Natural Killer cells. Several transcription factors have been described to be necessary for Natural Killer cell development. Mice lacking these transcription factors often have a deficit in Natural Killer cells *in vivo*. Here we illustrate a role for the forkhead transcription factor, Foxk1, in Natural Killer cell development. $Foxk1^{-/-}$ mice have significantly fewer Natural Killer cells than do wild-type mice and their remaining Natural Killer cells have decreased cytotoxicity. An increase in the percentage of cells in a developmentally important expansion stage indicates that Foxk1 acts there. However, Foxk1 seems to play no role in the thymic development of Natural Killer cells; cells with phenotypic characteristics of

thymus-derived Natural Killer cells are present in Foxk1$^{-/-}$ mice. Our studies show a clear role for Major Histocompatibility Complex class I and Foxk1 in the development of functionally mature Natural Killer cells in mice.

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PRIOR PUBLICATIONS

Moody LA, Alexander MS, Mooney JM, Gehlbach KA, Garry DJ, Schatzle JD, M Bennett (2007). Foxk1 is required for NK cell development and function. (Paper submitted)

Moody LA, Gandhi NA, Devora G, Arora V, Schatzle JD, M Bennett (2007). MHC class I-NK cell interactions regulate NK cell development. (Manuscript in preparation)

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β2m:	beta-2 microglobulin
γc:	common gamma chain
[^{125}I]dU:	5-iodo [^{125}Iodine]-2'-deoxyuridine
^{138}Cs:	Cesium-138
^{51}Cr:	Chromium-51
ABC:	adenosine triphosphate binding cassette
ADCC:	antibody-directed cellular cytotoxicity
AICD:	activation-induced cell death
bHLH:	basic helix-loop-helix
BIM:	BCL-2-interacting mediator of cell death
BLAME:	B lymphocyte activating macrophage expressed
BLS:	bare lymphocyte syndrome
BM:	bone marrow
BMC:	bone marrow cell
CD62L:	CD62 ligand
cdk:	cyclin-dependent kinase
CFSE:	5-(and 6)-carboxyfluorescein diacetate succinimidyl ester
CLP:	common lymphoid progenitor
CRACC:	CD2-like receptor activating cytotoxic cells
CRD:	carbohydrate recognition domain

CS-1:	see CRACC
CTL:	cytotoxic T lymphocyte
CTLA4:	cytotoxic T lymphocyte associated antigen-4
DAF-16:	decay accelerating factor-16
DAP-10/12:	DNAX-activating protein of 10/12 kilodaltons
DC:	dendritic cell
DN:	double negative
DNA:	deoxyribonucleic acid
DP:	double positive
Eat-2:	EWS-Fli1-activated transcript-2
EBV:	Epstein Barr Virus
EM:	effector memory
ER:	endoplasmic reticulum
ERK-1/2:	extracellular signal-related protein kinase-1/2
FACS:	fluorescence-activated cell sorting
FasL:	Fas ligand
FGF:	fibroblast growth factor
fhl3:	four and a half LIM 3
FL:	flt3 ligand
Fox:	forkhead box
GATA-3:	GATA binding protein-3

GITR: glucocorticoid-induced TNF receptor-related protein

Gran: granulocyte

HSC: hematopoietic stem cell

i.p.: intraperitoneal

IFN-γ: interferon-gamma

IFN-α: interferon-alpha

IgSF: immunoglobulin superfamily

IL: interleukin

IL-15R: IL-15 receptor

ILF: interleukin binding factor

ILT: immunoglobulin-like transcript

IRF-2: interferon regulatory factor-2

ITAM: intracellular tyrosine-based activation motif

ITIM: intracellular tyrosine-based inhibition motif

ITSM: intracellular tyrosine-based switch motif

LAIR: leukocyte-associated immunoglobulin-like receptor

LCMV: lymphocytic choriomeningitis virus

LIR: leukocyte immunoglobulin-like receptor

LN: lymph node

Mac: macrophage

MCMV: murine cytomegalovirus

MEF:	myeloid Elf-like factor
MHC:	major histocompatibility complex
MIR:	monocyte/macrophage immunoglobulin-like receptor
MNF:	myocyte nuclear factor
MULT-1:	murine UL-16 binding protein-like
MZ:	marginal zone
NCR:	natural cytotoxicity receptor
NF-κB:	nuclear factor-kappa B
NK:	natural killer
P13 kinase:	phosphotidylinositol-3 kinase
PD1:	programmed death 1
PIR:	paired immunoglobulin-like receptor
Poly I:C:	polyinosinic polycytidylic acid
Rae-1:	retinoic acid early transcript-1
RER:	rough endoplasmic reticulum
RNA:	ribonucleic acid
RT-PCR:	reverse transcription-polymerase chain reaction
SAP:	SLAM associated protein
SCID:	severe combined immune deficiency
SEM:	standard error of the mean
SH2:	Src homology 2

SHIP: SH2 domain-containing inositol phosphatase

SHP-1/2: SH2-containing phosphatase-1/2

SLAM: signaling lymphocytic activation molecule

SLE: systemic lupus erythematosus

T1/T2: transitional stage 1/2

TAP: transporter associated with processing

TCF-1/LEF-1: T cell factor-1/lymphoid enhancing factor-1

TCR: T cell receptor

TGF-β: transforming growth factor-beta

Th1/Th2: T helper cell type 1/2

TNF-α: tumor necrosis factor-alpha

TRAIL: TNF-related apoptosis-inducing ligand

T_{reg}: regulatory T cell

XLP: X-linked lymphoproliferative disease

CHAPTER I

INTRODUCTION

Innate immunity serves as an initial line of defense and therefore is vital to the health of an organism. Cellular components of the innate immune system function to provide early defense against pathogen invasion and also serve to promote the induction of the acquired immune response. Natural Killer (NK) cells, specifically, act as a bridge between innate and acquired immunity first by providing the organism with protection against transformed cells and virus-infected cells through cytotoxic mechanisms and then through the secretion of cytokines that modulate the acquired immune response to act against these insults. Understanding the development and function of NK cells can lead to a better understanding of the immune system as a whole and how each of its components functions together to maintain the health of the organism. This dissertation will focus on the development of functionally mature murine NK cells in two systems: MHC class I^+ NK cells developing in an MHC class I^-environment and the $Foxk1^{-/-}$ mouse.

I. NK CELL IMMUNOBIOLOGY

A. Background

Natural Killer (NK) cells are large, granular lymphocytes of hematopoietic origin that arise from a pluripotent progenitor cell in the bone marrow and are generally defined by their expression of NK1.1 or CD49b (1-3). NK maturation in the bone marrow is

dependent upon IL-15 and cell contact with the surrounding stromal cells (3-8). Mature NK cells from the bone marrow exit to the peripheral lymphoid organs, the spleen, liver, uterus and peripheral blood. As the name natural killer implies, NK cells are cytotoxic lymphocytes that require no prior sensitization. As such, unlike T and B cells, NK cells do not possess rearranged antigen receptors (2). NK cells possess receptors that recognize MHC class I and class I-like molecules that allow them to recognize perturbations in MHC levels (9-15). NK cells provide an early defense against transformed cells and virus-infected cells, which they recognize as abnormal through the down-regulation of MHC class I and the expression of stress ligands on the surface of the target cells (16-21). They perform their cytolytic functions through the release of cytolytic granules or through death receptor pathways; these mechanisms will be discussed in more detail later. NK cells are also very important early in the course of an infection to provide cytokines to modulate the adaptive arm of the immune system in order to drive the proper response to the infection (22). Through viral infection or transformation, MHC class I levels can be reduced on the cell surface, rendering them susceptible to NK killing (16, 23-25). Allogeneic cells are also good targets

for NK cell killing due to their lack of self MHC class I expression. In these cases, the NK cell does not receive negative signals through its inhibitory receptors; therefore, the activating signals that the NK cell receives results in lysis of the target cell. The phenomenon by which NK cells recognize and kill cells that lack MHC class I is called “missing self” (26). The missing self hypothesis was first put forth when it was discovered that NK cells specifically lysed tumor cells that lacked MHC class I expression (24, 27).

NK cells derive their specificity for target cell recognition through cell-surface receptors for class I MHC. In the mouse, these receptors are the Ly49 receptors and the CD94/NKG2 heterodimers, members of the c-type lectin family (11, 28). These receptors can be inhibitory or activating. An important aspect of NK cell biology is that inhibitory signals generally override activating signals, preventing NK cells from indiscriminately killing self cells. In the F1 progeny of mated allogeneic strains of mice, parental bone marrow is rejected through the process termed hybrid resistance (29-31). This occurs due to the expression of Ly49 receptors in non-overlapping patterns on the NK cells in these F1-hybrid mice, indeed, in all mice, which enables the existence of NK cells that lack inhibitory receptors for parental MHC class I but retain expression of non-MHC class I-recognizing activating receptors. Thus, NK cells expressing an inhibitory receptor for one parent, but not the other, will reject cells of the parental strain for which they have no inhibitory receptors.

NK cells can be activated through dendritic cell (DC) recognition of antigenic determinants on virus-infected cells. NK cells and DCs interact in the paracortex of draining lymph nodes (32), where NK cells are recruited during an infection. The mechanism of DC-NK cell crosstalk is both cell contact dependent as well as cytokine mediated (33, 34). DCs are critical for stimulation of NK cell activity through the cross-presentation of IL-15 (33, 35). NKG2D on NK cells has been shown to interact with its ligands (Rae-1, H60 and MULT-1) on DCs, inducing the expression of IFN-γ by NK cells to aid in the development of T helper type I (Th1) cells (36). NK cells have also been shown to modulate the activity of DCs through their interaction, in effect dampening the adaptive response through the secretion of TGF-β, which leads to down-modulation of MHC class II on DCs (37). When fewer NK cells are present, NK cells regulate DC maturation and upregulation of co-stimulatory molecules (37, 38). In higher concentrations of NK cells, DCs are lysed, perhaps as a mechanism of diminishing the immune response (37, 39). Immature DCs that are peptide-loaded have been shown to be eliminated through the interaction with TRAIL on NK cells (39) and immature DCs are lysed primarily through their interaction with the NK-triggering receptor NKp30 (40, 41). Through interaction with NKp30, TNF-α and IFN-γ are released, leading to the maturation of DCs (42). NK cells have also been shown to interact with B cells, resulting in antibody class-switch in the B cell and cytokine secretion by the NK cell (43).

B. Function of NK cells

NK cells are vital to host defense against viral infections and transformed cells. They are also responsible for the rejection of incompatible bone marrow grafts. They perform these functions through the release of cytolytic granules and through the death receptor pathways. NK cells also secrete cytokines in response to these stimuli to regulate the adaptive immune response. Discussed below are the individual components of the NK cell response.

i. Granule Exocytosis

The granule exocytosis pathway is the main mechanism of NK cell-mediated target cell lysis. The components of this pathway include the pore-forming molecule perforin and the serine proteases granzyme A and B. NK cells contain stores of perforin and granzyme mRNAs that are processed upon activation in order to arm the cell. As a result, unactivated NK cells are only minimally lytic (44). Perforin-mediated killing happens very quickly in productive NK cell-target cell couples, with the perforin-mediated lysis of cells occurring within minutes (44, 45).

Perforin is made by NK cells and cytotoxic T cells (CTL) and is found in the secretory lysosomes that also contain granzymes. Perforin is released in a Ca^{2+} dependent manner following NK cell activation by a target cell. Perforin then binds to the cell surface of the target cell, recognizing the phosporylcholine

residues within the cell membrane. Monomers of perforin aggregate on the cell surface, forming pores that increase in size with the addition of perforin molecules (46, 47). The pores formed by perforin may allow the granzymes to enter the cell, causing lysis. However, perforin has also been shown to induce cell death in the absence of granzymes, suggesting that perforin has lytic capabilities on its own in extremely high amounts (44). While it was originally thought that the pores formed by perforin allowed the granzymes to enter the target cell, it has been suggested that perforin-mediated pore formation on target cells induces repair mechanisms within the cell that allow for the uptake of granzymes through endocytic pathways, making the cells more susceptible to granzyme-induced apoptosis (48, 49). Killing of target cells by perforin alone has also been observed *in vitro*, occurring in only minutes, suggesting that perforin-mediated cell death is necrotic, rather than apoptotic, in nature (49). Target cell lysis mediated by the activating receptor NKG2D has been shown to be dependent upon perforin (21). In perforin knock-out mice, tumor cells expressing the Rae-1 ligand for NKG2D were not lysed, whereas wild-type mice exhibited profound cytotoxicity against these targets (21). In addition to NKG2D, the lysis of target cells mediated by the activating receptors CD244, Ly49D and Ly49H is also perforin dependent (50-53)

Granzymes A and B are serine proteases that are taken into target cells, via the aforementioned process, following perforin-mediated pore formation (44, 54,

55). Granzyme B directly activates the caspase cascade, which eventually results in DNA fragmentation (56, 57). Granzyme A, when released into a target cell, induces apoptosis through the cleavage of an inhibitior of the tumor-suppressor NM23-H1, which promotes DNA degradation (56). Not only is inhibition of NM23-H1 relieved by granzyme A, but targets of granzyme A are responsible for the transcription of genes involved in the DNA repair response; cleavage of these proteins ensures the death of the cell by preventing repair mechanisms (55).

ii. Death Receptor Pathways

In the perforin knockout and granzyme knockout mice, some killing can be observed (58). This residual killing is mediated by the death receptor ligands that are expressed on NK cells and bind the death receptors themselves on target cells. NK cells express FasL, Tumor Necrosis Factor- (TNF) α and TNF-related apoptosis-inducing ligand (TRAIL), all of which can induce apoptosis in cells that express their respective receptors (59-63). These ligands bind members of the TNF-receptor (TNFR) family, Fas, TNFR1 and TNFR2 and TRAIL- receptor, respectively (62-64). Ligand binding of the TNFRs results in activation of the mitochondrial caspase pathway and apoptosis (59). The Fas-FasL pathway is necessary for the self-removal of an expanded lymphocyte population following an immune response by activation-induced cell death (AICD) (65-70). Fas- and FasL-deficient mice develop lymphoproliferative disorders and autoimmunity due to their inability to undergo AICD (71-74). In NK cells, the killing mediated by

Fas-FasL interactions has been the most widely studied of the death receptor pathways. Whereas perforin- and granzyme-mediated cell death occur within minutes, Fas-FasL induced cell death occurs over a span of hours and requires multiple Fas-FasL interactions (45, 59, 61).

iii. *Cytokine Secretion*

In addition to their cytotoxic properties, NK cells also secrete cytokines that act either locally or in a paracrine fashion (75, 78). NK cells have been shown to secrete interferon-γ (IFN-γ), TNF-α, transforming growth factor (TGF)-β, granulocyte-macrophage colony stimulating factor (GM-CSF), and the interleukins (IL) IL-3, -10 and -13 (25, 76). These cytokines are produced in response to interaction with target cells and other cells of the immune system, as well as through the interaction with other soluble mediators and modulate the functions of cells with the proper cytokine receptors. NK cells are the major source of IFN-γ during viral infections (76, 79) and IFN-γ has been shown to directly inhibit viral replication and infectivity (80). Cytokine secretion by NK cells in the pregnant uterus is essential for proper vascularization of the placenta (81-83). IFN-γ is also necessary for the polarization of $CD4^+$ T cells into T helper type 1 cells (36). This has been shown using intravital microscopy, which followed the recruitment of NK cells to the lymph node after immunization where the IFN-γ they secreted was essential for Th1 development (32). The generation of IFN-γ by NK cells in the lymph node is directly dependent upon interaction

with DCs (36, 84). The IFN-γ secreted by NK cells not only induces Th1 cell development, but also leads to the induction of $CD8^+$ T cells to become CTL in a $CD4^+$ T cell-independent fashion (85). B cells have been shown to induce the expression of IFN-γ (a Th1 cytokine) as well as IL-13 (a Th2 cytokine) in NK cells (43, 86). IFN-γ secretion by NK cells has been shown to aid in switch recombination to the IgG2a isotype (87). In addition, NK cells have been shown to promote the production of IgG1 in B cells, an isotype associated with T_H2 responses, although this has not been attributed to IL-13 production by NK cells (86). Thus, cytokines produced by NK cells have far-reaching effects on the immune response.

C. NK Cell Development

NK cells develop from a common lymphoid progenitor (CLP), which then further differentiates into the NK/T cell progenitor, mainly in the bone marrow. Recent studies have detailed the development of a small subset of NK cells in the thymus as well (88, 89). This dissertation will focus primarily on the development of NK cells, so the specifics of NK cell development will be discussed here.

i. NK cell development in the bone marrow

The bone marrow of the adult mouse is a rich source of hematopoietic stem cells (HSC). HSCs can give rise to the erythroid, myeloid and lymphoid

lineages. The processes by which these cell lineages are derived are only partially understood. It is thought that HSCs symmetrically divide, meaning that they generate two daughter HSCs or two daughter differentiated cells (90-92). With each step of differentiation HSCs become more committed to a specific cell lineage until they reach a stage of total lineage commitment, at which they can no longer self-renew. This process for NK cell development from HSCs will be discussed in detail below.

The acquisition of flt3 by $lin^-c\text{-}kit^+sca\text{-}1^+$ HSCs is the first step toward NK cell lineage commitment; once flt3 expression begins, the stem cells are no longer capable of self-renewal (93). These oligopotent lin^-flt3^+ cells are termed common lymphoid progenitors (CLP). Flt3-ligand (FL) is essential in order for the CLP to differentiate further and $Flt3^-$ cells fail to develop functionally mature NK cells *in vitro* (6). The signaling through Flt3 after ligation by FL leads to the survival of the CLP; mice lacking Flt3 have severely reduced numbers of CLPs (94, 95). The expression of the IL-7 receptor has also been shown to be dependent upon Flt3-Flt3L signaling and aids in conferring lymphoid potential onto the cells (96). The CLP then differentiates further to generate a common T/NK precursor cell, which has been defined by the expression of $Lin^-Sca\text{-}1^+c\text{-}kit^+B220^+CD19^-CD62L^+$ and has been identified in the fetal liver, where a significant proportion of early lymphopoiesis occurs, and in the adult bone marrow (97, 98). These cells have the potential to differentiate into both T and NK cells *in vitro*, but lack the ability

to become myeloid cells or B cells, despite the expression of B220 (98). The expression of CD122 (the IL-2/15 receptor β), CD2, CD244 and CD11a (and the down-modulation of B220) represent the phenotype of NK lineage committed precursors (99, 100). These cells have lost the ability to become T cells and retain the ability to differentiate only into NK cells.

Several *in vitro* systems have been described that define the factors necessary for NK cell development. $Lin^{-}CD122^{-}c\text{-}kit^{+}Sca\text{-}2^{+}$ cells were found to become IL-15 responsive after culture with IL-6, IL-7, stem cell factor and FL through the upregulation of CD122 on the cell surface of the progenitors (3). Only after CD122 is expressed on the NK progenitor cells are they able to survive in culture with IL-15 and fully develop into $NK1.1^{+}$, lytic NK cells (3). These cells are devoid of Ly49 receptors, though. The generation of NK cells that express Ly49 receptors *in vitro* is absolutely dependent upon cell contact with stromal cells (6). These experiments have definitively shown the necessity of NK cell development for IL-15 and stromal cell interactions.

NK committed precursors undergo five discrete steps of differentiation in order to become fully mature, lytic NK cells (Table 1.1, 99). Stage I cells that express CD122 require interaction with IL-15 for their survival and proliferation (3, 4, 8, 99, 101). IL-15 is vital for the homeostasis of NK precursors and for the generation of lytic NK cells (3, 102). After the lineage commitment stage (stage I), pre-NK cells gain the expression of the NK1.1 antigen (only expressed in the

C57BL/6 mouse strain), which delineates the NK cell phenotype, and the CD94/NKG2 receptors, which provide the NK precursor cells with the ability to recognize non-classical MHC class I and class I-like molecules (stage II). In stage III of NK cell development, the precursor NK cells gain expression of Ly49 receptors through stromal cell contact (6) and gain the expression of c-kit as well as low level expression of CD49b (DX5), CD11b (Mac-1) and CD43. The acquisition of Ly49 receptors is an important step in NK cell maturation, in that they are critical for the development of functional NK cells, as will be discussed under the heading "NK cell tolerance mechanisms." NK cells that lack Ly49 receptors, such as neonatal NK cells, have the ability to distinguish between MHC class I^+ and MHC class I^- cells, but they cannot discern one MHC class I allele from another (3, 103). This implies that CD94/NKG2A is required for the global recognition of MHC class I levels through the invariant class Ib MHC, but that they play no role in allorecognition (104, 105). In the fourth stage of NK cell development (stage IV), the expression of CD51 is down-modulated and the expression of CD49b is upregulated, generating cells that are $NK1.1^+CD49b^{high}CD51^{low}$. Cells in stage IV of development undergo a massive expansion, generating a large pool of immature NK cells that in stage V of development acquire high levels of CD11b and CD43 and lose the expression of c-kit and CD51 entirely, generating mature NK cells that have a full lytic potential (99).

After mature NK cells are generated in the bone marrow, they exit to the periphery where they interact with potential target cells in the spleen, liver and peripheral blood. NK cells, as previously discussed, can also be recruited to the lymph nodes through their expression of CD62 ligand (CD62L, L-selectin, 32). Bone marrow-derived NK cells represent roughly 90% of the total NK cell population in the spleen and peripheral blood and about 50% in the lymph node (88). These NK cells represent a highly lytic population of NK cells that are vital for the organism to act quickly in the presence of virus infection and tumor cell transformation.

ii. NK cell development in the thymus

Until recently it was assumed that all NK cell development occurred in the bone marrow in adult mice or the fetal liver during fetal development. However, thymus-derived NK cells have been recently described (88, 89). Generation of thymus-derived NK cells occurs from the T/NK oligopotent progenitor cell that is recruited to the thymus through its expression of CD62L (98).

In the thymus a majority of NK/T common progenitor cells develop into T cells, which is not surprising in light of the fact that the thymus is primarily devoted to development of T cells. However, in NK/T progenitor cells in which the transcription factor Id3 is activated, NK cells develop (106). Id3 expression in these progenitor cells prevents the rearrangement of the T cell receptor (TCR) genes and NK cells develop; the lack of TCR rearrangement is unlikely to account

for the development of NK cells, rather it is the expression of specific factors that lead to NK cell development after Id3 expression (106). Thymus-derived NK cells also require the activity of transcription factors that are necessary for T cell development, such as GATA-3 (88, 89). In fact, GATA-3^+ NK cells found in the periphery can be attributed to thymic development and not bone marrow development. NK cells developed in the thymus require IL-7 in order to survive and proliferate; thus, thymus-derived NK cells express CD127, the IL-7 receptor-α chain, whereas bone marrow-derived NK cells do not. The gene for CD127 is transcribed after initiation by GATA-3 (88). IL-7, like IL-15 and IL-2, signals through the common-γ chain (γc). The γc requires the association with β- and α-subunits that are specific to IL-7 in order to transmit the signals. NK cells derived from the thymus have been shown to be superior cytokine producers, greatly contributing to the development of Th1 cells and the overall immunity against virus infection (88, 89). These cells seem to be specialized for the production of cytokines, since they exhibit much reduced lytic capability and even have reduced expression of granzyme B compared to bone marrow-derived NK cells (89). It is thought that the reduced lytic ability of thymus-derived NK cells may be due to a more immature phenotype; these NK cells express lower levels of the inhibitory Ly49s Ly49G2, A and C/I as well as lower levels of the activating Ly49D (88). In summary, NK cells derived from the thymus are a vital component of the NK cell-mediated immune response, in that they provide cytokines for the defense

against pathogens and they can be readily distinguished from BM-derived NK cells.

D. Murine Major Histocompatibility Complex (MHC)

The mouse major histocompatibility complex (MHC) genes are located on chromosome 17. This region of the genome is highly polymorphic and encodes the MHC class I and II genes as well as the non-classical MHC genes (107). Class II MHC, although important in the generation of adaptive immunity, does not directly impact NK cell responses so it will not be discussed further. MHC class I molecules are comprised of a heavy chain and a light chain. The light chain consists of an N-terminal α_1 domain linked via a disulphide bond to the α_2 domain that is connected to the membrane-spanning α_3 domain, which contains the cytoplasmic domain of the class I MHC. The light chain of MHC class I consists of the entirely extracellular β2-microglobulin (β2m) polypeptide, which is noncovalently bound to the heavy chain (107).

The most polymorphic region of class I MHC is within the α_1 and α_2 domains. This polymorphic region that spans the area between these two domains is the peptide binding groove, where processed peptides that are to be presented to T cells bind. Cytosolic antigens that are to be presented on MHC class I are processed by the proteasome to generate peptides that are nine amino acids in length (107). Peptides are transported into the lumen of the endoplasmic

reticulum (ER) by two ATP-binding cassette (ABC) transporter proteins, transporter associated with antigen processing- (TAP) 1 and 2. These proteins form a heterodimeric assembly that, in an ATP-dependent manner, transports peptides into the lumen of the ER. A partially formed class I MHC molecule, consisting of the incompletely folded α and β2m chains, is associated with the TAP transporter after release from the chaperone, calnexin, and accepts the newly translocated peptide. This peptide-MHC complex is released from the TAP molecules and then completes its folding. The properly folded peptide-MHC complex is then transported to the cell surface via the golgi export pathway (107).

Several mouse models deficient for MHC class I have been developed to study the impact of class I MHC on cellular development and tolerance mechanisms. A lack of β2m prevents class I MHC from being expressed on the cell surface. Therefore, mice lacking β2m fail to express MHC class I. Cells from β2m-deficient mice are more susceptible to lysis by NK cells (24). It has also been shown that NK cells from β2m-deficient mice exhibit decreased cytotoxicity against β2m-deficient targets, indicating that self tolerance mechanisms are still intact within these mice (24, 108). Other models for class I MHC deficiency include mice lacking the transporter proteins TAP-1 and TAP-2. The lack of class I MHC expression in TAP-1 or TAP-2 deficient mice emphasizes the importance of peptide being bound to the MHC for proper cell

surface expression. In TAP-deficient mice, partially folded class I MHC molecules are still assembled in the ER, but they never make it to the golgi due to the lack of bound peptide. NK cells from TAP-deficient mice exhibit the same decreased cytotoxicity as seen in β2m-deficient mice (108-110). Finally, mice have been generated that lack a specific class I MHC locus, such as H-2K$^{b-/-}$ knockout mice. To completely remove the class I MHC expressed in these mice, double-knock out mice have been generated, which remove the genes from both the D and K loci (H-2K^{b}D$^{b-/-}$, 111). All of these models provide a means by which to study NK cell cytotoxicity, in that MHC class I^{-} cells have increased susceptibility to NK cell lysis. They also provide a means by which to study NK cell self tolerance, since self tolerance is maintained in these mice, despite the lack of MHC class I.

E. NK Cell Receptors

Murine NK cells express cell surface receptors that provide activating and inhibitory signals to the cell to determine whether an interaction with a target cell will result in lysis of that cell or promote cytokine secretion. The receptors derive from two families of cell surface receptors: the C-type lectin superfamily of receptors and the immunolglobulin superfamily of receptors (IgSF). In the mouse, the majority of NK receptors are of the C-type lectin family; The Ly49s and the CD94/NKG2 receptors are in this family. The IgSF receptors on murine

NK cells contain the CD2/SLAM family and are not MHC class I-specific. Instead, these receptors bind ligands within their own family. These receptors are discussed in greater detail here, centering on murine NK cell receptors, as this dissertation will focus entirely on murine NK cells.

i. C-type lectin superfamily

C-type lectins are carbohydrate-recognizing proteins that are Ca^{2+} dependent. They are organized into two main groups, the collectins, like mannose-binding protein, and the selectins, like CD62L. The collectins are soluble proteins that mediate pathogen recognition, whereas the selectins mediate lymphocyte trafficking and homing to sites of infection through interaction with carbohydrate moieties on endothelial cells (reviewed in 112). On NK cells, the main C-type lectins that are expressed are the Ly49 family and the CD94/NKG2 family. The genes for these receptors are all found within the NK gene complex on mouse chromosome 6.

a. Ly49 receptors

Ly49 receptors were identified as being a part of the C-type lectin superfamily due to their C-terminal carbohydrate recognition domain (CRD). The ability of Ly49s to recognize carbohydrates is questionable, though, since these receptors have been shown to interact with other proteins and they lack the Ca^{2+} interaction domain that is normally associated with the CRD in C-type lectins, causing the receptors to be termed C-type lectin-like (112). Structurally, Ly49

receptors are type II transmembrane proteins that contain a C-terminal CRD, linked to a neck region that varies with the individual Ly49 receptor. The neck region is attached to an anchor sequence in the membrane, followed by an N-terminal cytoplasmic tail (112). The cytoplasmic tail of Ly49s determines the function of the Ly49, whether it will be inhibitory or activating. Potential inhibitory Ly49s have been identified based on their cDNA sequences. These include the genes for *Ly49a, b, c, e, f, g, i, j, o, q, s, t* and *v*, although only Ly49A, C, G and I have been examined in detail. Inhibitory Ly49s are characterized by an immunoreceptor tyrosine-based inhibition motif, or ITIM, that confers their inhibitory function (113). Activating Ly49s (identified through cDNA as *Ly49d, h, k, l, m, n, p, r, u* and *w*) do not contain an ITIM in their cytoplasmic domains; instead, activating receptors contain a positively-charged amino acid residue in their transmembrane domains that confers the ability of these receptors to interact with the DNAX-activating protein of 12-kD, DAP 12, that contains an immunoreceptor tyrosine-based activation motif or ITAM (14). Ly49 receptors are expressed on the cell surface as homodimers, giving each inhibitory receptor two ITIMs and each activating receptor two DAP 12-interaction domains.

Ly49s appear on the cell surface of NK cells post-natally over the course of weeks, reaching adult levels by 21 days of age (114). The acquisition of Ly49 receptors is thought to be influenced by MHC class I expression; mice transgenic for inhibitory Ly49s have altered expression of the Ly49 repertoire when self-

specific Ly49s are expressed early in development (115, 116). The Raulet and Sentman groups identified the sequential acquisition of Ly49 receptors and that the levels of Ly49s can be altered based on the MHC class I environment (117, 118). Ly49 receptor acquisition is not dependent on MHC class I interaction, however; mice deficient in MHC class I express increased levels of Ly49s, indicating that perhaps Ly49-MHC interactions negatively shape the Ly49 repertoire in NK cells (119). Ly49s recognize classical class I MHC molecules on the surface of potential target cells. In B6 mice, inhibitory Ly49s A and G2 and activating Ly49D recognize allogeneic H2-D^d, whereas the inhibitory Ly49s C and I recognize syngeneic H2-D^b (104, 120). When inhibitory Ly49s receive a signal through interaction with MHC class I, the tyrosine residue in the ITIM becomes phosphorylated, which recruits the phosphatases SHP-1, SHP-2 and SHIP (121). The resultant signals are predominantly negative or inhibitory to the NK cell, preventing lysis of the interacting cell. The interaction of activating Ly49s with their ligands results in the phosphorylation of two tyrosine residues in the ITAM of DAP-12, leading to the recruitment of the kinases ZAP-70 or Syk, which gives an activation signal for the NK cell, directing the lysis of the target cell in the absence of a cognate inhibitory signal or in the presence of an overriding activating signal (122, 124).

Some Ly49s have been shown to recognize MHC-like molecules that are encoded by viruses. Ly49H specifically binds the murine cytomegalovirus-

(MCMV) derived protein m157. This was discovered when the resistance of B6 to MCMV was mapped to the NK complex region of chromosome 6. The gene responsible for this resistance was found to be Ly49H. Indeed, *in vitro* studies show that Ly49H exhibits specific binding to m157 and results in an activation signal, leading to the lysis of the infected cell (17, 125-132). MCMV-susceptible 129/J mice, which are share an MHC with B6 mice but lack the Ly49H gene due to a separate NK cell gene complex, were found to have an inhibitory Ly49, $Ly49I^{129}$, that bound to the m157 protein and conferred an inhibitory response to the NK cell, preventing lysis of infected cells in 129 mice (132). The m157 molecule, which is MHC class I-like in structure, containing $\alpha 1$, $\alpha 2$, and $\alpha 3$ domains, likely evolved as a means of protecting MCMV-infected cells from lysis by NK cells. Ly49H may have been selected for in mice as a mechanism to eliminate virus-infected cells through the activation of NK cells.

b. CD94/NKG2 receptors

CD94 and NKG2 receptors are expressed on the cell surface of NK cells as heterodimers, with one CD94 molecule paired with one NKG2 molecule. These receptors are also type II transmembrane proteins that are C-type lectin-like molecules, in that they do not bind carbohydrates but they share a structural homology with the C-type lectins in the CRD (112). CD94 has the ability to pair with multiple different members of the NKG2 family, such as NKG2A, NKG2C and NKG2E. NKG2 receptors cannot reach the cell surface without co-

expression of CD94 (112). Like Ly49s, CD94/NKG2 receptors can be either inhibitory or activating. NKG2A is the only member of the family that contains an ITIM in its cytoplasmic tail, making it inhibitory. CD94/NKG2C and NKG2E lack an ITIM and instead associate with the adapter molecule DAP-12 to transmit activating signals to the NK cell (112).

CD94/NKG2 receptors recognize invariant non-classical class I MHC. The binding of these receptors to their respective ligands is contingent upon the presence of the peptide Qdm, derived from the leader sequences of classical class I MHC, within the peptide binding groove (13). NKG2A, C and E have been shown to bind Qa-1b bound to Qdm (13, 133). The expression of Qdm on the cell surface has been shown to be dependent upon TAP activity (112), indicating that CD94/NKG2 receptors may play a role in the recognition of cells that have down-modulated their expression of MHC class I.

In fetal and neonatal NK cells that do not express Ly49 receptors, CD94/NKG2A is expressed on nearly all NK cells (104, 105). This ubiquitous expression of the inhibitory CD94/NKG2A is thought to provide inhibitory signals to the developing NK cells to allow them to determine overall levels of MHC class I on potential target cells (103, 105). This could be an important mechanism for providing NK cell self tolerance before Ly49-dependent tolerance mechanisms are in place.

NKG2D is an activating member of the NKG2 family, but it functions differently than the other NKG2 members. Unlike the other members of the NKG2 family, NKG2D does not heterodimerize with CD94 (134). Instead, NKG2D homodimerizes, negating the need for CD94. In addition, NKG2D can associate with either DAP-12 or DAP-10 to mediate its activation signals (112). NKG2D is quite divergent from the other family members, sharing only a 21% sequence homology and having its own set of ligands. The ligands for NKG2D are MHC-like in structure and include retinoic acid early transcript (Rae1), the murine UL-16 binding protein-like (MULT-1) and the minor histocompatibility antigen H-60; these ligands are inducible upon cellular stress, transformation and infection, making cells susceptible to NKG2D-mediated lysis (18, 135-138). In NK-DC interactions, upregulation of NKG2D ligands on DCs after infection leads to NK cell-mediated induction of DC IL-12 production, in turn leading to the development of cytotoxic T lymphocytes (CTL, 38, 85). NKG2D has been found to be the major mechanism by which the NK-sensitive tumor, YAC-1, is lysed (139). In MHC class I-positive tumors, the expression of NKG2D ligands renders them susceptible to killing by NK cells, indicating that the signaling through NKG2D is stronger than the inhibitory signals and is able to breach the threshold required for killing (136, 139). Ligands for NKG2D have been found to be upregulated on some bone marrow allografts, making them susceptible to NKG2D-mediated lysis 140; this provides a mechanism by which host-type NK

cells can become activated to reject allogeneic BMC. The rejection or lysis of NKG2D-sensitive cells is mediated entirely through perforin, not the death receptor pathways (21). The cytolytic function of NKG2D was determined to be due to its association with and subsequent signaling through DAP10. DAP12 signaling was found to be necessary for cytokine secretion by NK cells (141). In fact, differential splicing of the NKG2D gene results in the production of a long and a short isoform of NKG2D; the long isoform only associates with DAP10, whereas the short isoform can associate with both adapters (142). Thus, given its role in CTL generation, tumor and bone marrow rejection, NKG2D is a vital part of the NK-cell response mechanism.

ii. The immunoglobulin superfamily of NK cell receptors

The IgSF in NK cells is represented by the Siglec family of receptors, the Ig-like transcript (ILT) family, the leukocyte-associated Ig-like receptors (LAIR), the paired Ig-like receptors (PIR), the natural cytotoxicity receptors (NCR), and the CD2/SLAM family of receptors. Like Ly49 receptors, ILT (also known as leukocyte Ig-like receptor (LIR) and monocyte/macrophage Ig-like receptors (MIR)) bind MHC class I molecules and can be inhibitory or activating. They are expressed on human lymphocytes and myeloid cells, however, their expression in mice is unknown (143). The Siglecs, a very diverse family of sialic acid-binding receptors, are expressed in the mouse and the human on various lymphoid and myeloid cells; the only Siglec expressed on murine NK cells is the inhibitory

Siglec-E (144). The ITIM in Siglec-E has been shown to associate with SHP-1 and -2 to potentiate inhibitory signals after binding of sialic acid (145). In mice, the LAIR family of receptors primarily consists of LAIR-1, which is expressed on NK cells in addition to most other leukocytes (144). Its ligand, however, is unknown. The NCR play an important role in the natural cytotoxicity of human NK cells, but their role in murine NK cells is less well understood (146-149). To date, only NKp46 has been shown to be expressed on murine NK cells (148). The PIR family of receptors, especially the activating PIR-A and the inhibitory PIR-B, has a broad expression pattern in humans; they are found on NK cells, B cells, DCs and cells of the myeloid lineage (150, 151). In mice, their expression is somewhat less expansive. Although they are expressed on murine B cells, DCs and myeloid cells, they are only expressed on the T/NK progenitor cell in the thymus (152), perhaps indicating a role for PIRs in the selection of T cells during development and in thymic NK cell development. The ILT, Siglec, LAIR and NCR families of receptors are largely not understood in murine NK cell function and are currently under investigation. The CD2/SLAM family of receptors has been much more intensively studied in mice.

Members of the CD2/SLAM family are found on mouse chromosome 1 with the exception of CD2 and BLAME, which are found on chromosome 3. This family includes signaling lymphocytic activation molecule (SLAM), CD2, CD48, CD58, CD244, CD84, Ly9, Ly108, CD2-like receptor activating cytotoxic cells

(CRACC, CS-1), CD84-H1 and B lymphocyte activating macrophage expressed (BLAME) (reviewed in 153). In the mouse, only CD2, CD48, CD84, Ly9 (minimally), Ly108 and CRACC are expressed on NK cells (153). These receptors form homotypic or heterotypic receptor ligand pairs, with members of the family serving as both receptor and ligand, in some cases the ligands are the same molecule as the receptor. The CD2/SLAM family of cell surface receptors is characterized by the expression of an Ig-variable-like (V) domain at the N-terminus and an Ig-constant (C2) domain at the C-terminus of the extracellular region of the molecule.

The intracellular domain of CD244, CD84, Ly9, Ly108 and CRACC all contain immunoreceptor tyrosine based switch motifs (ITSM), which enable the association of signaling adapters that contain SH2 domains, such as SH2D1A, or SLAM-associated protein (SAP) and SH2D1B, or EWS-Fli1-activated transcript-2 (Eat-2), although CRACC does not associate with these adapter molecules (153). A mutation in the gene for SAP results in X-linked lymphoproliferative disease (XLP), which is characterized by an uncontrolled polyclonal activation of lymphocytes, generally after exposure to Epstein Barr virus (EBV). In normal individuals, EBV infection is controlled by $CD8^+$ T cells specific for the virus even when latent infection remains (153). In mice, SAP-deficiency results in a XLP-like phenotype. EBV does not infect mice, so other infection models have been established to study the effect of SAP-deficiency in mice. Specifically, mice

infected with an acute strain of lymphocytic choriomeningitis virus (LCMV) results in clearance of the virus, but with increased T cell numbers remaining following infection (154, 155). Infection with a chronic strain of LCMV results in uncontrolled lymphoproliferation, leading to the death of all SAP knockout mice infected (154, 155). The murine gammaherpesvirus-68 leads to increased numbers of virus-specific T cells as well as tissue infiltration and damage (156). These models allow the study of SAP involvement in lymphocyte responses to virus infection and the mechanisms involved in development of lymphoproliferation in XLP.

CD244 expression and function on murine NK cells has been intensively studied since its discovery in 1993. CD244 has been shown to produce two different isoforms that differ in their cytoplasmic tail, with the short isoform having one ITSM and the long form having four (153). The long isoform of CD244 has been shown to exhibit an inhibitory effect when engaged by its ligand, CD48; this inhibition by CD244 is SAP-independent (157). The short isoform of CD244 has been shown to potentiate an activating signal that is SAP-dependent (153, 158). In mice the long isoform is the predominantly expressed form of CD244, indicating that CD244 may play an inhibitory role in mice. An inhibitory CD244 could be potentially important for NK cell tolerance mechanisms during early NK cell development, since CD244 is one of the first cell surface receptors expressed in B6 mice.

F. NK Cell Self-tolerance Mechanisms

Like T and B lymphocytes, NK cells must maintain tolerance to the host environment. Whereas a breach in T and B cell tolerance directly leads to autoimmunity, a breach in NK cell tolerance may indirectly lead to autoimmunity through the activation of T and B cells against self antigens due to the regulatory role of NK cells (159, 160). Therefore, mechanisms to ensure NK cell tolerance to self are of the utmost importance. It was proposed that NK cell tolerance was sustained through the presence of "at least one" inhibitory receptor for self MHC class I on every NK cell. However, the recent discovery of mature NK cells that lack self-inhibitory receptors has brought this hypothesis into question. Below are discussed new theories for the mechanism of NK cell tolerance, the NK cell licensing hypothesis and the disarming hypothesis.

i. NK cell licensing

The NK cell licensing model for NK cell tolerance was first proposed in 2005 by the Yokoyama group. This model, also termed NK cell "arming", addresses the induction of self-tolerant NK cells. NK cell licensing to acquire lytic capability is dependent upon inhibitory Ly49-class I MHC interactions, providing a reasonable mechanism by which NK cells from class I MHC-deficient mice lack cytotoxic ability (Figure 1.1).

NK cells activated through cross-linking of the NK1.1 molecule produce significant amounts of IFN-γ, even in NK cells that have not been activated by cytokine (161, 162). To address the issue of NK cell activation in mice with or without class I MHC expression, activation by NK1.1 cross-linking and subsequent IFN-γ secretion were measured. IFN-γ was produced by wild-type, B6 mice, but not by mice lacking MHC class I. This led the investigators to question the role of class I MHC in the development of NK cell cytotoxicity. Because Ly49 receptors bind MHC class I, the role of Ly49-MHC interactions was examined.

Activating Ly49 receptors were found not to be involved in the development of lytic NK cells because NK cells from DAP-12-deficient mice produced normal amounts of IFN-γ in response to NK1.1 cross-linking (161). Therefore, the inhibitory Ly49s were considered for a role in arming of NK cells. It was found that self inhibitory Ly49-expressing NK cells in MHC class I^+ mice but not MHC class I^- mice were able to generate IFN-γ, indicating that MHC class I expression is necessary for effector maturation of NK cells. In fact, when NK cells from transgenic mice expressing a single chain trimer for an individual MHC molecule were tested for their ability to generate IFN-γ, it was found that only those NK cells that expressed an inhibitory Ly49 that was specific for the MHC of the mouse could produce IFNγ; cells lacking self-specific Ly49s could not (161).

However, the self inhibitory receptor-negative NK cells could be induced to produce IFN-γ after poly inosinic poly cytidylic acid (poly I:C) or IL-2 stimulation. Mice expressing mutant self inhibitory Ly49s that lacked a functional ITIM could not secrete IFN-γ in response to NK1.1 stimulation, indicating that signaling through the Ly49 receptor was necessary for licensing of the NK cell; this was not, however, dependent on the phosphatase SHP-1, which is thought to play a dominant role in ITIM signaling. The inability of inhibitory receptor-negative NK cells to become activated in response to NK1.1 cross-linking is not due to these cells being immature, as they express similar levels of the maturation markers CD11b and CD43 as inhibitory receptor-positive cells (161).

These data indicate the necessity of MHC-Ly49 interactions in generating a pool of NK cells that are capable of becoming activated. Kim, et.al. clearly show, through the use of single chain MHC molecules *in vivo*, that NK cells with inhibitory receptors to self MHC class I become activated when taken *ex vivo* and stimulated with NK1.1, whereas NK cells that lack self inhibitory receptors remain significantly less able to become activated (161).

ii. The disarming model for NK cell tolerance induction

The Raulet group proposed another mechanism for NK cell tolerance, also in 2005. In their studies, they too examined the ability of MHC class I^- cells and MHC class I^+ cells lacking self inhibitory receptors to become activated in

response to stimulation. Their model for the induction of tolerance in NK cells lacking self inhibitory receptors was termed the “disarming” model (Figure 1.2, 163).

The ability of NK cells to become activated was determined by IFN-γ secretion in response to tumor targets as well as by direct cytotoxicity of tumor targets. Again, NK cells from MHC class I^- mice were found to be hyporesponsive compared with NK cells from class I^+ mice (108, 163). NK cells that were self inhibitory receptor negative had significantly decreased IFN-γ secretion and cytotoxicity compared with NK cells expressing self inhibitory receptors when stimulated with tumor cells that expressed NKG2D ligands. Like in the Yokoyama study, hyporesponsive NK cells could be induced to become cytotoxic with poly I:C or IL-2 stimulation as well as with *Listeria monocytogenes* infection. MHC class I^- bone marrow graft rejection was found to be almost entirely through the subset of cells that expresses self inhibitory receptors. These data led the investigators to conclude that NK cell hyporesponsiveness is an active process by which cells that lack self inhibitory receptors have their activating signals repressed to prevent them from indiscriminately lysing self cells (163). This is likely due to a signaling mechanism, because levels of activating receptors remain constant between inhibitory receptor positive and negative subsets of NK cells (163). Potentially, inhibitory receptors specific for non-MHC molecules could be responsible for the

down-modulation of the activating signals in cells that lack self-specific inhibitory Ly49 receptors.

iii. Discussion of the two models of NK cell tolerance

Both the NK cell licensing model and the disarming model propose mechanisms by which NK cells become tolerant to self. Each model advocates the necessity for MHC class I expression to generate self tolerant yet potentially active NK cells, however the licensing model specifically states that inhibitory signals generated by the interaction of self MHC class I with Ly49 receptors is the mechanism for this (161, 162), whereas the disarming model proposes no such mechanism. On the other hand, the disarming model indicates an active mechanism for NK cell hyporesponsiveness that the licensing model proposes to be a passive process (161, 163). Therefore, it would seem that these two models are not mutually exclusive; indeed, it would seem that they may be complimentary. Perhaps the actual model of NK cell tolerance induction is a synthesis of the two proposed models, whereby NK cells that express self inhibitory receptors that encounter self MHC class I are effectively armed for future activation and the NK cells that lack self inhibitory receptors are actively disarmed until activation after infection enables them to respond.

G. Transcription Factors in NK Cell Development

The distinct factors that drive each step of NK cell development are largely unknown, although recent advances have improved our understanding of the transcription factors that are necessary for NK cell development in general. Discussed here are the known transcription factors involved in NK cell development and their proposed functions in NK cells.

i. Id3

Basic helix-loop-helix transcription factors, as their name would imply, are classified by the presence of two domains that are homologous between all family members. One domain is composed mainly of basic residues which enable the transcription factor to bind to its consensus sequence. The second homologous domain is the helix-loop-helix domain, which enables these proteins to interact and form homo- or heterodimers (164). The basic helix-loop-helix (bHLH) transcription factor Id3 has been implicated in the development of the immune system. Specifically, Id3 has been found to be a transcriptional repressor of TCR rearrangement in developing T cells (106). Id3 specifically prevents the D-J rearrangement of the TCR genes. It was determined that through the inhibition of TCR rearrangement in the T/NK bipotent progenitor cell that NK cell development was promoted, making Id3 activity necessary for the generation of NK cells (106).

ii. Ets family

Several members of the Ets family of transcription factors have been implicated in development and functional regulation of the immune system. The Ets family members Ets-1, myeoid-elf-1-like factor (MEF) and PU.1 have been shown to regulate the development of NK cells, specifically. These transcription factors play a vital role in generating mature, functional NK cells.

a. Ets-1

Ets-1 is expressed in splenic NK cells (165) and Ets-1 deficient mice have significantly reduced numbers of peripheral NK cells and the remaining NK cells have greatly reduced cytotoxicity. *in vivo*, Ets-1 deficient mice develop tumors when injected with the RMA-S MHC class I deficient tumor cell line, which is rejected by wild type mice (166). Recently, Ets-1 has been implicated in the transcription of CD244, which may account for the developmental defects of NK cells in Ets-1 deficient mice, since CD244 is one of the earliest expressed receptors on developing NK cells and may therefore be involved in NK cell development (167, 168). However, the fact that CD244 knockout mice develop NK cells normally would indicate that Ets-1 may be responsible for the development of other molecules necessary for NK cell development.

b. MEF

MEF is a transcriptional transactivator in the Ets family of transcription factors and has been shown to regulate NK cell development and function. MEF-deficient mice have severely reduced numbers of peripheral NK cells and reduced

cytotoxicity against tumor targets (169-171), similar to Ets-1 deficient mice; it is possible that MEF and Ets-1 play redundant roles in the development of NK cells, although no distinct functions for MEF in NK cell development have been described. MEF has also been shown to directly activate the transcription of perforin. A lack of perforin is therefore thought to be responsible for the reduced cytotoxicity in MEF-deficient mice. The transcription of perforin is due to MEF and not Ets-1, as shown by transcriptional reporter assays (172). Therefore, MEF is a vital transcription factor for the development and function of murine NK cells.

c. PU.1

The transactivating Ets transcription factor family member PU.1 has been shown to be necessary for many areas of hematopoiesis. T cell and B cell as well as NK cell development are affected by a deficiency in PU.1 (173, 174). Whereas T and B cells are completely absent in PU.1-deficient mice, NK cells are present, but at reduced numbers. The NK cells that remain in PU.1-deficient mice have reduced expression of inhibitory Ly49 receptors and the cytokine receptors for stem cell factor and IL-7, which may explain the low numbers of circulating NK cells; fewer NK cells develop because they are not receiving the signals that they require to mature (173). The circulating NK cells also fail to proliferate when exposed to IL-2 or IL-12, indicating that PU.1 is necessary for proper response to certain cytokine signals (173). These data show that PU.1 is required for proper

NK cell development and that this requirement is separate from its requirement in T and B cell development. However, recent studies indicate that the development of NK cells is modulated by the lack of either T or B cells, with a T cell-deficiency resulting in accelerated NK cell development and an increased NK compartment and B cell-deficiency resulting in reduced splenic NK cell survival (175). These studies highlight the possibility that the function of PU.1 in T or B cells alone may impact the development of NK cells without a separate function of PU.1 in NK cells.

iii. Interferon Regulatory Factor-2 (IRF-2)

IRF-2 deficient mice have reduced numbers of mature, peripheral NK cells. In *in vitro* studies, IRF-2 was implicated in NK cell responses to IL-15 signals (176). However, this does not bear out *in vivo*. Developing NK cells in the bone marrow of IRF-2 deficient mice that have nearly reached maturity exhibit increased apoptosis, perhaps explaining why the periphery is lacking in mature cells (176). The NK cells found in the periphery have an immature phenotype, suggesting that the immature cells from the bone marrow escape to the periphery, whereas the mature cells apoptose before they can leave (176, 177). Based on comparisons with IL-15-deficient mice, which have a more severe NK cell deficiency than the IRF-2 deficient mice that extends into the bone marrow compartment, it was determined that IRF-2 is not necessary for relaying IL-15 signals (176). If IRF-2 was necessary for IL-15 signaling, it would be expected

that the mice that are deficient in IRF-2 would exhibit a similar phenotype to the IL-15 deficient mice, however, the IRF-2 deficient mice have a clearly defined phenotype that is separate from that seen in IL-15 knockouts (176). Thus, it seems that IRF-2 is necessary for a final maturation step in NK cell development that enables the cells to exit to the periphery.

iv. T cell factor/lymphoid enhancer factor (*TCF-1/LEF-1)*

TCF/LEF transcription factors specifically bind lymphoid enhancer elements and transactivate their target genes when bound to β-catenin of the *wnt* pathway (178). TCF-1 was originally found to be necessary for T cell development (179, 180). Subsequent studies identified LEF-1 as a redundant factor in T cell development, as TCF-1 knock-down mice have reduced numbers of T cells, although TCF-1/LEF-1 double knock-out mice have fewer T cells (179). LEF-1 single-deficient mice do not have as severe a lack of T cells as TCF-1 knock out mice (179). Similar roles for TCF-1 and LEF-1 have been discerned for NK cell development (178). A non-redundant role for TCF-1 in NK cell development seems to be the acquisition of some Ly49 receptors (181). Ly49A acquisition is specifically regulated by TCF-1 (182, 183); Ly49-MHC class I interactions are not necessary for this (178). Ly49D is also transactivated by TCF-1 (181). The transcription of Ly49G2 and Ly49I are actually repressed by TCF-1, indicating that TCF-1 may be an early-acting factor in the acquisition of Ly49 receptors, since Ly49A is the earliest Ly49 acquired (181). The role of

TCF-1 in Ly49 development may clarify the reason for the decrease in NK cell number in TCF-1 deficient mice. Since Ly49-MHC interactions are necessary for proper development, dysregulation of Ly49 acquisition may impact NK cell development negatively.

H. Forkhead Box (Fox) Transcription Factor Foxk1

Forkhead/*winged*-helix (Fox) transcription factors play an important role in the development of several body systems, including the immune system. Members of the forkhead family are identified based on their structure, not their function. 15 subfamilies have been described within the Fox family, termed Foxa-Foxo (184). Within each subfamily, each particular gene is denoted by a number, for example, Foxa1. The DNA-binding region is highly conserved between family members and has been crystallized, showing what has been called a "winged-helix" structure that is characterized by three α-helical domains and two "wings" of β-sheets (104, 184, 185), resembling the wings of a butterfly. Fox family members can exhibit activating or inhibitory function; the transactivation or repression domains of these proteins are highly divergent. Very little is known about how the transactivation and repression domains influence transcription. The nomenclature of Fox genes differs by species. In the mouse, when naming the gene, the first letter is capitalized and the others are lowercase and italicized (i.e. *Foxa1*). When naming the protein, the name is not italicized (Foxa1).

Currently, over 100 Fox family members have been identified based on sequence homology in the DNA-binding domain. Several Fox factors have been associated with the development and function of the immune system. These transcription factors will be discussed below, as will the transcription factor Foxk1, which we have studied in the development of NK cells.

i. Forkhead transcription factors in the immune system

Over the past decade and a half, Fox transcription factors have been intensely studied. Several immune dysfunctions have been shown to be due to mutations in Fox genes. In this section, the Fox transcription factors Foxn1, Foxp3, Foxj1 and Foxo3a will be discussed.

a. Foxn1

The development of a thymus is essential to the development and selection of T cells. The nude mouse, so called because of its hairlessness, lacks a thymus and hence T cells due to a mutation in Foxn1. The mutation in Foxn1 is caused by a single nucleotide deletion that results in a shift of the reading frame of Foxn1, which causes a loss of the DNA-binding domain (185, 186). This mutation results in a lack of all types of thymic endothelial cells due to the arrest of the thymic endothelial precursors prior to their differentiation into mature cells (186). The N-terminus of FOXN1 was found to be necessary for thymus development in an N-terminal Foxn1 deficient mouse (187). While most of the distinct gene targets for FOXN1 in the generation of the thymus are largely

unknown, it is thought that it regulates the expression of fibroblast growth factor (FGF) receptors as well as programmed death 1 (PD1) ligand (185). PD1 ligand-PD1 interactions are thought to play a role in positive selection of thymocytes. The importance of these pathways to thymic development remains unclear, however. Foxn1 activity has been found to be regulated by bone morphogenetic proteins (BMP), members of the transforming growth factor- (TGF-) β superfamily, and members of the WNT family of proteins (188, 189). Over-expression of β-catenin, a downstream target in the WNT-signaling pathway, resulted in upregulation of Foxn1 (190). Foxn1 expression is also repressed; it has been shown that extracellular signal-regulated kinase (ERK) 1 and 2 activation results in the prevention of Foxn1 transcription (191). Understanding the mechanisms by which Foxn1 regulates thymus development will provide insights into the interactions necessary for T cell development and selection.

b. Foxp3

The *scurfy* mouse is characterized by excessive proliferation of $CD4^+$ T cells that results in the infiltration of multiple organs by these cells. *Scurfy* was found to be caused by an X-linked recessive mutation; this was later determined to be a mutation in the Foxp3 gene (192). Like the nude mouse, the mutation in Foxp3 that results in the *scurfy* mouse is caused by a frameshift in Foxp3; this results in a truncated protein, lacking the DNA-binding domain (185, 192).

Scurfy mice seemed to lack an important component of the immune system responsible for dampening the immune response. Recently, a population of $CD4^+$ T cells named regulatory T cells (T_{reg}) has been described. T_{reg} cells represent between 5-10% of the $CD4^+$ T cells in a mouse and are characterized by the expression of Foxp3 (193, 194). T_{reg} cells are thought to be necessary to regulate autoinflammatory disorders and transplant rejection by T cells. Mice deficient in Foxp3 have a lymphoproliferative disorder nearly identical to that seen in *scurfy*. Transduction of Foxp3 into Foxp3-negative cells results in the upregulation of GITR (glucocorticoid-induced TNF-receptor-related protein) and CTLA4, which are found on T_{reg} cells, although it is not known whether Foxp3 directly regulates the expression of these genes (195-197). Foxp3-transduced T cells are also able to inhibit untransduced T cells *in vivo* (194). Thus, it appears that Foxp3 is sufficient to confer T_{reg} properties onto T cells. Foxp3 is thought to be a transcriptional repressor, in that Jurkat T cells that have been transduced with Foxp3 produce less IL-2 than their mock-transduced counterparts (196). Foxp3 can be induced upon TCR-stimulation, implying that T_{reg} cells are produced during an immune response, perhaps to control the proliferation of expanding effector cells. Further work is needed to identify target genes of Foxp3 as well as the mechanisms that regulate its expression.

c. Foxj1

Foxj1 was originally identified as a transcription factor specific to ciliated tissues and was well described for its role in developing those tissues (184, 185). Foxj1-deficiency is embryonic lethal, resulting in a reversal of internal organs and a complete lack of ciliated tissues (198). The involvement of Foxj1 in the immune system was only recently observed when mice that are prone to develop systemic lupus erythematosus (SLE) exhibited a marked loss of the gene in lymphocytes (199). This observation prompted the investigation into whether Foxj1 is involved in the prevention of autoimmune responses. Due to the embryonic lethality of Foxj1 deficiency, a chimeric fetal liver, in which $Foxj1^{-/-}$ hematopoietic stem cells were grafted onto a $RAG^{-/-}$ fetal liver, was generated to study the development of the immune system in the absence of Foxj1 (200). These chimeric mice exhibited systemic autoimmune inflammation and their $CD4^+$ T cells were hyper-activated. These $CD4^+$ T cells were skewed toward the T_H1 type (200). In wild-type $CD4^+$ T cells, Foxj1 has been found to inhibit the nuclear factor-κB (NF-κB) activity that is necessary for the generation of the T_H1 phenotype (200, 201), perhaps describing its regulatory role in preventing autoimmunity. Foxj1 expression may be regulated through a negative-feedback mechanism in which NF-κB regulates its own down-modulation (200). Thus, it appears that Foxj1 may be a potential therapeutic target for autoimmune disorders in which NF-κB is constitutively activated.

d. Foxo3a

The Foxo sub-family of transcription factors has been studied in a variety of cellular processes. A Foxo homolog in *Caenorhabditis elegans*, DAF-16, has been reported to regulate longevity in that organism (202). Foxo3a has also been shown to negatively regulate oocyte development through the induction of the cyclin dependent kinase (cdk) inhibitor $p27^{KIP}$ (203); $Foxo3a^{-/-}$ mice have accelerated oocyte development (203, 204). Many Foxos have redundant functions and although Foxo3a seems to be the main Foxo that is expressed in cells of the immune system, it is likely that other Foxos also serve a function. In the immune system, Foxo3a has been shown to regulate lymphocyte homeostasis. $Foxo3a^{-/-}$ mice develop a lymphoproliferative disease that leads to multiple organ infiltration and widespread inflammation at eight months of age (205). As with Foxj1, Foxo3a activity was significantly reduced in autoimmune-prone mice, indicating that Foxo3a may have a role in regulating the immune response (199, 205). A direct target of Foxo3a is cyclin G2, which inhibits cell-cycle progression and has been shown in lymphocytes to negatively regulate proliferative responses (205). It is unclear, however, whether the induction of cyclin G2 in lymphocytes is due to regulation by Foxo3a. Expression of survival factors in the immune system, including IL-2 and IL-3, leads to the inactivation of Foxo3a. This mechanism is dependent upon phosphorylation of Foxo3a through

the phosphatidylinositol 3- (PI3) kinase and AKT pathways (205, 206). The inactivation of Foxo3a in B cells results in B cell proliferation, indicating that Foxo3a may keep B cells in a quiescent state. In addition, Foxo3a has been shown to directly activate the transcription of several apoptotic genes, including FasL and Bcl-2-interacting mediator of cell death (BIM); this has also been shown in NK cells (205-208). Although the work describing the role of Foxo3a in the immune system is largely still ongoing, thus far is seems as though Foxo3a serves an important regulatory role in the homeostasis of the immune cells.

ii. Foxk1

Foxk1, originally termed myocyte nuclear factor (MNF), was first identified in the skeletal muscle (209), although it is expressed in a wide variety of tissues throughout the mouse. These tissues include the heart, lung, spleen, liver, thymus, brain and testes. Foxk1 is enriched in myogenic stem cells. Studies using Foxk1$^{-/-}$ mice have shown that cells lacking Foxk1 enter the G_0/G_1 phase of the cell cycle and cannot progress through the cell cycle. This has been shown to be due to the requirement of Foxk1 to repress the transcription of the cdk inhibitor p21^{CIP}. In the absence of Foxk1, p21 levels rise, preventing cell cycle progression (210). Originally, Foxk1 was thought to act as a transcriptional activator; it was shown to transactivate the myoglobin gene in skeletal muscle cells (209, 211), although this initial report has not been repeated.

The regulation of Foxk1 expression in myogenic progenitor cells is being defined. Using a transcriptional reporter, consisting of the promoter for Foxk1 attached to the gene for LacZ (that encodes β-galactosidase), it has been shown that in myogenic progenitors that Foxk1 is expressed. To define the elements that regulate this, the promoter was examined and a Sox binding site was revealed (212). It was then demonstrated that Sox15 binds this region of the Foxk1 promoter and transcriptionally activates Foxk1 expression (212). In fact, by mutating the Sox-binding site in the Foxk1 promoter, Foxk1 transcription ceased. The binding of Sox15 to the Foxk1 promoter was shown to recruit the Four and a half LIM (Fhl) family member Fhl3, which serves as a co-activator of Foxk1 expression. Fhl3 alone cannot bind or transactivate Foxk1, but it synergizes with Sox15 to increase the transcription of Foxk1 (212). Thus, the regulation of Foxk1 in skeletal muscle progenitor populations has been described. The activity of Foxk1 in other cells is less well defined.

There are no reports indicating a role for Foxk1 in the immune system, but the expression of Foxk1 in lymphoid tissues (spleen, liver and thymus) indicates that it may affect the development and maintenance of immune cells, due to the role of Foxk1 and other forkheads in development. To investigate this possibility, we have studied the effects of Foxk1 in the immune system. These results will be reported in chapter IV.

I. Objectives

In the course of our investigation into the components necessary for proper NK cell development, we have used two different models. We have examined the importance of MHC class I interactions with NK cells in the development of functionally mature NK cells using irradiation chimeras of B6→TAP1$^{-/-}$ mice. We have also investigated the requirement for Foxk1 in the development of functionally mature NK cells by using the Foxk1$^{-/-}$ mouse. These models and their relevance to NK cell maturation will be discussed below.

i. MHC class I^{+} NK cells developed in an MHC class I^{low} environment

The necessity for MHC class I molecules to generate a fully functional pool of NK cells is widely acknowledged. In this study we have attempted to determine why NK cells from TAP1$^{-/-}$ mice are functionally deficient. This will be discussed further in Chapter III.

ii. The role of Foxk1 in the development and function of NK cells

Determining the transcriptional regulators that are required for NK cell development is essential for a full understanding of these processes. In this vein,

we have studied the role of the transcription factor Foxk1 in NK cell development and function. The results of these studies will be discussed in chapter IV.

Developmental Markers	Acquisition of Markers				
	I	II	III	IV	V
CD122	X	X	X	X	X
CD2	X	X	X	X	X
CD244	X	X	X	X	X
CD11a	X	+/-	+/-	+/-	+/-
NK1.1	--	X	X	X	X
CD94/NKG2	--	X	X	X	X
Ly49	--	--	X	X	X
c-kit	--	--	X	X	+/-
CD51	--	high	high	Low	--
CD49b	--	low	low	High	High
CD11b	--	low	low	Low	High
CD43	--	low	low	Low	High

Table 1.1 The expression pattern of cell surface receptors and integrins throughout the five stages of developing NK cells. X denotes expression, +/- denotes minimal expression, -- denotes no expression, high denotes high expression and low denotes low expression of the molecule. Adapted from Kim, et. al., 2002.

Forkhead Transcription Factor	Role in Immune System
Foxn1	Thymic development
Foxp3	Regulatory T cell development
Foxj1	Inhibition of NF-κB in autoimmune disease
Foxo3a	Regulation of lymphocyte homeostasis

Table 1.2 Forkhead transcription factors and their roles in the immune system. Represented are the known forkhead transcription factors involved in the immune response.

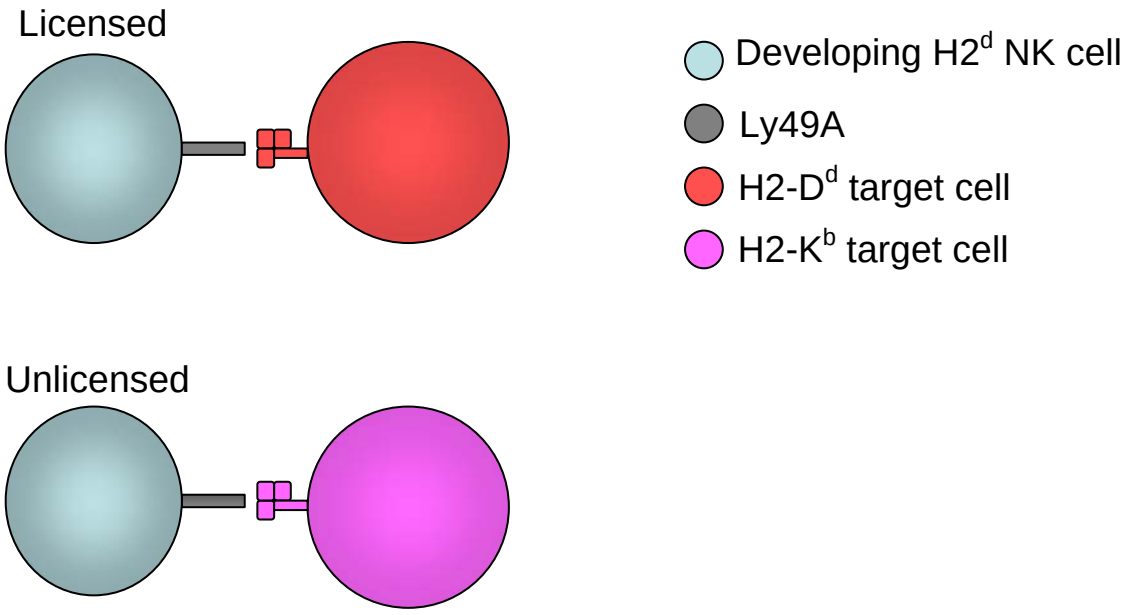


Figure 1.1 Licensing model of NK cell tolerance. NK cells that express inhibitory Ly49 receptors for self MHC class I that receive signals from interaction with self MHC class I become licensed to for NK cell cytotoxicity (top). NK cells with inhibitory Ly49 receptors that do not encounter self MHC class I remain unlicensed.

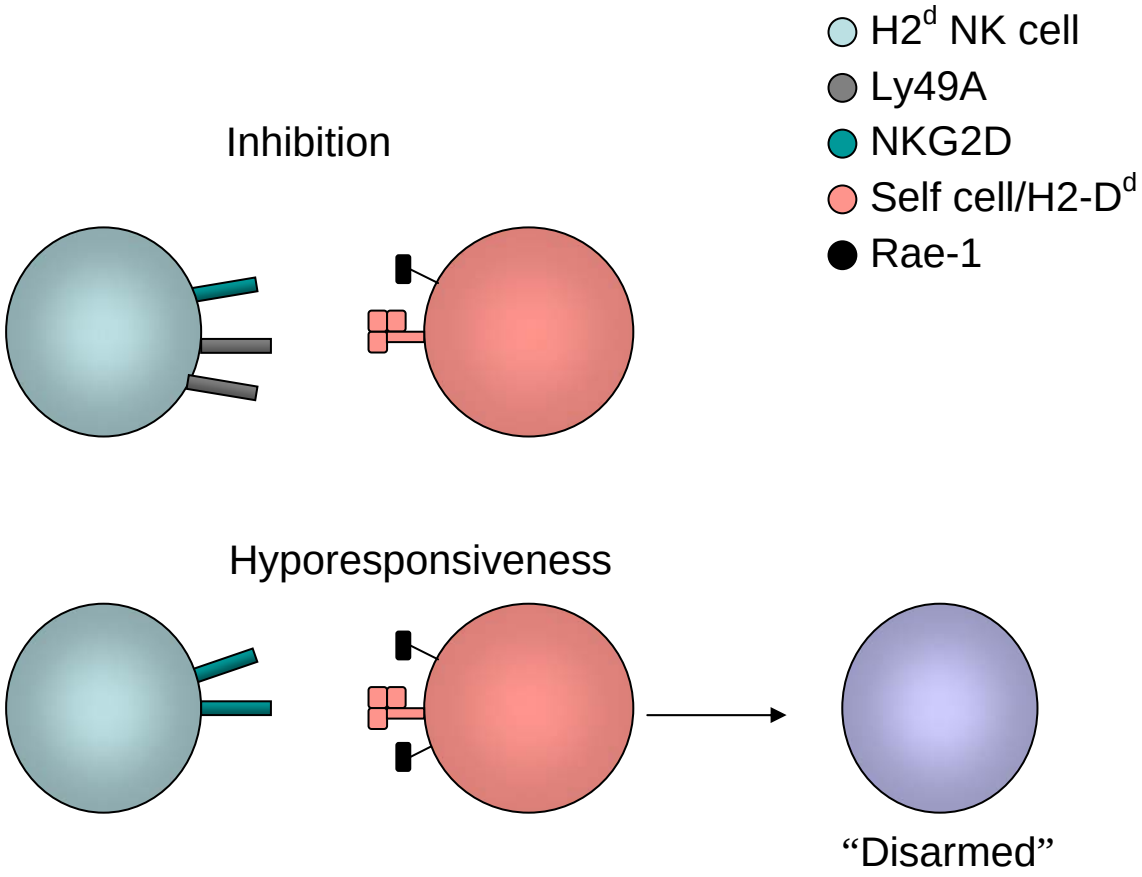


Figure 1.2 The disarming model of NK cell tolerance. In NK cells that have sufficient inhibitory receptors to prevent lysis of self cells, inhibition occurs (top). In NK cells where activating signals outnumber inhibitory signals, disarming occurs (bottom).

CHAPTER II

MATERIALS AND METHODS

I. Mice

All mouse strains, [129,C57BL/6 TAP1$^{-/-}$ (TAP1$^{-/-}$), (BALB/c X C57BL/6) F1 (CB6F1)$^{+/+}$ and (C.B-17 X B6)F1 (CB6F1) $^{scid/scid}$, D8, BALB/c, C57BL/6 (B6), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (B6.CD45.1) and MNF$^{-/-}$ (Foxk1$^{-/-}$)] used in this study were either bred at The University of Texas Southwestern Medical Center at Dallas or were purchased from the National Cancer Institute (Rockville, MD; B6) or from Jackson Laboratories (Bar Harbor, ME; TAP1$^{-/-}$). Foxk1$^{-/-}$ mice were the kind gift of Daniel Garry, M.D. Foxk1$^{-/-}$ mice were generated by targeting of the *Mnf* gene with a neomycin expression cassette (211). Mice were housed in either conventional or specific pathogen free facilities. All mice were maintained according to institutional standards and procedures. Mice of both sexes were used and similar results were obtained with either sex. Mice were 6 weeks to 4 months old at the time of use.

II. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was isolated from skeletal muscle, heart, liver, kidney, spleen, brain, lung and testis using the TriPure Isolation Kit (Roche, Indianapolis, IN) as previously described (210). PCR conditions for Foxk1 were as follows: 94ºC 1.5', (94ºC 15s, 62ºC 15s, 72ºC 15s) x 30 cycles, 72ºC 7'. Foxk1 PCR was

performed using the following primer pair: Forward-5' GCAGGAAGGTGGAGTGAAAC 3', Reverse-5' CCATGGGAGGACAGGAGATA 3'. 18S Ribosomal RNA, as control for RNA content, was detected using the following primer pair: Forward-5'CTCAACACGGGAAACCTCAC 3' AND Reverse-5' TGCCAGAGTCTCGTTCGTTAT 3'. PCR products were resolved on 2% agarose gels.

III. Generation of radiation chimeras

To generate B6→TAP1$^{-/-}$ primary chimeras, bone marrow cells (BMC) from the long bones and spines of donor mice were harvested by crushing the bones in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, Penicillin, Streptomycin, non-essential amino acids and sodium pyruvate (Invitrogen, Carlsbad, CA). $6x10^6$ whole BMC were infused into recipient B6 or TAP1$^{-/-}$ mice that had been previously irradiated with between 8-9 Gy ^{138}Cs-Gamma irradiation. BMC were allowed to reconstitute for 6-10 weeks before the chimeras were used. Chimerism was determined by peripheral blood staining for H2^b in B6→TAP1$^{-/-}$ chimeras. Secondary chimeras were generated by infusing 'primary' chimera BMC into recipient mice as described above.

Bone marrow from B6.CD45.1 and Foxk1$^{-/-}$ mice was harvested and transferred as described above. Briefly, 5x10^6 B6.CD45.1 BMC were injected into Foxk1$^{-/-}$ mice irradiated with 850 rads. 5x10^6 Foxk1$^{-/-}$ BMC were injected into B6.CD45.1 mice irradiated with 850 rads. Mice were given acidified water for three weeks post transplant. The bone marrow and spleen were analyzed for NK cell development and content six to eight weeks post transplant.

IV. Bone marrow transplant assays

Primary chimeras (B6→B6 and B6→TAP1$^{-/-}$) in groups of 5 mice were challenged with 2.5x10^6 donor or host and third-party BMC (harvested as above) 1-3 months post generation. The assay for BMC engraftment was to measure the splenic incorporation of the thymidine analog 5-iodo [^{125}I]-2'-deoxyuridine ([^{125}I]dU). Five days post transplant, groups of 5 mice were injected with 25 μg 5-flouro-2'-deoxyuridine (Sigma, St. Louis, MO) intra-peritoneally (i.p.) to inhibit host thymidylate synthase to lower endogenous thymidine levels. After 30-60 minutes, 0.3 μCi [^{125}I]dU (Amersham, GE Health Sciences, Piscataway, NJ) was injected i.p. The mice underwent $C0_2$ euthanasia 2 h later, and spleens were removed and soaked in 70% ethanol overnight to elute non-DNA radioactivity. The ^{125}I radioactivity was measured in a gamma scintillation counter. The percent (%) splenic uptake was calculated. The values presented are geometric means (95% confidence limits). Two-tailed Student's *t* tests were

performed to detect statistical differences between geometric mean values of various groups, using Microsoft Excel. *p* values <0.05 were considered significant.

Secondary chimeras were similarly challenged with $2.5x10^6$ $TAP1^{-/-}$ BMC.

V. In vivo cytotoxicity assay

A second assay for BMC survival or rejection in host mice was used in irradiated neonatal host mice, because endogenous uptake of [^{125}I]dU is quite high in such mice. Donor BMC were labeled with 5-(and-6-)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Invitrogen, Carlsbad, CA), using the previously described method, with slight modifications (213). Briefly, BMC were isolated from BALB/c, $TAP1^{-/-}$ and B6 mice, as described above. The cells were counted and 1 X 10^8 cells were removed, washed and re-suspended in either 0.4 μM (B6 host-strain samples) or 4 μM CFSE ($TAP1^{-/-}$ or BALB/c donor-strain samples). The cells were incubated at 37 ºC for 10 minutes and washed twice with PBS supplemented with 2% FCS. The cells were re-suspended in sterile PBS and 1 X 10^7 cells of each type were mixed together before injection in a volume of 200 μl via a tail vein. All recipient mice were 13-17 day old B6 mice that were exposed to 6 Gy ^{137}Cs-gamma irradiation. A sample of the injection mixture was analyzed using a FACScan or FACScaliber flow cytometer (Becton Dickenson, San Jose, CA) for reference. Twenty-four hours after injection, the

recipient mice were sacrificed and their spleens were harvested. The spleen cells were dispersed into PBS supplemented with 2% FCS and splenocytes were filtered through 100-μm nylon bolt cloth. The cells were then washed and re-suspended in PBS/2% FCS. Erythrocytes were lysed with isotonic Tris-ammonium chloride. Splenocytes were immediately analyzed. At least 8,000 CFSE-labeled cells were collected in each sample. The survival of donor-type CFSE-labeled cells was calculated by determining the ratio, % cells per spleen/% cells injected, ($^{\text{CFSE-high (spleen)}}/_{\text{CFSE-high (injected)}}$ cells)/($^{\text{CFSE-low (spleen)}}/_{\text{CFSE-low (injected)}}$ cells). The mean ± SEM ratio values were measured and calculated. The two-tailed Student's *t* test, using Microsoft Excel, determined any significant differences ($p < 0.05$).

VI. Flow cytometry and antibodies

Bone marrow, spleen, thymus, lymph node and peripheral blood were harvested and single cell suspensions were prepared. Cells were pre-incubated at 4ºC with anti-CD16/32 (2.4G2) to block non-specific antibody binding. Cells were then stained with pre-determined dilutions of antibody for four-color analysis with fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Perdinin Chlorophyll Protein (PerCP)-Cy5.5, Allophycocyanin (APC) and PE-Cy5 conjugated antibodies. Biotin conjugated antibodies were detected by secondary staining with streptavidin-conjugated PE, APC, PerCP or PE-Cy5 antibodies

(Becton Dickenson, San Jose, CA). The following markers were examined for the analyses: CD3 (145-2C11), CD21/35 (7G6), CD62L (MEL-14), CD8 (53-6.7), CD45R (RA3-6B2), CD19 (1D3), CD25 (7D4), CD11b (M1/70), CD43 (S7), CD11a (M17/4), Ly-6G (Gr-1) and Ly6-C (RB6-8C5), Ly49G2 (4D11), Ly49C/I (5E6), Ly49C/I/H (1F8), Ly49D (4E5), Ly49A (A1), CD94 (18d3), CD117 (2B8), Ly-6A/E (E13-161.7; D7), CD122 (TM-β1), CD51 (RMV-7), CD244 (2B4), CD23 (B3B4), CD4 (GK1.5), CD69 (H1.2F3), CD49b (DX5), NK1.1 (PK136), IgM (R6-60.2), CD44 (IM7), CD90.2 (53-2.1), Ter119 (Ly-76) and CD127 (B12-4) (Becton Dickenson, San Jose, CA). 1F8 antibody was purified and conjugated in our laboratory. Data was collected using a FACScan, FACsCalibur or an LSRII (Becton Dickenson, San Jose, CA) and analyzed with CellQuest (Becton Dickenson, San Jose, CA) or FlowJo software (Tree Star, Ashland, OR).

VII. In vitro cytotoxicity assays and preparation of effectors

Cytotoxicity assays were performed as described (214). Briefly, $2x10^6$ YAC-1 target cells were labeled with 100 μCi ^{51}Cr in 500 μl RPMI for two hours. $5x10^4$ target cells were then incubated with effector cells at various effector:target ratios for four hours at 37ºC. Percent specific lysis, represented as the release of ^{51}Cr gamma radiation into the supernatant, was determined by scintillation counting.

To generate effector cells, B6 and Foxk1$^{-/-}$ mice were injected with 300µg poly inosinic acid: poly cytidylic acid (poly I:C; Calbiochem, Darmstadt, Germany) 24 hours prior to harvesting spleens. Single cell suspensions were prepared and cells were incubated at 4ºC with 2.4G2 and then with biotinylated antibodies for CD3, CD4, CD8, CD11b, Ly-6G and CD19. Antibody-bound cells were incubated with DM streptavidin microbeads (Becton Dickenson, San Jose, CA) and placed in a magnetic field to remove the magnetic subset of cells, resulting in >80% pure NK cells. The purified NK cells were either used directly in ^{51}Cr-release assays against YAC-1 target cells or washed and incubated with biotinylated CD49b antibody and then with DM streptavidin microbeads. The magnetic fraction was obtained, resulting in greater than 90% purity. The doubly-purified NK cells were put into culture at 3x10^{6} cells/ml with 500 U/ml rIL-2 or 150 ng/ml rhIL-15 for five days and then used as effectors in ^{51}Cr-release assays. Purified NK cells from previously unmanipulated mice were cultured with 100 µg/ml IL-12 and IL-18 for five days and used as effectors against YAC-1 targets.

Chapter III

MHC CLASS I-NK CELL INTERACTIONS REGULATE NK CELL DEVELOPMENT

Objective

It has been shown that NK cell self tolerance is generated and maintained through Ly49 receptors and self-MHC class I interactions during NK cell development (117, 118, 161, 215, 216). In these studies we wanted to determine whether MHC class I^{high} NK cells generated in an MHC class I^- environment would become functionally mature in the absence of Ly49-MHC interactions. We also sought to determine whether neonatal NK cells, which lack Ly49 receptors, still maintain the ability to reject BMC grafts. We performed these studies to gain insight into the reason for the functional deficiency of NK cells in $TAP1^{-/-}$ mice.

Introduction

Natural Killer (NK) cells are lymphocytes largely responsible for clearance of virus infected cells as well as tumor cells (22, 76, 217). They also play a critical role in the acute rejection of allogeneic bone marrow (BM) grafts (29, 218). In tissue transplantation, generation of BM chimeras has been shown to improve solid tissue engraftment (219-221), making the tolerance of NK cells to the BM grafts of utmost importance. However, the mechanism governing NK cell tolerance to self has yet to be fully elucidated.

NK cells develop in the bone marrow from a multipotent progenitor cell that requires interactions with the stromal cells of the bone compartment as well as various cytokines, such as IL-15 for their development (3, 6). Mice with a depleted bone marrow microenvironment, such as following ^{89}Sr treatment, fail to develop NK cells or develop NK cells that lack Ly49 receptors (1). Ly49 receptor expression on NK cells is fundamentally important to the ability of NK cells to distinguish self from non-self (114, 115, 222-224). *in vitro* studies using neonatal NK cells that lack Ly49 receptors underline the importance of CD94/NKG2A receptors in distinguishing MHC class I^+ cells from MHC class I^- cells (103-105). Neonatal NK cells, however, lack the ability to lyse allogeneic MHC class I^+ targets (103).

Recognition of self by mature NK cells that express Ly49 receptors cannot account for NK cell self-tolerance, however. Mice that lack surface expression of MHC class I molecules have self-tolerant NK cells, even though the NK cells in these mice have normal or somewhat higher levels of Ly49 expression (119). DL6 (H-2^b, D^d) mice, in which the D^d gene exhibits mosaic expression, fail to lyse or reject cells of B6 or D8 origin. When NK cells are sorted from DL6 mice for D^{d+} and D^{d-} subsets and cultured with IL-2, this results in the D^{d+} cells now being able to lyse D^d- cells (225). This implies that NK cell tolerance requires continued contact between the NK cells and cells expressing self MHC class I, in

this case both D^b and D^d. Supporting this study is the observation that fully mismatched B6 to BALB/c chimeras accept both B6 and BALB/c BMC grafts. However, after culture of the NK cells in IL-2, the tolerance is reversed (226). This implies that contact with cells other than NK cells is needed to engender this tolerance and to maintain it. The bone marrow stroma most likely contains the necessary elements for generation and maintenance of NK cell tolerance. NK cells developed *in vitro* fail to acquire Ly49 receptors in the absence of bone marrow-derived stroma (3, 6, 102). In addition, bone fragments from allogeneic mice placed under the kidney capsule of irradiated mice enabled the generation of tolerance to BMC of the allogeneic strain

In this study we have examined the need for the bone marrow microenvironment in the development and maintenance of NK cell tolerance. We chose to study the tolerance of NK cells in a MHC class I^{low} environment, as these NK cells are inherently self-tolerant and the development of chimeras in this environment gives us a unique opportunity to observe the effects of the MHC class I^{low} environment on MHC class I^{high} hematopoietic cells.

Results

Growth of MHC class I^{high} BMC in irradiated $TAP1^{-/-}$ recipients results in chimeras unable to reject histoincompatible BMC grafts

To determine the rejection capability of chimeras generated in MHC class I deficient mice, B6→B6 and B6→TAP1$^{-/-}$ chimeras were challenged with B6 BMC. Five days later, both groups of chimeric mice were shown to accept the graft (Fig. 3.1a). As expected, B6→B6 chimeras reject TAP1$^{-/-}$ and BALB/c BMC grafts when challenged (Fig. 3.1a). B6→TAP1$^{-/-}$ chimeras accepted the TAP1$^{-/-}$ graft, also as expected. However, they also accepted the BALB/c (Fig. 3.1a) and D8 (H2^{b}, D^{d}) (Fig. 3.1b) BMC grafts. This was unexpected, as the NK cells in these mice had never seen the H2^{d} antigen before and would therefore not be expected to recognize it as 'self' and should not be tolerant to these targets.

To rule out the possibility that there may be a subset of T cells, perhaps regulatory T cells, that are functioning to suppress the NK cells in these mice in an as yet unknown manner, we generated chimeras between CB6F1$^{+/+}$ or CB6F1$^{scid/scid}$→TAP1$^{-/-}$ mice and determined their rejection capability to H2^{b} BMC. One group of CB6F1$^{scid/scid}$→TAP1$^{-/-}$ mice were reconstituted with thymocytes prior to the BM transplant. CB6F1 mice that are given H2^{b} BMC exhibit hybrid resistance, rejecting the parental strain; generating chimeras between CB6F1 mice and TAP1$^{-/-}$ mice prevents hybrid resistance, i.e., the parental strain is accepted (Fig. 3.2). This is an entirely NK cell-dependent phenomenon. T cells are unnecessary, as is seen when thymus cells are reconstituted in CB6F1$^{scid/scid}$→TAP1$^{-/-}$ chimeras.

Knowing that T cells were not responsible for the 'tolerance' exhibited by the NK cells in the B6→TAP1$^{-/-}$ chimeras, we decided to examine the NK cells themselves. Chimerism was determined in the B6→TAP1$^{-/-}$ chimeras and >60% of the NK cells were of B6 origin (peripheral blood stains >60% H-2^{b+}, data not shown). We chose to examine the Ly49 repertoire of the B6→B6 and B6→TAP1$^{-/-}$ chimeras' NK cells to determine whether there is skewing of the repertoire in the B6→TAP1$^{-/-}$ mice that may be the source of the tolerance that we observed. When compared to the B6→B6 chimeras, the B6→TAP1$^{-/-}$ chimeras showed no difference in their Ly49 expression (Fig. 3.3a and b). This was true both in receptor expression and in cell surface density. Therefore, altered Ly49 expression on these chimeras cannot explain the lack of cytolytic activity of B6→TAP1$^{-/-}$ NK cells.

Host BM microenvironment determines ability of NK cells to reject BMC grafts

We next wanted to address whether stem cells from chimeric mice would be able to generate functional NK cells when generated in MHC class I^{+} or MHC class I^{-} mice; this was done to test the contribution of the environment in which NK cells develop and to determine whether interaction with MHC class I^{+} cells of hematopoietic origin is sufficient for the development of lytic NK cells. To test this, secondary chimeras were prepared by infusing BMC from B6→B6 or

B6→TAP1$^{-/-}$ chimeras into B6 and TAP1$^{-/-}$ mice. These secondary chimeras were then challenged with TAP1$^{-/-}$ BMC. In summary, regardless of the origin of the BMC from which NK cells are derived, TAP1$^{-/-}$ BMC when given to B6 hosts (i.e. B6→B6→B6 or B6→TAP1$^{-/-}$→B6) were rejected. Conversely, TAP1$^{-/-}$ BMC given to TAP1$^{-/-}$ hosts (i.e. B6→TAP1$^{-/-}$→TAP1$^{-/-}$ or B6→B6→TAP1$^{-/-}$) were accepted (Fig. 3.4). These results indicate that the bone marrow microenvironment is essential for the development of NK cells capable of rejecting BMC grafts as well as determining the specificity of the rejection.

Neonatal NK cells reject MHC class I^{low} but not MHC class I^{high} bone marrow grafts

Previously it has been shown that neonatal NK cells, lacking Ly49 receptors, are unable to lyse MHC class I^{high} targets in a ^{51}Cr-release assay (103). Neonatal NK cells that are derived from MHC class I^{+} environments exhibit a lack of lytic ability against MHC class I^{+} targets and targets expressing non-self MHC, suggesting that the acquisition of Ly49 receptors is a critical developmental stage that enables the rejection of allogeneic cells. These observations led us to investigate whether neonatal mice had rejection capability *in vivo*. To assay for engraftment in such young mice (13-17 days old) we needed to employ a technique other than the standard ^{125}[I]dU-uptake assay. We chose to

use a CFSE-based approach (213). Irradiated neonatal B6 mice were injected with CFSE-labeled B6 and either BALB/c or TAP1$^{-/-}$ BMC. Splenocytes were analyzed by FACS 24 hours later for the percentage of B6 and either BALB/c or TAP1$^{-/-}$ cells remaining. As shown in Figure 3.5, TAP1$^{-/-}$ BMC survived significantly less well than did BALB/c BMC. This indicates that neonatal B6 mice have the ability to reject MHC class I^{low} but not MHC class I^{high} BMC. This has not been previously shown.

Discussion

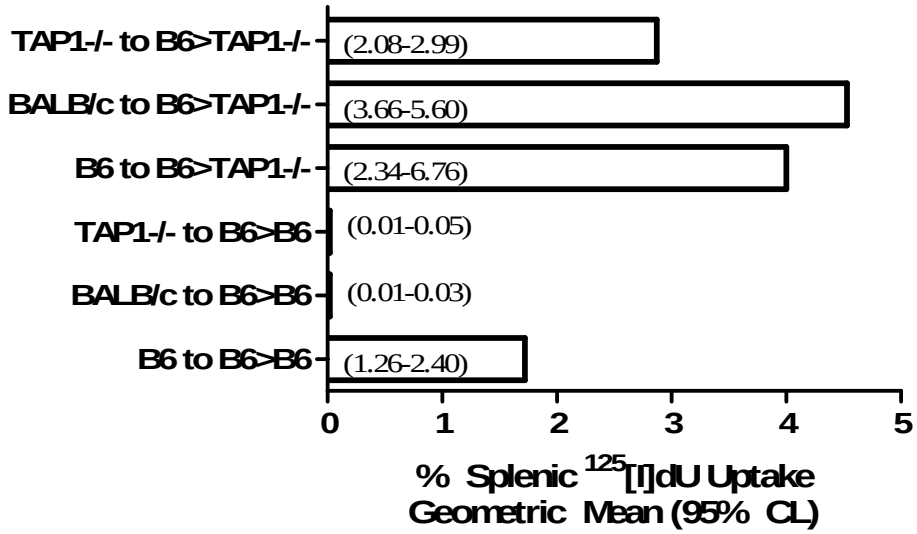
Recent advances in the field of NK cell tolerance have proposed mechanisms for the self-tolerance of NK cells (108, 161, 163). It has been shown that NK cells do exist that lack inhibitory receptors for self MHC class I molecules; these NK cells are somehow not autoreactive. These cells are hyporesponsive in that they do not lyse autologous targets and are only able to lyse allogeneic targets at an impaired level (161, 163). In our studies, we observe that NK cells developed in TAP1$^{-/-}$ mice also exhibit a hyporesponsive phenotype, whether they be of TAP1$^{-/-}$ or B6 origin. In unpublished data, we have observed that NK cells freshly isolated from B6→B6 and B6→TAP1° mice lyse the same targets unequally; B6→TAP1° NK cells do not lyse RMA (H-2K^{b}) or RMA-S (MHC class I^{-}) tumor targets, although they exhibit some lytic capability against the NK-sensitive target YAC-1. This target they lyse 4-fold less than their B6→B6 counterparts. This answers an important question about the generation of

NK cell self-tolerance: it is the result of developmental education and derives from the bone marrow microenvironment, not from the hematopoietic cells themselves, as the tolerance cannot be transferred.

One question still remains to be answered: by what mechanism are hyporesponsive NK cells generated? Kim, et. al. propose an active process for the generation of functional NK cells, which they term "licensing" (161). In this process, cells that express Ly49 molecules for self MHC class I are actively "licensed" to respond to potential target cells, while those that do not express self-specific Ly49 molecules are hyporesponsive. Our data indicate that this licensing process may indeed be necessary to generate functionally active NK cells; NK cells from MHC class I^- mice, regardless of the MHC class I expression level of the NK cells themselves, exhibit a hyporesponsive phenotype. Recently, Ly49-MHC class I interactions in *cis* have been described (227-231). Our data would indicate that *cis* interactions between Ly49s and MHC class I are not sufficient to generate functionally mature NK cells because although the NK cells that are generated in B6→TAP1$^{-/-}$ mice express MHC class I, these NK cells remain hyporesponsive. In addition, our data indicate that any interaction, even in *trans*, between MHC class I on hematopoietic cells and Ly49s are insufficient to promote functional maturation of NK cells. In another model of NK cell tolerance generation, NK cells lacking self-specific Ly49 receptors become hyporesponsive when NK cells receive greater activating signals than inhibitory

signals from self cells, resembling T cell anergy (108, 163). This mechanism also potentially occurs in our model; the NK cells generated in a MHC class I$^-$ environment do not receive inhibitory signals, allowing them to receive only activating signals. The results from our chimera studies do not necessarily rule out one or the other of these mechanisms. Our study with the neonatal mice suggests that CD94/NKG2A, an inhibitory receptor that is expressed on all neonatal NK cells (105) that recognizes non-classical MHC class I, may be sufficient to render NK cells lytic against cells that lack MHC class I. The ability of NK cells to lyse allogeneic targets occurs only after the acquisition of Ly49 receptors, implying that Ly49-MHC interactions are necessary for the generation of NK cells that can lyse allogeneic targets. It would be beneficial to the field to determine whether this is indeed the case.

A.



B

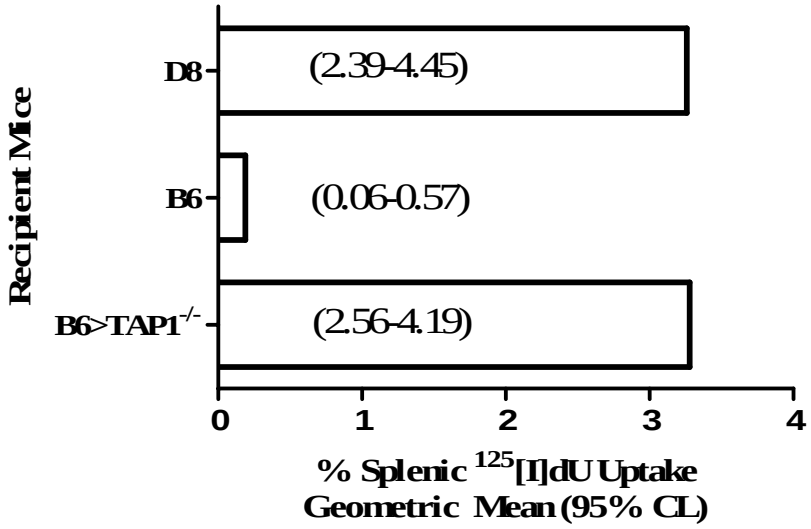


Figure 3.1 B6→TAP1$^{-/-}$ chimeras accept parental and third party BM grafts. B6→B6 and B6→TAP1$^{-/-}$ (TAP1 above) were exposed to 8Gy and inoculated with 2.5x10^6 B6, TAP1 and BALB/c BMC (a). B6, D8 and B6→TAP1$^{-/-}$ mice were irradiated (8Gy) and challenged with 2.5x10^6 D8 BMC (b). (a,b) Results are the percent splenic uptake of ^{125}[I]dU, expressed as the geometric mean. Ranges on the chart represent the 95% confidence limits. Asterisks indicate $p<0.05$ per the Student's *t* Test.

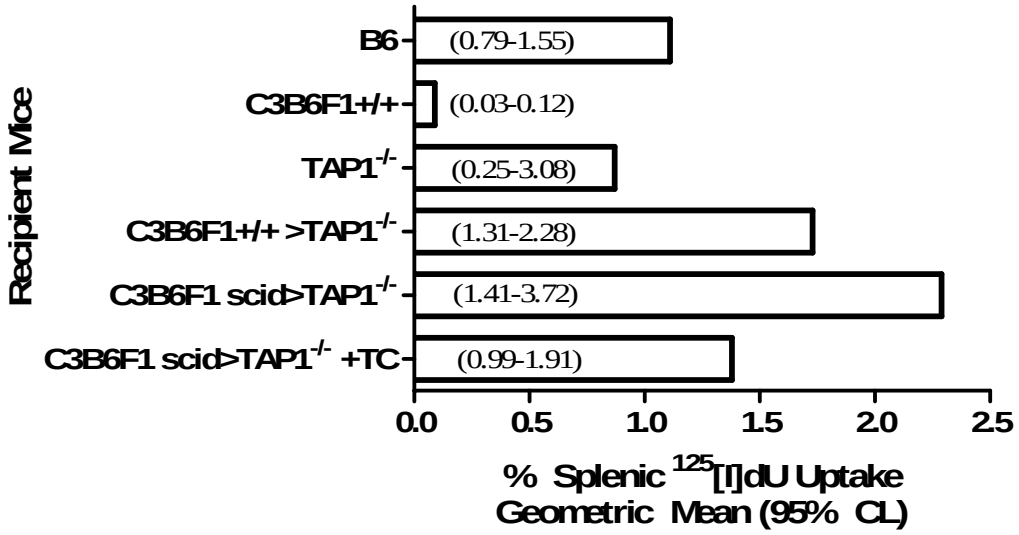
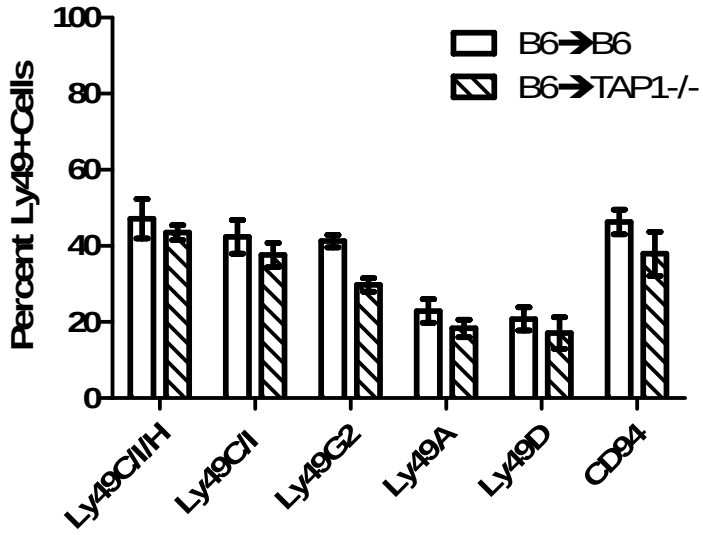


Figure 3.2 NK cell tolerance is T Cell independent.
B6, TAP1, C3B6F1$^{+/+}$→TAP1, C3B6F1$^{scid/scid}$→TAP1 and C3B6F1$^{+/+}$ were irradiated and challenged with TAP1 BMC. One group of C3B6F1$^{scid/scid}$→TAP1 chimeras received TC (thymus cells) prior to challenge. Results are the percent uptake of ^{125}[I]dU, expressed as the geometric mean. The ranges represent the 95% confidence interval. An asterisk indicates a $p<0.05$ as indicated by the Student's *t* Test.

A


Percent Ly49+Cells
100
80
60
40
20
0
B6→B6
B6→TAP1-/-
Ly49CI/I/H
Ly49CI/I
Ly49G2
Ly49A
Ly49D
CD94

B

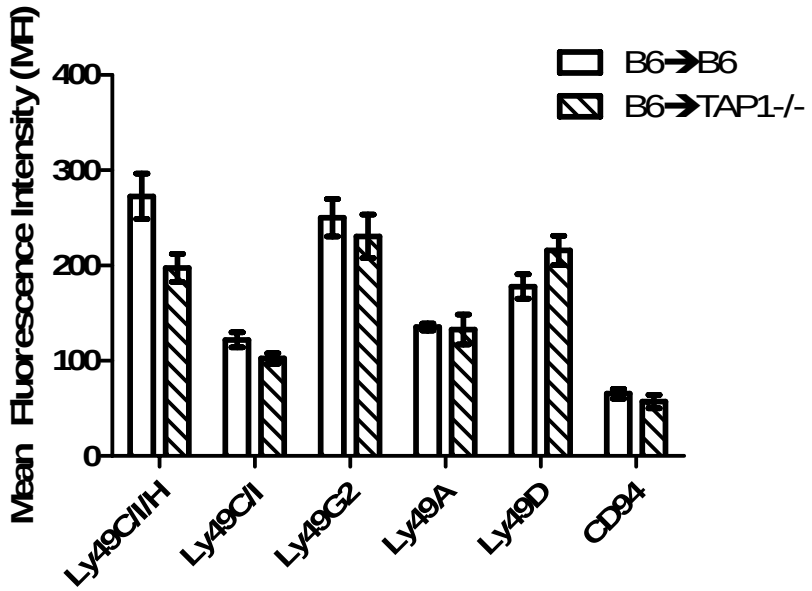

Mean Fluorescence Intensity (MFI)
400
300
200
100
0
B6→B6
B6→TAP1-/-
Ly49CI/I/H
Ly49CI/I
Ly49G2
Ly49A
Ly49D
CD94

Figure 3.3 Ly49 expression is unaltered in B6→TAP1$^{-/-}$ chimeras.
B6→B6 and B6→TAP1$^{-/-}$ splenocytes were examined for NK1.1, Ly49 and CD94 expression by flow cytometry. Data is represented as the percentage of cells that express NK1.1 (a) or the Mean Fluorescence Intensity (MFI, b). Open bars, B6→B6. Filled bars, B6→TAP1$^{-/-}$ (a,b).

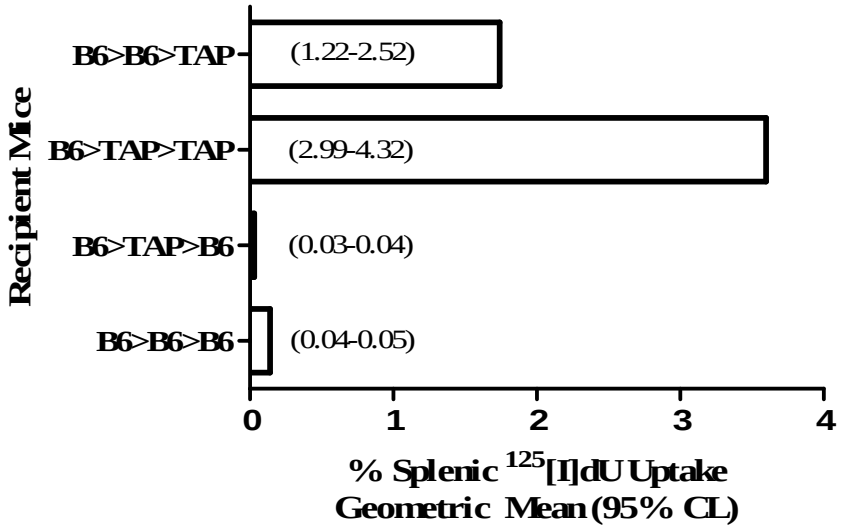


Figure 3.4 Bone marrow microenvironment is necessary for generation of functional NK cells. Secondary chimeras (B6→B6→B6, B6→TAP1$^{-/-}$→B6, B6→TAP1$^{-/-}$→TAP1$^{-/-}$, B6→B6→TAP1$^{-/-}$) were irradiated and challenged with TAP1$^{-/-}$ BMC. Data is represented as the percent splenic uptake of ^{125}[I]dU, expressed as the geometric mean. The 95% confidence interval is shown as a range.

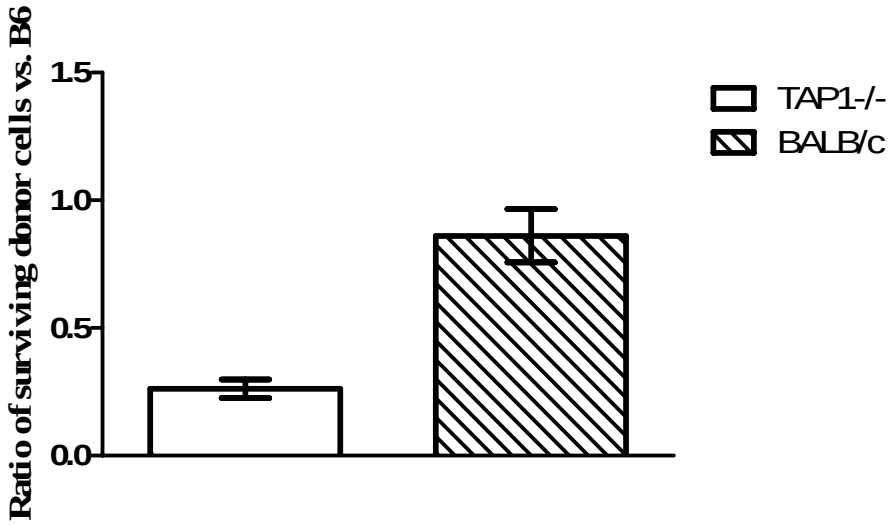


Figure 3.5 Neonatal mice cannot reject MHC class I^{high} BMC.
13-17 day old B6 mice were irradiated with 6Gy and injected with equal parts B6 and TAP1$^{-/-}$ or BALB/c BMC that were CFSE labeled. 24 hours later the spleens were removed and splenocytes were examined by flow cytometry for the percentage of surviving cells of both B6 and TAP1$^{-/-}$ type. Data is represented as the ratio of surviving donor type cells vs. surviving B6 cells. An asterisk indicates $p<0.05$ as determined by the Student's *t* Test.

CHAPTER IV

FOXK1 IS REQUIRED FOR NK CELL DEVELOPMENT AND FUNCTION

Objective

Specific stages of NK cell development in the bone marrow have been recently described. This knowledge has enabled investigators to determine factors that regulate NK cell development in a stage-specific manner. In this study we have investigated the role of the transcription factor Foxk1 in NK cell development using Foxk1$^{-/-}$ mice. We have attempted to define how NK cells are affected by a lack of Foxk1 as well as the contribution of Foxk1 to development of NK cells in the thymus.

Introduction

Natural Killer (NK) cells provide a first line of defense against virus infected and tumor cells through their cytotoxic effector function (22, 76, 232) and they provide cytokines to modulate the adaptive response (76), making them an essential component of the immune response. Murine NK cell development mainly occurs in the bone marrow (99), although a subset of thymically-derived NK cells has been recently described (88, 233). In the bone marrow, NK cells develop from a common lymphoid progenitor and progress through a series of five well-defined stages, culminating in a large expansion to become mature NK cells that can exit the bone marrow and enter the periphery. The stages of NK cell

development in the bone marrow are characterized phenotypically by the expression pattern of various cell surface receptors and integrins (99). The first stage of NK cell development is termed lineage commitment and is characterized by the expression of CD2, CD122, CD244, and CD11a. Cells in stage I of development do not possess any effector potential, nor do they possess any MHC class I-specific receptors. The acquisition of cell surface receptors that are specific for MHC class I defines stages II and III of NK cell development. In stage II, the CD94/NKG2 receptors first appear, enabling the developing NK cell to interact with non-classical class I. Stage III of NK development is characterized by the expression of the Ly49 family of MHC class I-specific receptors. The combination of receptors expressed during stages II and III enable the developing NK cell to distinguish self from non-self through the recognition of MHC class I. The expansion stage of development, stage IV, is characterized by the proliferation of the immature pool of developing NK cells, which in stage V upregulate the activation marker CD43 and become mature NK cells that exit the bone marrow and exhibit full effector potential (99). NK cells derived from the thymus are distinguished from bone marrow-derived cells by their expression of CD127 and the transcription factor, GATA-3 (88, 89). Although recent advances have been made in our understanding of transcription factors that regulate NK cell development, a complete picture has yet to be determined.

Several transcription factors that regulate NK cell development have been defined. The Ets family of transcription factors has been implicated in several aspects of NK cell development (165-167, 170, 172, 234). Specifically, Ets-1 has been shown through the use of an Ets-1-deficient mouse to be absolutely necessary for proper NK cell development and maturation (166). Ets-1 has more recently been described in the transcriptional regulation of the CD244 gene, one of the earliest receptors expressed on NK-committed precursors (99, 167, 168, 235). The Ets-family members PU.1 and MEF have been associated with later stages in development (169, 171, 172). Studies using $MEF^{-/-}$ mice have shown that MEF is required for the development of mature NK cells; MEF has also been shown to bind directly to and activate the Perforin promoter (172). In fetal thymic organ cultures, overexpression of the dominant negative bHLH factor Id3 has also been shown to promote NK cell development from the bi-potential NK/T precursor by preventing D-J rearrangement, thereby activating a default pathway toward NK cell and not T cell development (106). A developmental stage-specific event, the acquisition of several Ly49 receptors, was determined to be regulated by the high mobility group protein, tcf-1 (178, 181-183). GATA-3, most notably known for its role in the generation of the Th2 subset of $CD4^{+}$ T cells, has been recently shown, through knockout studies, to be necessary for the development of thymus derived NK cells; these cells have decreased cytotoxicity, increased cytokine secretion and reduced expression of Ly49 receptors compared

to their bone marrow-derived counterparts (88, 233). Even with these recent advances in our understanding of NK cell developmental biology, the definition of transcription factors that regulate discrete steps of NK cell development are unknown.

Many forkhead transcription factors have been implicated in the development of the immune system (184, 185). Currently, over 100 forkhead transcription factors have been identified based on their unique "winged-helix" DNA binding domain (185). Foxp3 is necessary for regulatory T cell development and can be used as a marker to distinguish regulatory from effector T cells (192-194). Foxn1-deficient mice lack a thymus, showing the necessity of Foxn1 for thymic and thereby T cell development (186, 191). Foxo3a in B cells results in an accumulation of B cells in the G_0/G_1 phase of the cell cycle (206, 236). In addition, Foxo3a has been shown to directly activate the transcription of several apoptotic genes, including Fas-ligand and Bcl-2-interacting mediator of cell death (BIM, 207); IL-15 prevents this action of Foxo3a and promotes survival of NK cells (208).

Foxk1, originally termed myocyte nuclear factor, or MNF, was discovered in murine muscle as a transcriptional repressor (209-211, 237). Since its discovery, Foxk1 has been shown to regulate the myogenic stem cell population by promoting cell cycle progression through the transcriptional inactivation of the cdk-inhibitor, $p21^{CIP}$ (210, 238). $Foxk1^{-/-}$ mice have decreased numbers of muscle

stem cells, impaired cell cycle kinetics and impaired muscle regeneration (211). In this study we have examined the role of Foxk1 in the immune system using Foxk1-deficient mice ($Foxk1^{-/-}$). Examining the lymphocyte populations in these mice revealed that $Foxk1^{-/-}$ mice have reduced numbers of circulating mature NK cells, with only marginal perturbations in B and T cell numbers. The development of NK cells in $Foxk1^{-/-}$ mice is also impacted, with defects seen in stages I and IV of bone marrow development. The existing mature NK cells in the spleen of $Foxk1^{-/-}$ mice also exhibit defective cytolytic function, recoverable with IL-2 and IL-15. Finally, we show here that the mature NK cells in $Foxk1^{-/-}$ mice are likely thymus-derived, indicating that the bone marrow but not the thymic pathway of NK cell development is defective in $Foxk1^{-/-}$ mice. Taken together, these data suggest that Foxk1 is an essential regulator of NK cell development, impacting the functional capacity of mature NK cells.

Results

Phenotypic analysis of immune cells in Foxk1$^{-/-}$ mice

Foxk1 has been associated with the development and maintenance of skeletal muscle (209, 239, 240). We used semi-quantitative RT-PCR analysis and observed Foxk1 expression in the skeletal muscle (lane 1) and spleen (lane 2) of B6 mice (Figure 1A). Based on the importance of forkhead transcription factors in the development of the immune system, we wanted to determine the impact of Foxk1-deficiency on cells of the immune system.

Utilizing Foxk1$^{-/-}$ and age-matched B6 mice we performed flow cytometric analysis of the spleen, bone marrow, peripheral blood and thymus. T cells, B cells and NK cells were examined (Table 1). The total cellularity of the spleen in Foxk1$^{-/-}$ mice was reduced ($72.0x10^6$±8.49) compared to B6 ($103.68x10^6$±7.68), although this was not surprising due to the decreased size of Foxk1$^{-/-}$ mice in general (211, 239). Due to consistently decreased lymphocyte compartments in Foxk1$^{-/-}$ mice, likely due to their smaller size, the percentages of each cell type more accurately reflect the differences between the B6 and Foxk1$^{-/-}$ mice. In the spleen there was a significant decrease in the CD8$^+$ effector memory T cell population (CD3$^+$CD8$^+$CD62L$^-$CD44$^+$CD69$^+$) of Foxk1$^{-/-}$ mice (70.40±4.24 for B6 vs. 43.55±2.97 for Foxk1$^{-/-}$) as well as an increase in total B cell percentages (53.85±0.14 for B6 vs. 61.42±1.37 for Foxk1$^{-/-}$). The percentage of

NK cells was decreased in the spleen as well, although the difference was not significant, as is seen in bone marrow and circulating populations of NK cells (Figure 1b, 3.47±0.49 for B6 vs. 1.51±0.73 for Foxk1$^{-/-}$). In the bone marrow, NK cell progenitors (NK1.1^{+}c-kit^{+}CD244^{+}) of Foxk1$^{-/-}$ mice were significantly decreased compared to B6 (Figure 1b, 17.08±4.33 for B6 vs. 8.41±2.14 for Foxk1$^{-/-}$). Analysis of the thymus showed no differences in T cell populations (data not shown). NK cells were decreased in the peripheral blood of Foxk1$^{-/-}$ mice (Figure 1b, 5.15±0.71 for B6 vs. 1.24 for 0.30). Given our laboratory's interest in NK cell development, we decided to focus on the NK cell population within Foxk1$^{-/-}$ mice. Throughout the bone marrow and peripheral blood there were consistent significant decreases in NK cell percentage in Foxk1$^{-/-}$ mice and a consistent but not significant reduction in the spleen (Figure 1b), indicating that Foxk1 might play a role in either NK cell development or homeostasis, prompting us to explore this possibility further. We initially examined the developmental stages of NK cells in B6 and Foxk1$^{-/-}$ mice.

NK cell development is altered in Foxk1$^{-/-}$ mice

The five discrete stages of NK cell development in the bone marrow are characterized phenotypically by the specific expression pattern of integrins and cell surface receptors (99). We hypothesized that Foxk1$^{-/-}$ NK cells may have impaired development that would account for the decrease in NK cells in the

periphery. We examined each individual stage (lineage commitment (I), CD94/NKG2 acquisition (II), Ly49 acquisition (III), expansion (IV), mature (V)) by flow cytometry. Bone marrow cells from B6 and Foxk1$^{-/-}$ mice were examined and CD122^{+}CD2^{+}CD244^{+}CD11a^{+} cells were defined as lineage committed precursors. There was no significant difference between the number of B6 (unshaded bars, 61.20±3.60) and Foxk1$^{-/-}$ (hatched bars, 56.65±3.85) lineage committed NK precursors (Figure 2A). We did observe a phenotypic difference in the lineage committed precursor population in Foxk1$^{-/-}$ mice. Foxk1$^{-/-}$ cells (-/-, bottom panels) lacked expression of a CD11a^{high} population that was present in the B6 lineage committed precursor cells (+/+, top panels, Figure 2B). Reduced expression of CD11a, an adhesion molecule, on developing NK cells may prevent proper interaction with the stromal cell environment and cause further developmental defects.

Stages II and III of NK cell development, acquisition of CD94/NKG2 and Ly49 receptors, respectively, were also examined. Stage II cells (Figure 2C) were defined by their expression of NK1.1^{+}c-kit^{-}CD11b^{-} (data not shown) and there were no significant differences observed between B6 (81.7±0.6) and Foxk1$^{-/-}$ (50.6±13.1). Stage III cells (Figure 2D) were defined by their expression of NK1.1^{+}c-kit^{+}CD11b^{-} (data not shown) and no significant differences were detected between B6 (15.6±0.4) and Foxk1$^{-/-}$ (42.6±9.8).

Expansion stage (IV) cells were characterized by the expression of $NK1.1^{+}CD49b^{high}CD51^{low}$. $Foxk1^{-/-}$ bone marrow cells had an increased percentage of expansion stage cells (Figure 2E, 59.2±7.2, -/-, bottom panel) compared to B6 (22.4±5.3, +/+, top panel). There was also a decreased percentage of mature NK cells, defined by $NK1.1^{+}CD49b^{+}CD43^{+}$ (Figure 2F, 0.87±0.01 for B6 vs. 0.12±0.01 for $Foxk1^{-/-}$). This implies that Foxk1 directly impacts NK cells during the expansion stage of development and prevents them from progressing to the mature stage of development.

To determine whether $Foxk1^{-/-}$ NK cells had altered expression of Ly49 receptors, we examined $Foxk1^{-/-}$ (hatched bars) and B6 (unshaded bars) NK cells for Ly49A, G2, C/I and D by flow cytometry (Figure 2G). Gated $NK1.1^{+}CD3^{-}$ $Foxk1^{-/-}$ NK cells had significantly reduced expression of Ly49s G2 (47.97±1.85 for B6 vs. 10.99±2.99 for $Foxk1^{-/-}$) and C/I (36.7±1.85 for B6 vs. 20.93±5.18 for $Foxk1^{-/-}$) compared to B6. Ly49D expression was slightly decreased on $Foxk1^{-/-}$ NK cells (28.87±1.40 for B6 vs. 22.67±13.35 for $Foxk1^{-/-}$), although not significantly. Ly49A expression was unaltered (22.91±3.13 for B6 vs. 16.78±14.01 for $Foxk1^{-/-}$), although $Foxk1^{-/-}$ mice had widely varying levels of Ly49A. These differences in Ly49 expression in $Foxk1^{-/-}$ mice may impact NK cell effector function or further stages of development.

Reduction in NK cells in $Foxk1^{-/-}$ mice is hematopoietic cell intrinsic

It was possible that Foxk1 was acting directly in NK cells to exert its effects on NK cell development or it could be a necessary transcription factor for stromal cells that aid in NK cell development. To determine whether the reduction of NK cells in Foxk1$^{-/-}$ mice is hematopoietic cell intrinsic or whether it is due to an environmental or stromal cell type, we generated B6.CD45.1→Foxk1$^{-/-}$ and Foxk1$^{-/-}$→B6.CD45.1 bone marrow cell chimeras and examined the spleens of these chimeras for NK cell content eight weeks post transplant. The splenocytes from the chimeras were examined for the CD45.1 marker to determine the level of reconstitution from either the B6.CD45.1 or the Foxk1$^{-/-}$ hematopoietic compartment. B6.CD45.1→Foxk1$^{-/-}$ chimeras had significantly higher percentages of NK cells (4.35±0.175) than did Foxk1$^{-/-}$→B6.CD45.1 chimeras (0.925±0.625), which had virtually no NK cells (Figure 3). These data show that Foxk1 functions directly in cells of the hematopoietic lineage to impact the development of NK cells.

Foxk1$^{-/-}$ NK cells have reduced cytotoxicity in vitro

To determine the lytic capability of the few NK cells that do exist in the periphery of Foxk1$^{-/-}$ NK cells, we performed standard ^{51}Cr-release assays using the NK-sensitive YAC-1 target cell line. NK cell numbers were adjusted to ensure that there were equivalent numbers of NK cells from B6 and Foxk1$^{-/-}$ mice per well. No killing was detectable directly *ex vivo* for either B6 or Foxk1$^{-/-}$ NK

cells (data not shown). To polyclonally activate NK cells, Poly I:C was given to B6 and Foxk1$^{-/-}$ mice one day prior to harvest of their NK cells. Poly I:C-activated Foxk1$^{-/-}$ NK cells were unable to lyse YAC-1 targets, whereas B6 NK cells lysed the targets in a dose-dependent manner (Figure 4A). The inability of Foxk1$^{-/-}$ NK cells to respond to Poly I:C stimulation implies a defect either in the cytokine environment of the Foxk1$^{-/-}$ mouse, in the ability of the Foxk1$^{-/-}$ NK cells to effectively transduce cytokine signals or an inherent lytic defect. To determine the ability of Foxk1$^{-/-}$ NK cells to respond to cytokines, we performed ^{51}Cr-release assays using LAKs activated with either IL-2 (Figure 4B), IL-15 (Figure 4C) or IL-12 and IL-18 (Figure 4D). In the case of IL-2 and IL-15 (Figures 4B, C), which share a similar signaling pathway, Foxk1$^{-/-}$ NK cell lytic capability was equivalent to that of B6. However, IL-12 and IL-18 failed to activate Foxk1$^{-/-}$ NK cells to lyse YAC-1 targets to B6 levels (Figure 4D). IL-2 and IL-15 stimulation could possibly result in greater proliferation of NK cells, which may be necessary for the increased cytotoxicity of NK cells cultured with these cytokines and may explain why IL-12 and IL-18, which do not promote the proliferation of NK cells, do not rescue the ability of Foxk1$^{-/-}$ NK cells to lyse YAC-1 targets. However, there was a consistent lack of proliferation seen in the Foxk1$^{-/-}$ NK cells after culture (with IL-2, IL-15 or IL-12 and IL-18) when compared to the B6 NK cells. This implies that the proliferation of the Foxk1$^{-/-}$ NK cells, when cultured with IL-2 or IL-15, is not responsible for their increased cytotoxicity. Taken together,

these data indicate that Foxk1$^{-/-}$ NK cells are deficient in their ability to respond to IL-12 and IL-18 signaling and that there is no inherent defect in their lytic function.

Foxk1 is required for bone marrow but not thymic NK cell development

NK cells that develop in the thymus differ from NK cells that develop in the bone marrow in that they have reduced expression of Ly49s and they are less cytotoxic than bone marrow-derived NK cells (88, 89). Noting that the peripheral NK cells that are detected in Foxk1$^{-/-}$ mice exhibited a similar phenotype to thymically-derived NK cells, we hypothesized that Foxk1 functions in bone marrow and not in thymic NK cell development. The result of this would be a greater proportion of thymus-derived, or CD127^{+}, NK cells in the periphery of Foxk1$^{-/-}$ mice. We examined the CD127 expression of CD3^{-}CD49b^{+}NK1.1^{+} cells from the spleen, lymph node (LN) and thymus of B6 and Foxk1$^{-/-}$ NK mice. In the thymus (Figure 5A, right panels, Figure 5B), no difference was detected in CD127 expression on NK cells between B6 (+/+, top panels) and Foxk1$^{-/-}$ (-/-, bottom panels) mice; virtually all NK cells expressed CD127, as expected. Examination of the LN (middle panels) revealed no difference in the percentage of NK cells expressing CD127 (Figure 5A, B), however the Foxk1$^{-/-}$ NK cells seemed to have a population of CD127^{low}-expressing cells that did not alter the overall count (Figure 5A). There were significantly higher levels of CD127^{+} NK

cells in Foxk1$^{-/-}$ spleens (21.06±2.41) than in B6 spleens (7.63±0.93). These data indicate that thymic development of NK cells in Foxk1$^{-/-}$ mice is intact, although the thymic pathway of NK cell development does not account for the entire pool of circulating peripheral NK cells in Foxk1$^{-/-}$ mice, as not all splenic NK cells express CD127.

Discussion

In this study, we show that the forkhead transcription factor, Foxk1, is required for NK cell development and lytic function. Foxk1$^{-/-}$ mice have significantly reduced numbers of mature peripheral NK cells. A severe reduction of NK cells, such as that observed in Foxk1$^{-/-}$ mice, is a hallmark of the loss of a factor involved in NK cell development and has been seen in other transcription factor knockout models in which NK cell development is effected (106, 165, 169, 170, 172, 182, 241). Foxk1$^{-/-}$ mice seem to have a specific defect in stage IV of bone marrow NK development that leads to the decrease in NK cell number in the periphery. The absence of Foxk1 in the developing NK cells leads to an accumulation of stage IV cells and a corresponding decrease in fully mature stage V cells. One possible explanation for this is the role of Foxk1 as a negative regulator of the cyclin dependent kinase (cdk) inhibitor, p21^{CIP}. In muscle stem cells, Foxk1 represses the transcription of p21, allowing the cells to progress into S phase of the cell cycle. When Foxk1 is absent, p21 levels do not regress, leaving the muscle progenitors in G_0/G_1 phase (210). It is possible that the same mechanism is functional during stage IV of NK cell development, a time of high proliferation. Although the p21 levels in mature circulating NK cells from Foxk1$^{-/-}$ mice are equivalent to those of B6 mice (L.A.M., unpublished observations), it is possible that the cells that do progress to maturity have normal levels of p21

due to compensatory mechanisms (242-244). Perhaps, because thymus-derived NK cells do not undergo the type of expansion that is required in bone marrow development, p21 levels are not altered in these cells or perhaps are not as important for their development. Therefore, future studies will attempt to determine the expression level of p21 in developing NK cells.

NK cells perform their lytic function through two major mechanisms: the release of cytolytic granules including perforin and granzymes and through death receptor pathways, such as Fas-FasL (59, 61, 65, 66). In order to become highly lytic cells, NK cells require activation by cytokines, such as IL-15, IL-12 and IL-18 (245-249). Foxk1$^{-/-}$ NK cells have a decreased ability to become activated by the cytokines IL-12 and IL-18. These are potent antiviral interleukins that are also produced in response to the double-stranded RNA mimic, poly I:C (250). The cdk-inhibitor p21 may also play a role in cytokine responses. Although mature NK cells in Foxk1$^{-/-}$ mice have normal expression of p21 (by RT-PCR), it has been shown that p21 is upregulated by the expression of IFN-α, causing entry into G_1 of the cell cycle (251, 252). It is possible that in the context of poly I:C stimulation that IFN-α is released, leading to increased p21 levels and loss of cell proliferation. Levels of IFN-α have been found to be decreased in the absence of IL-12 after poly I:C treatment, indicating a possible connection between the two cytokines (253). Another possibility to describe the decreased activation of Foxk1$^{-/-}$ NK cells by IL-12 and 18 is that Foxk1 directly regulates the expression

of genes that are necessary for IL-12 and IL-18 receptor signal transduction. We show that *in vitro* culture with IL-2 or IL-15 rescues the ability of Foxk1$^{-/-}$ NK cells to lyse susceptible target cells. However, due to the fact that the defect in Foxk1$^{-/-}$ mice is hematopoietic cell intrinsic and IL-15 is largely produced by non-hematopoietic cells, it seems unlikely that a lack of IL-15 in Foxk1$^{-/-}$ mice would explain the lytic or developmental NK cell defects in these mice. Proper signaling through the IL-15R requires cross-presentation of IL-15 on the IL-15Rα on an accessory cell (33, 254). One possible explanation for the developmental and functional NK cell defects in Foxk1$^{-/-}$ mice is that there is a defect in the cross-presentation of IL-15.

Thymus-derived NK cells can be distinguished from bone marrow-derived NK cells based on their expression of CD127 and the transcription factor GATA-3 (88, 89). Here we show that Foxk1 is not necessary for the development of thymus-derived NK cells; this may be due to a lack of expression of Foxk1 in thymic NK cells. In the future, we plan to investigate whether Foxk1 is expressed in thymic NK cells. A lack of Foxk1 in thymus-derived NK cells would indicate separate developmental pathways for NK cells in the bone marrow and in the thymus.

	Spleen	
	B6 % (absolute number)	Foxk1-/- % (absolute number)
Total Cellularity (x10^6)	103.68±7.67	72±8.49
CD3-NK1.1+	3.47±0.49 **(3.60±0.62)**	1.51±0.73 **(1.09±0.65)**
CD3+	30.266±3.76 **(31.38±4.89)**	26.60±2.99 **(19.16±2.41)**
CD3+CD4+CD8-	65.03±2.34 **(61.20±6.99)**	57.97±2.27 **(41.7±1.84)**
CD3+CD4-CD8+	28.69±2.59 **(29.74±2.91)**	34.69±3.15 **(24.97±2.54)**
CD3+CD8+CD62L-CD44+CD69+	**70.40±4.24 (72.99±11.56)**	**43.55±2.97 (31.36±2.39)**
B220+	**53.85±0.14** (55.83±0.18)	**61.42±1.37** (44.22±1.21)

	Bone Marrow	
	B6 % (absolute number)	Foxk1-/- % (absolute number)
Total Cellularity (x10^6)	(46.44±9.70)	(32.35±3.08)
NK1.1+c-kit+2B4+	**17.08±4.33 (7.91±2.26)**	**8.41±2.14 (2.71±0.77)**
IgM+B220+	6.39±1.09 (2.97±0.59)	3.51±0.73 (1.14±0.27)

	Peripheral Blood (per 10^4 cells)	
	B6 %, absolute number x 10^2	Foxk1-/- %, absolute number x 10^2
CD3-NK1.1+	**6.65±1.74**	**1.06±0.42**

Table 4.1 Lymphocyte content of B6 and Foxk1$^{-/-}$ organs. B6 and Foxk1$^{-/-}$ spleen, bone marrow and peripheral blood were examined for lymphocyte content. Subsets of T cells were examined in the spleen. n=3-5 mice for each organ examined. Bold data represents $p<0.05$.

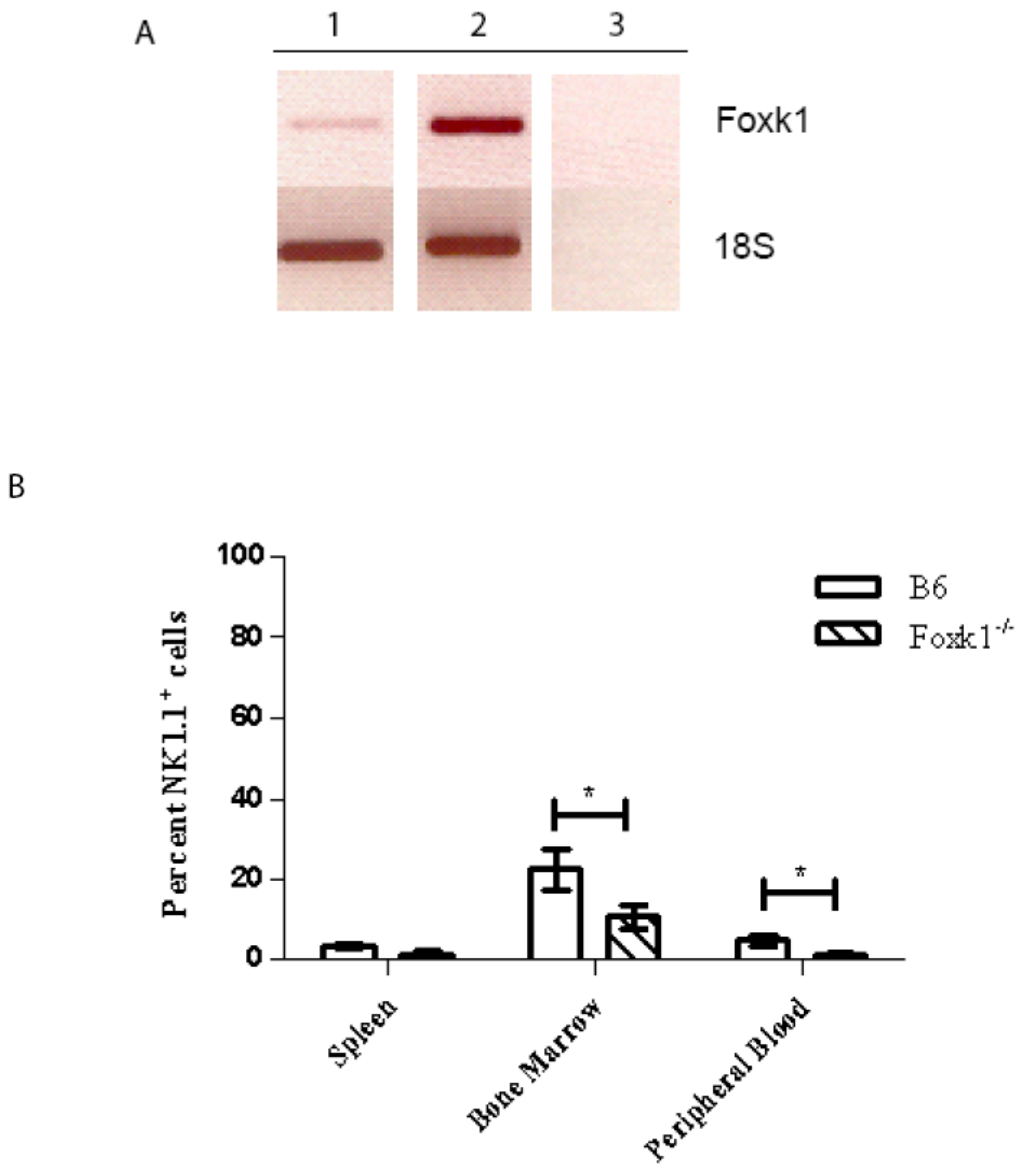
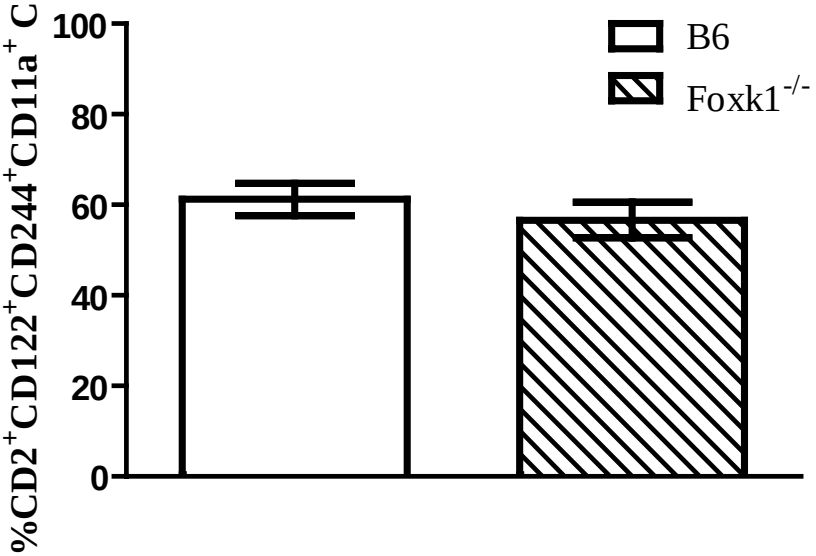


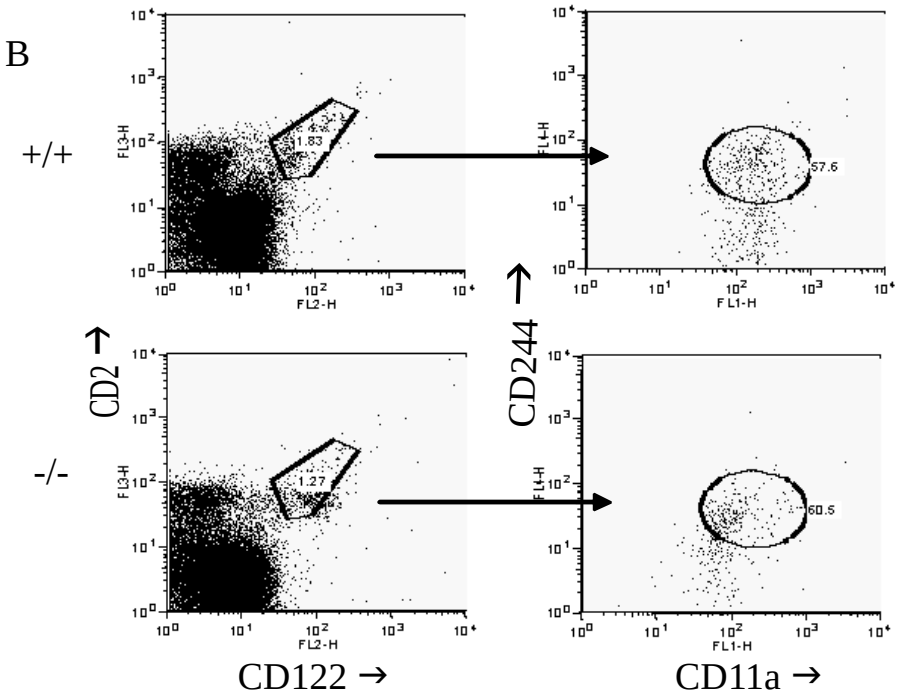
Figure 4.1 Foxk1$^{-/-}$ mice have reduced NK cell numbers. A, Foxk1 expression in skeletal muscle and spleen. Lane 1, skeletal muscle; lane 2 spleen; lane 3 no reverse-transcriptase control. 18S ribosomal RNA was used as a control for cDNA loading. B, Spleen, bone marrow and peripheral blood were examined by flow cytometry for NK cell content. A decrease in NK cell percentage was seen in all tissues examined. Asterisks indicate significant differences between B6 and Foxk1$^{-/-}$ mice. In spleen p=0.057, in bone marrow p=0.035, in peripheral blood,

p=0.003. n=3-5 mice for each organ examined. Statistics were determined using a two-tailed Student's t-test, three to six mice were examined.

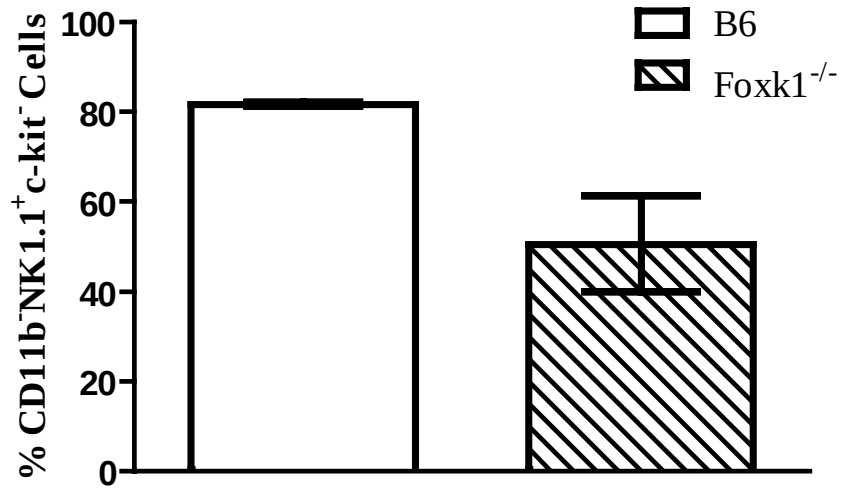
A


%CD2+CD122+CD244+CD11a+ Cells
100
80
60
40
20
0
B6
Foxk1-/-

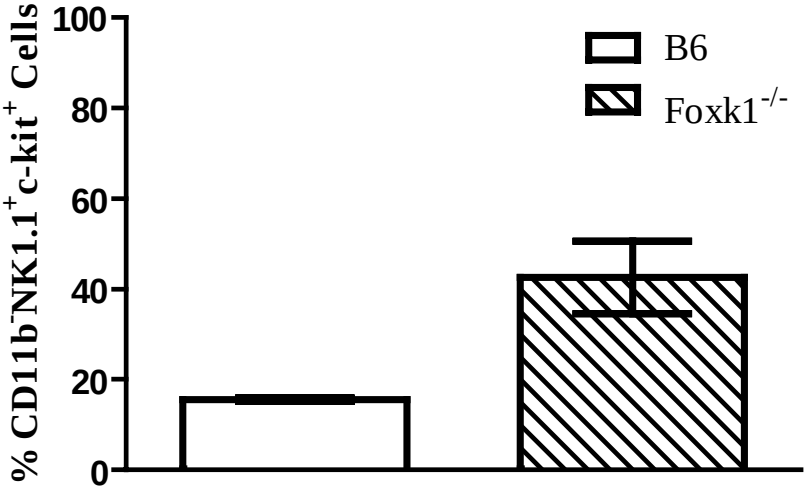
B


+/+
-/-
CD2 →
CD244 →
CD122 →
CD11a →
FL3-H
FL2-H
FL1-H
1.83
57.6
1.27
60.5

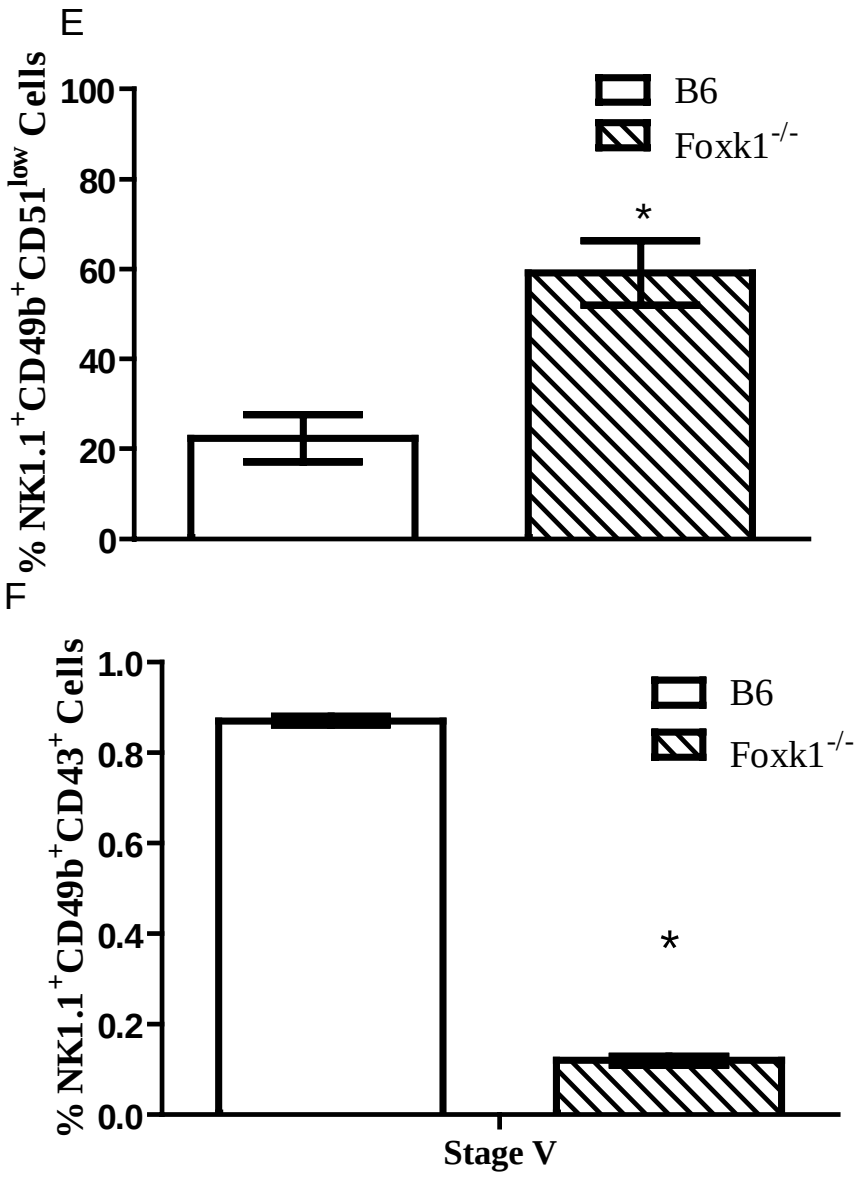
C


% CD11b-NK1.1+c-kit- Cells
100
80
60
40
20
0
B6
Foxk1-/-

D


% CD11b-NK1.1+c-kit+ Cells
100
80
60
40
20
0
B6
Foxk1-/-

E


% NK1.1+CD49b+CD51low Cells
100
80
60
40
20
0
B6
Foxk1-/-
*
% NK1.1+CD49b+CD43+ Cells
1.0
0.8
0.6
0.4
0.2
0.0
B6
Foxk1-/-
*
Stage V

G

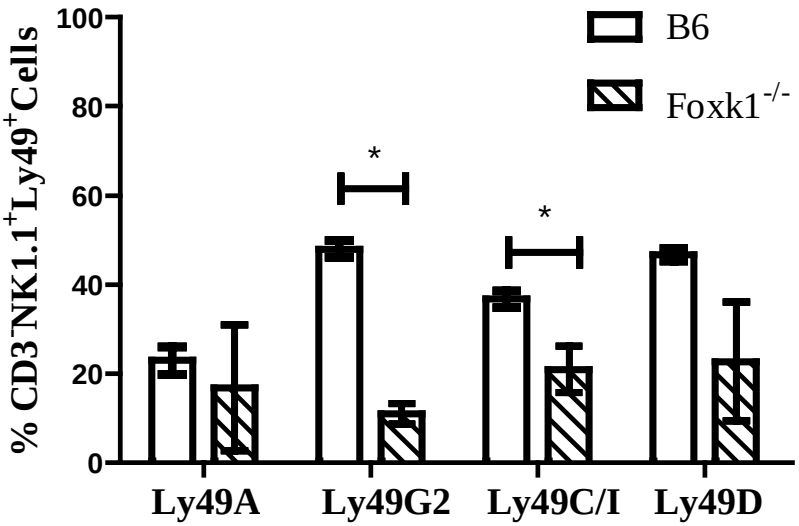


Figure 4.2 NK cell development is altered in Foxk1$^{-/-}$ mice. NK cell development was examined in B6 and Foxk1$^{-/-}$ mice in the bone marrow. Asterisks indicate significant differences between B6 mice (unshaded bars) and Foxk1$^{-/-}$ mice (hatched bars). A, lineage committed NK precursors (CD2^{+}CD122^{+}CD11a^{+}CD244^{+}) were represented equally between B6 and Foxk1$^{-/-}$ mice. B, lineage committed precursors from Foxk1$^{-/-}$ mice lack the CD11a^{high} population of cells. Representative plots showing B6 (+/+, top panels) and Foxk1$^{-/-}$ (-/-, bottom panels) bone marrow cells were gated on CD122^{+}CD2^{+} (left panel) cells and then examined for CD11a^{+}CD244^{+} (right panel) cells. C, stage II of development is unaltered in Foxk1$^{-/-}$ mice. D, stage III of development is unaltered in Foxk1$^{-/-}$ mice. E, Foxk1$^{-/-}$ mice have a greatly increased expansion stage (stage IV) of development, p=0.03. F, mature NK cells (stage V) are reduced in the bone marrow of Foxk1$^{-/-}$ mice, p=0.01. G, Ly49 receptor expression on B6 (unshaded bars) and Foxk1$^{-/-}$ (hatched bars) NK cells. NK cells from Foxk1$^{-/-}$ mice have decreased expression of Ly49G2 (p=0.002) and Ly49C/I (p=0.045). n=4 mice for statistical analysis. Statistics were performed using a Student's t-test.

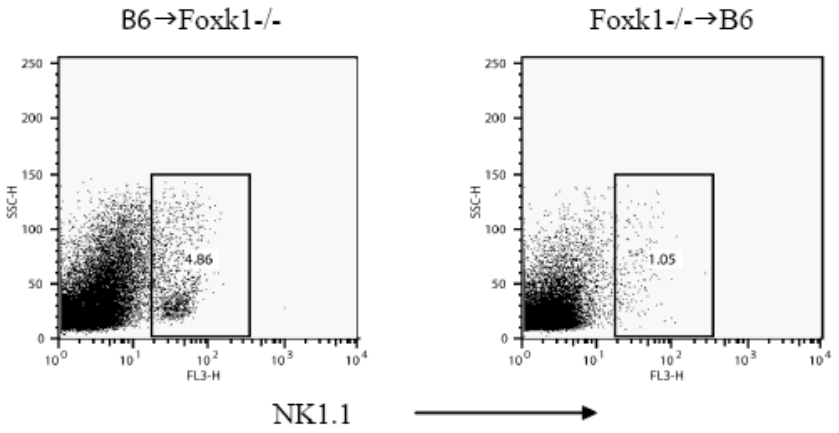
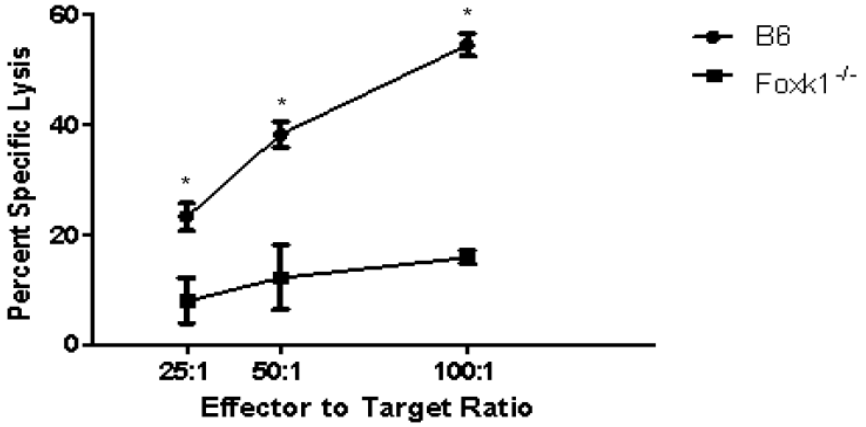
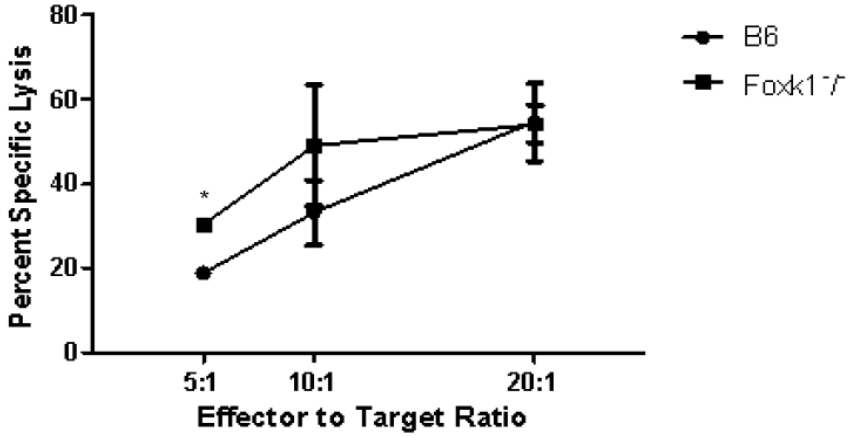


Figure 4.3 Foxk1$^{-/-}$ NK cell deficiency is hematopoietic cell intrinsic. B6.CD45.1→Foxk1$^{-/-}$ and Foxk1$^{-/-}$→B6.CD45.1 chimeras were generated and A, splenic NK cell content and B, bone marrow developmental stages were determined by flow cytometry. Five mice of each chimera type were examined. Shown are representative dot plots examining NK1.1 expression and side scatter characteristics from one mouse of each type (B6.CD45.1→Foxk1$^{-/-}$, left panel; Foxk1$^{-/-}$→B6.CD45.1, right panel). Foxk1$^{-/-}$→B6.CD45.1 chimeras consistently had greatly reduced numbers of NK cells. n=3 for each chimera.

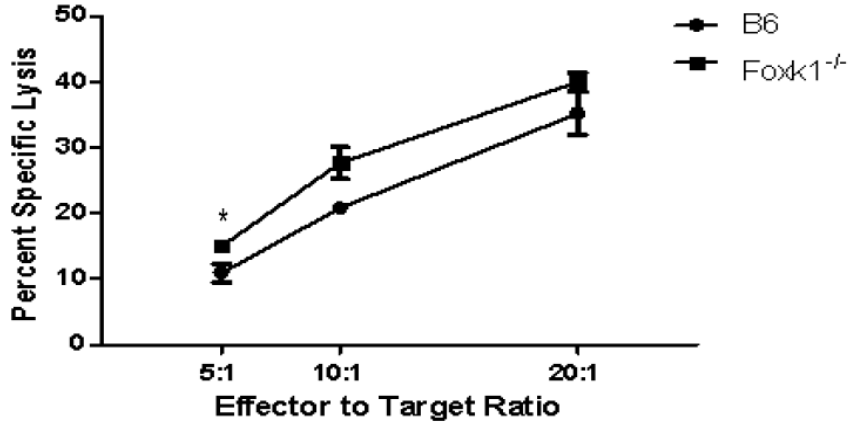
A


Percent Specific Lysis
60
40
20
0
25:1
50:1
100:1
Effector to Target Ratio
B6
Foxk1 -/-
*
*
*

B


Percent Specific Lysis
80
60
40
20
0
5:1
10:1
20:1
Effector to Target Ratio
B6
Foxk1 -/-
*

C


Percent Specific Lysis
50
40
30
20
10
0
5:1
10:1
20:1
Effector to Target Ratio
B6
Foxk1 -/-
*

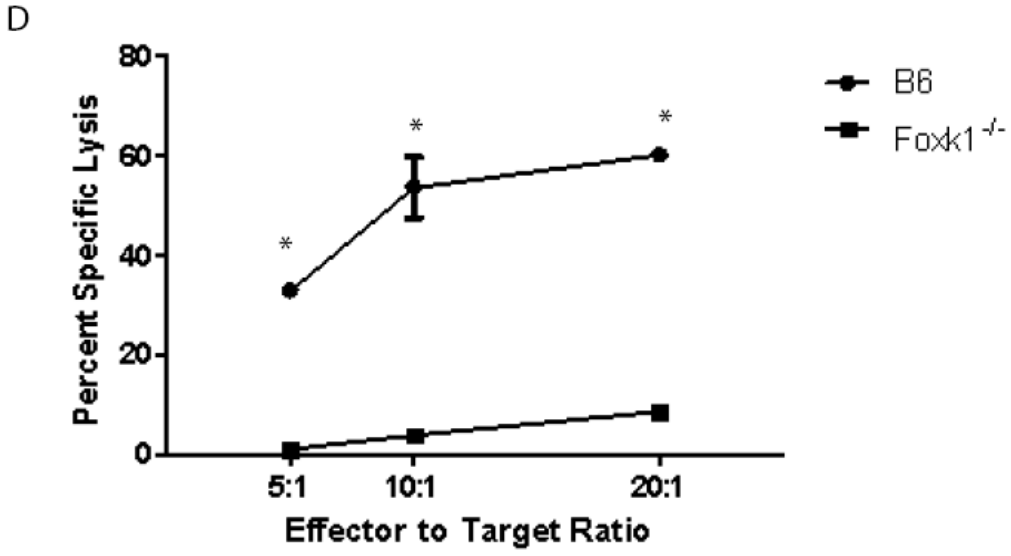


Figure 4.4 Foxk1$^{-/-}$ have reduced cytotoxicity in vitro. B6 (filled circles) and Foxk1$^{-/-}$ (filled squares) NK cells were tested for their ability to lyse the NK-sensitive target cell line, YAC-1, by standard four-hour ^{51}Cr-release assay. A, Poly I:C-activated Foxk1$^{-/-}$ NK cells exhibit decreased lytic capability compared to B6 NK cells. B, IL-2-activated B6 and Foxk1$^{-/-}$ NK cells lyse YAC-1 cells equally well. C, IL-15-activated B6 and Foxk1$^{-/-}$ NK cells equally lyse YAC-1 targets. D, Culture with IL-12 and IL-18 combined fail to promote lysis of YAC-1 targets by Foxk1$^{-/-}$ NK cells. n=3 for each assay. Asterisks indicate $p<0.05$. Statistics were performed using a Student's t-test.

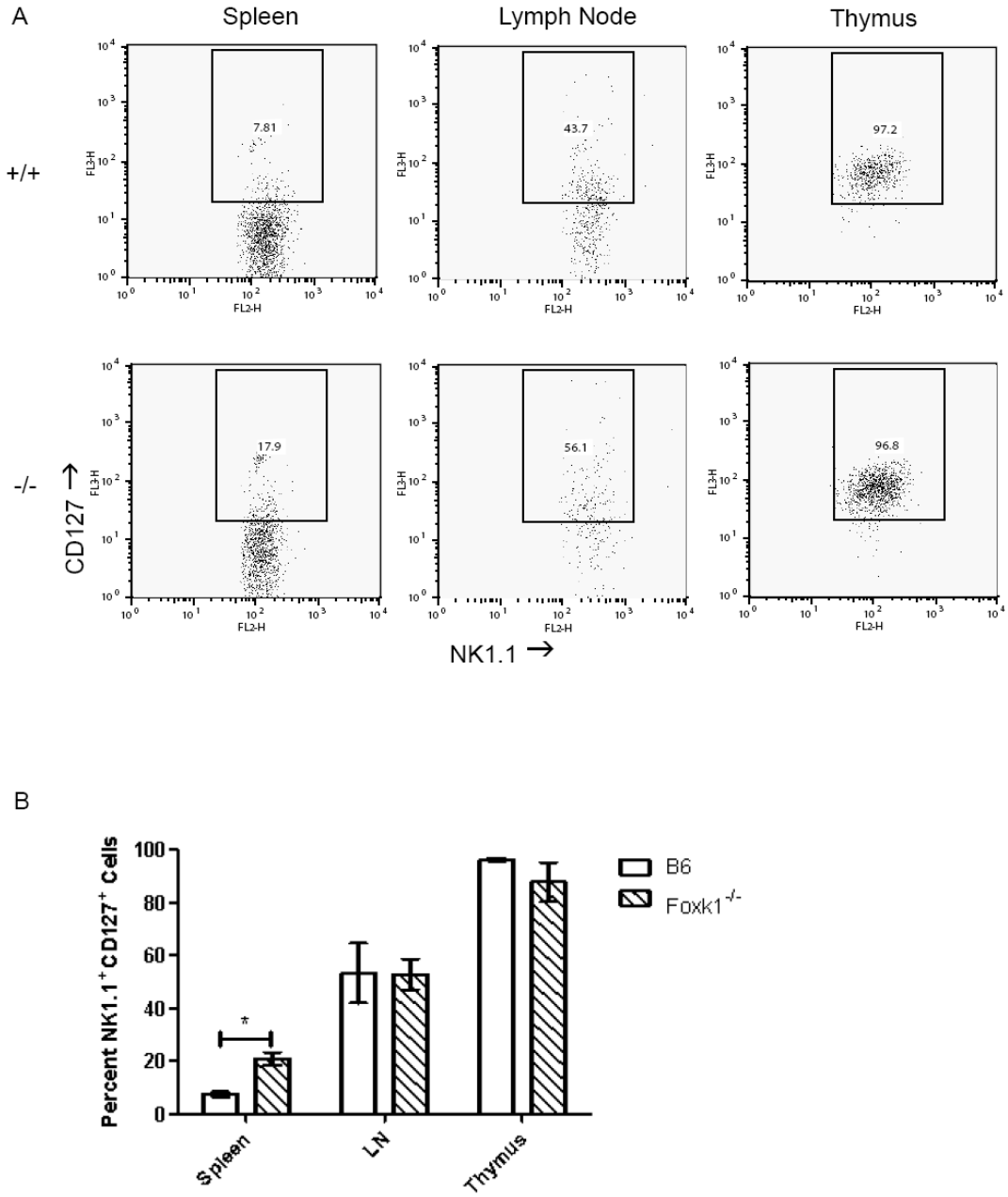


Figure 4.5 Foxk1 is required for bone marrow but not thymic NK cell development. B6 and Foxk1$^{-/-}$ mice were examined for CD127 expression on CD3^{-}NK1.1^{+} cells from the thymus, lymph nodes (LN) and spleen. A, representative plots from B6 (+/+, top row) and Foxk1$^{-/-}$ (-/-, bottom row) spleen (left panels), LN (middle panels) and thymus (right panels). CD127^{+} cells are gated, the percentage of positive cells is noted within the gate. B, Analysis of

$CD127^+$ NK cells in spleen (left), LN (middle) and thymus (right) of B6 (unshaded bars) and $Foxk1^{-/-}$ (hatched bars) mice. $Foxk1^{-/-}$ mice have a significantly greater proportion of $CD127^+CD3^-NK1.1^+$ cells in the spleen than do B6 mice, $p=0.01$. $n=4$ mice for each organ examined. Statistics were determined using a Student's t-test.

CHAPTER V

DISCUSSION

I. Development of MHC class I^+ NK cells in MHC class I^{low} mice

A. Summary

In these studies we have addressed the question of whether NK cells that express MHC class I, when developed in an environment devoid of MHC class I, will develop the ability to lyse allogeneic or MHC class I^- cells unlike MHC class I^- NK cells. We have shown that MHC class I^+ NK cells that develop in an environment lacking MHC class I are tolerant to MHC class I^- cells and are unable to reject allogeneic bone marrow cells. This is despite the fact that the B6 NK cells developed in this environment have a Ly49 repertoire that resembles that of normal B6 NK cells, again distinct from MHC class I^- NK cells. The functional impairment of these NK cells was found to be truly a developmental phenomenon as evidenced by the generation of cells from previously tolerant mice (B6→TAP1$^{-/-}$) in MHC class I^+ mice (e.g. B6→TAP1$^{-/-}$→B6) that resulted in NK cells that are tolerant to the environment in which they are developed but not to allogeneic or MHC class I^- cells. This implies that the functional defects of MHC class I^- and MHC class I^+ NK cells developed in a class I MHC$^-$ environment are due solely to the environment in which they are developed. This also implies that the ability to distinguish between self and allogeneic MHC class I is derived through interaction of NK cells with the MHC.

B. Implications

The importance of Ly49-MHC class I interactions in regulating NK cell function has been intensively studied. Mice that lack MHC class I possess NK cells with elevated levels of Ly49 receptors, particularly inhibitory Ly49s (119, 255). This was thought to be the reason why NK cells from these mice failed to lyse allogeneic targets, since the expanded inhibitory receptor repertoire would prevent the lysis of these cells (108, 109). However, the upregulation of inhibitory Ly49s on MHC-deficient NK cells does not explain why NK cells from wild-type mice that lack self-inhibitory receptors are still tolerant to self or why MHC class I^+ NK cells that are developed in MHC class I^- mice that have normal Ly49 expression patterns are unable to distinguish between $MHC^{+/-}$ targets. In these instances, the licensing hypothesis of NK cell self-tolerance generation provides a good answer. Following the licensing hypothesis, NK cells that are not exposed to MHC class I during their development, as is the case in MHC class I^- mice, fail to become functionally mature (161, 162). Thus, it appears that NK cells developed in a class I MHC^- environment remain unlicensed. The mechanism of this process is still in debate (162, 163, 256). Our data indicate that the licensing hypothesis is the likely mechanism for how NK cells gain the ability to become lytic. However, how the cells that do not get licensed become hyporesponsive is still in question. The idea that hyporesponsiveness is a sort of default pathway (161) seems incomplete. Rather, it seems as though there should

be a complimentary mechanism to regulate the progression of NK cells into the hyporesponsive state. Accordingly, the disarming hypothesis postulates that NK cells that become hyporesponsive undergo down-modulation of activating receptors or a dampening of activating signals in response to ligation of activating NK cell receptors by self ligands (163). Although no evidence for the down-modulation of activating receptors in unlicensed cells has been seen, it is possible that other compensatory mechanisms are in place. One possibility is that inhibitory receptors, such as Siglec-E or LAIR-1, that do not recognize MHC class I are upregulated to prevent unlicensed cells from becoming activated against self cells. It is equally possible that NK cells that receive very strong signals at a crucial point in their development become anergic, like in T cells. The synthesis of the licensing and the disarming hypotheses provides a complete mechanism for the generation of NK cell self tolerance in class I-bearing mice as well as in class I-deficient mice.

Recently, MHC class I-Ly49 interactions on the same cells, cis-interactions, have been described by two separate groups (228-230). Ly49A on an NK cell was found to form a stable interaction with H-2D^{d} on the same cell (228); this interaction prevented the Ly49 molecule from mediating inhibition through interaction with target cells (230). Other Ly49 molecules were found to also exhibit cis-binding (231). Based on our results that describe MHC class I^{+} NK cells in MHC class I^{-} mice, it appears as though this interaction is not

sufficient to license the NK cell for lytic activity. Cis-interaction may, however, provide a mechanism by which licensed NK cells are able to lyse transformed self-cells, such that inhibitory Ly49s are bound by MHC class I on the same cell in a non-productive manner, preventing the inhibition of that cell (230). This allows NK cells to receive greater activating than inhibitory signals.

Our results have several applications. NK cells have been implicated in aiding in breach of tolerance in autoimmunity in that recognition of activating ligands on self cells leads to the production of cytokines that can activate T and B cells with antigen receptors for self (257); understanding how NK cells gain tolerance can enable researchers to manipulate that mechanism to prevent a breach in tolerance. NK cells also secrete pro-inflammatory cytokines that may aid in tissue destruction in an autoimmune individual. In addition, human patients have been identified that have a mutation in the TAP1 gene that results in severely reduced expression of MHC class I, termed type I bare lymphocyte syndrome (BLS), a form of severe combined immune deficiency (SCID, 258-260). Patients with type I BLS first exhibit symptoms in late childhood; they develop multiple upper respiratory infections (259). NK cells from BLS patients have been determined to be only minimally cytotoxic; in fact, they are only capable of low levels of ADCC (258). BLS-patient NK cells that are activated become reactive against autologous targets, indicating that they may aid in autoimmunity (261, 262). $CD8^+$ T cells from these patients, although reduced in number, remain

cytotoxic (261) and $CD4^+$ and γδ T cells are increased in number and may therefore be capable of mounting a response to infections. A better understanding of the mechanisms behind NK cell tolerance induction in MHC class I^- environments would benefit these patients by enabling the generation of therapies to target the NK cell deficiency. Our data indicate that a bone marrow transplant would not significantly aid these patients because the NK cells that they possess would still be hyporesponsive due to a lack of MHC class I. Although hyporesponsive NK cells can be activated against infected cells, their activity is less than that of MHC class I^+-derived NK cells. Thus, studying the impact of MHC class I-Ly49 interactions may have clinical significance.

C. Future studies

Many areas concerning the tolerance induction of NK cells still need to be addressed. One important question that needs to be answered is whether unlicensed NK cells become unlicensed through an active mechanism or whether they simply remain hyporesponsive as a result of a lack of some as yet undefined signal. The importance of cis-interactions between MHC class I and Ly49 receptors in the tolerance induction of NK cells should also be examined, as cis-interactions may enable licensed NK cells to become activated, in that they prevent inhibitory receptors from providing productive inhibition to the NK cell. In addition, it remains to be seen by what mechanism hyporesponsive NK cells are enabled to become activated during infections and transformation. It should

be investigated whether there is a threshold for activating signals that is breached and allows virus infected and transformed cells to induce hyporesponsive cells to lyse the altered cell. The necessity of CD94/NKG2A on developing neonatal NK cells should also be examined to determine whether the negative signals received through this receptor are sufficient to prevent neonatal NK cells from lysing self cells and to allow them to distinguish MHC class I^+ cells from MHC class I^- cells, as has been shown (105).

A revised model of NK cell tolerance induction based on our current understanding and hypothesis is presented in figure 5.1. This model combines the previously proposed models for tolerance induction and suggests that there is not only an active mechanism for generating self tolerant but inducible NK cells, but that an active process regulates the generation of hyporesponsive NK cells during NK cell development.

II. The role of Foxk1 in NK cell development and function

A. Summary

Our studies have demonstrated a requirement for Foxk1 to ensure proper development of functionally mature NK cells. Foxk1-deficient mice have widespread defects throughout the lymphocyte compartment, however, our focus in these studies was on the severe reduction in NK cell numbers. NK cells from $Foxk1^{-/-}$ mice showed severely impaired killing ability after poly I:C or IL-12 and IL-18 stimulation, but improved cytotoxicity after IL-2 or IL-15 stimulation. This

implies a role for Foxk1 in the response to IL-12 and IL-18 signals. NK cell development is also altered in Foxk1$^{-/-}$ mice in that there is an accumulation of cells in stage IV of development and a reduction in the number of mature NK cells in bone marrow and periphery, indicating that a final maturation step in NK cell development is controlled by Foxk1. These NK cell defects in the Foxk1$^{-/-}$ mouse were found to be intrinsic to the hematopoietic compartment and may be inherent to NK cells, as wild type NK cells express Foxk1 (L.A.M., unpublished results). A significant proportion of the circulating NK cells in Foxk1$^{-/-}$ mice were found to exhibit hallmarks of thymus-derived NK cells, suggesting that Foxk1 does not function in the development of NK cells in the thymus.

B. Implications

Discovering a novel role for Foxk1 in NK cell development has broad implications. This is the first forkhead transcription factor to be linked to NK cell development, although other Fox factors have been shown to be important for development and maintenance of the immune system (184, 186, 191-194, 198-200, 205, 206). Like some of these transcription factors, Foxk1 seems to play a central role in the development of NK cells and not just in the development of certain receptors. This may be due to altered cytokine signaling in the Foxk1$^{-/-}$ mouse. Indeed, there is precedence for Fox transcription factors to be involved in cytokine pathways. Foxk2, a Fox transcription factor very similar to Foxk1 which

was originally termed interleukin binding factor (ILF), has been shown to regulate the expression of IL-2 in mouse and human (263). Although no defects in the response to IL-2 were seen in Foxk1$^{-/-}$ mice, the defective IL-12 and IL-18 response could indicate that Foxk1 regulates the response to these cytokines. It is also possible that the generation of IL-2 or IL-15 is aberrant in Foxk1$^{-/-}$ mice, however, that has not been investigated. Levels of IL-15 mRNA were determined to be conserved between wild-type and Foxk1$^{-/-}$ mice (L.M., unpublished results); however, IL-15 activity may be regulated at the protein level and is very difficult to detect.

A human ortholog of mouse Foxk1, FOXK1 has been described (264, 265). To date, no studies have investigated the role of FOXK1 in the human; however, it shares greater than 88% amino acid identity with the mouse protein (265, 266). Based on the homology of the proteins alone, it is thought that FOXK1 is involved in muscle repair mechanisms. Perhaps FOXK1 is also involved in the development of functional NK cells. Examining the function of human FOXK1 would be vital to our understanding of human NK cell development and would potentially validate the use of the Foxk1$^{-/-}$ mouse model for study of NK cell development in general.

In skeletal muscle, Foxk1 has been shown to regulate the expression of the CDK-inhibitor p21 (210, 238). Although it has not yet been determined whether this mechanism is in place in NK cells, there are clues that p21 may be involved

in the accumulation of stage IV NK cells in Foxk1$^{-/-}$ mice. The accumulation of stage IV cells in Foxk1$^{-/-}$ mice greatly resembles the accumulation of quiescent myogenic progenitors in these mice, leading us to question the roles of Foxk1 and p21 in the regulation of the cell cycle in NK cells. In T cells it has been shown that p21 negatively regulates T cell proliferation; p21$^{-/-}$ T cells that are stimulated with IL-2 have significantly increased proliferative capacity and are capable of mediating a lupus-like disease (367). p21 and p27^{KIP1} have been shown to be regulated by TGF-β1 and in the absence of these transcription factors, TGF-β1-induced G_1 arrest does not occur at equivalent levels (268). Responsiveness to IL-2 may be controlled by p21 and p27; T cells from p21$^{-/-}$p27$^{-/-}$ mice have increased responsiveness to IL-2, due in part to upregulation of the IL-2Rα on these cells (268). These studies provide some evidence for the activity of p21 in lymphocytes. However, despite these observations, it remains to be seen whether p21 is actively involved in the cell cycle regulation of developing NK cells and whether p21 is regulated by Foxk1 in these cells. Indeed, other transcription factors have been shown to regulate the expression of p21. The Ets transcription factor, Ets-1 has been shown to positively regulate p21 expression in smooth muscle (269). This regulation occurs through the binding of Ets-1 to a cis-element in the p21 promoter; Ets-1 was shown to be essential for the induction of p21 by mutating either p21 or Ets-1, which resulted in apoptosis of the cell, as opposed to quiescence (269). Ets-1 already plays a role in NK cell development,

in that NK cells that lack Ets-1 fail to mature (166) and it is required for the expression of CD244 (167, 168). In the absence of Foxk1, Ets-1 stimulation of p21 may proceed without negative regulation, leading to an accumulation of NK cells, like that seen in the expansion stage of Foxk1$^{-/-}$ mice. The transcription factor Sp1 has been shown to induce p21 expression in the presence of TGF-β (270). However, a role for Sp1 in the repression of p21 has also been found; Sp1 was shown to suppress the expression of p21 as well as the p21/cdk4/cyclin D complex that is necessary for cell proliferation (271). The repression of p21 by Sp1 was observed in smooth muscle cells, where five distinct Sp1 binding sites were found in the promoter of p21 (272). Ablation of some of these sites leads to activation of p21 by Sp1; indeed, in endothelial cells, only three of the binding sites are present, leading to activation of p21 by Sp1 (272). This indicates that the regulation of p21 by Sp1 is cell type specific and may be either repressive or activating in NK cells. Taken together, these studies show the importance of the regulation of p21 to cell cycle progression in several cell types; however, it is as yet unknown whether p21 regulation by Foxk1 plays a role in NK cell development, but it seems likely.

The identification of markers associated with thymic development of NK cells (88, 233) prompted us to study thymus-derived NK cells independently from bone marrow-derived NK cells. The presence of NK cells bearing markers that are specific for cells derived from the thymus in significantly increased

proportions and the coincidental decrease in bone marrow-derived NK cells in the Foxk1$^{-/-}$ mouse indicates that Foxk1 plays a role in the development of NK cells in the bone marrow but not the thymus. This provides evidence to further delineate the distinct pathways of bone marrow and thymic NK cell development. NK cells generated in the thymus have a very different functional profile from those developed in the bone marrow; thymus-derived NK cells preferentially secrete cytokines when activated and are only induced to become cytolytic in the course of an infection, whereas bone marrow-derived NK cells have a much greater cytolytic potential (89). The presence of functionally-deficient NK cells in the Foxk1$^{-/-}$ mouse suggests a role for Foxk1 in the development of lytic NK cells and may be essential in the maturation process by which bone marrow-derived NK cells gain their lytic function.

C. Future Directions

Describing the role of Foxk1 in NK cell development is merely the beginning of studies to understand the impact of Foxk1 in the immune system. Foxk1$^{-/-}$ mice have decreased numbers of effector memory CD8^{+} T cells and increased numbers of B cells as well as macrophages and granulocytes. While these alterations in the composition of the lymphoid pool could be due to the lack of NK cells, as cells from one lymphoid compartment have been shown to impact the homeostatic potential of other compartments (175), it is likely that Foxk1 also plays a role in these cell types. Therefore, it is necessary to investigate the

function of Foxk1 in these populations. A decrease in the effector memory population of $CD8^+$ T cells in $Foxk1^{-/-}$ mice implies that Foxk1 may act in the generation of T cell activation, proliferation or maintenance, potentially through the regulation of cytokine responses. It is possible that Foxk1 is generally involved in the response to cytokine signaling by effector cells. The lack of NK cell effector function after stimulation with IL-12 and IL-18 suggests a defect in this pathway. Therefore, a role for Foxk1 in cytokine signaling should be investigated. In addition, the generation of IL-2 in $Foxk1^{-/-}$ mice should also be examined, as IL-2 deficient mice exhibit impaired secondary T cell responses and decreased NK cell lytic ability (273). The fact that there are fewer effector memory cells could imply that proliferation or maintenance of this population is mediated by Foxk1. Also, Foxk1 and Foxk2 could provide redundant functions in the production of IL-2, leading to a less severe phenotype in the absence of just one factor.

Possible mechanisms for Foxk1 involvement in the expansion stage of NK cell development also need to be addressed. Earlier reports into the function of Foxk1 have described the regulation of the cdk-inhibitor, $p21^{CIP}$, by Foxk1 in skeletal muscle (210). Foxk1 acts to inhibit the transcription of p21, thereby allowing the cell cycle to progress. It is possible that this same mechanism is at work in developing NK cells, preventing stage IV cells (and perhaps effector memory T cells) from proliferating normally. It should be determined whether

there are excessive p21 levels in stage IV NK cells of Foxk1$^{-/-}$ mice and whether the stage IV cells that do not have excessive p21, if any, are able to proliferate and mature into functional NK cells. If some stage IV cells are able to proliferate normally and escape to the periphery as mature NK cells, it would explain the fact that not all of the mature NK cells in the periphery of Foxk1$^{-/-}$ mice are thymus-derived.

Another potential avenue of investigation is to examine the role of Foxk1 in the thymus. RT-PCR confirms Foxk1 expression in the thymus (M.S.A., unpublished observations), however its role is not clear. Due to the near normal or normal levels of T cells and the completely typical T cell development in the thymus of Foxk1$^{-/-}$ mice, it would appear that Foxk1 does not influence T cell or thymus development. The fact that there are significant numbers of potentially thymus-derived NK cells in the periphery (their over-representation likely being due to the lack of bone marrow derived NK cells) suggests that NK cell development in the thymus is unaffected by Foxk1-deficiency. However, the presence of Foxk1 in the thymus indicates that there may be a function for Foxk1 in the thymus and this should be explored further.

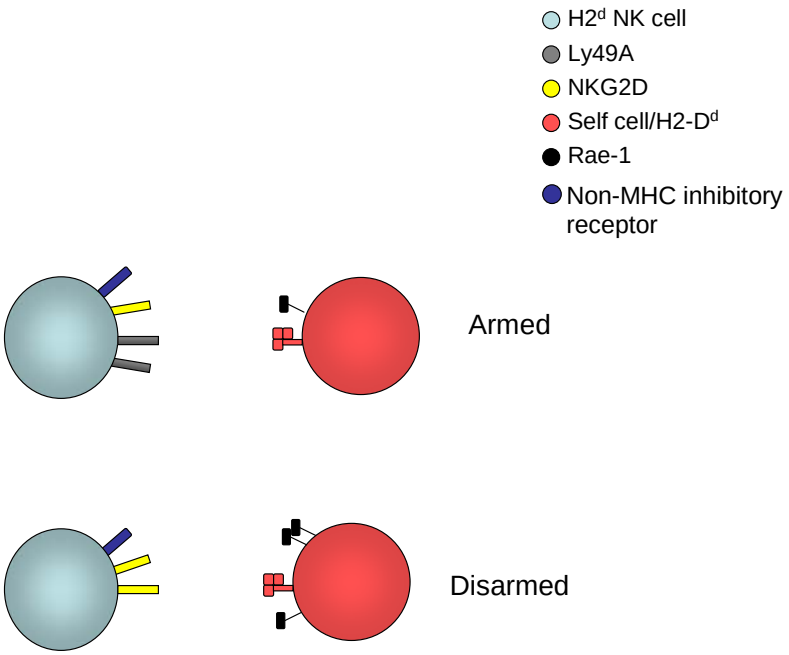


Figure 5.1 A synthesis of the arming, or licensing, and disarming models of NK cell tolerance development. Developing NK cells that receive greater inhibitory signals through self-MHC class I interaction with Ly49 than activating signals become armed. Cells that receive too strong of an activating signal become disarmed, perhaps through overriding inhibition by non-MHC recognizing receptors.

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