

Ly49 receptors and MHC-I interactions, and their implications in cancer immunosurveillance

Megan Mai Tu

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in Microbiology and Immunology

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

University of Ottawa



uOttawa

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Abstract

Natural killer cells are part of the innate immune response, and get their name ‘natural killer’ due to their unique ability to recognize and kill aberrant cells without prior sensitization. The Ly49 receptors on NK cells are involved in both their education process and mediating their ability to recognize aberrant cells. Virally-infected and tumor cells have been shown to exhibit decreased levels of MHC-I on their cell surface. As MHC-I is expressed on all nucleated cells, the Ly49 receptors on NK cells recognize this loss of MHC-I on the surface as a kill signal, leading to direct cytotoxicity through the release of perforin and granzymes. This recognition process by NK cells is termed “missing-self.” The interactions between MHC-I and Ly49 receptors are integral to NK cell function. The response of the NK cell to cells which it encounters is dependent on a balance of activating and inhibitory receptors. This signal in turn is dependent on the engagement and interactions of various ligands, such as MHC-I, by the activating and inhibitory Ly49s on the NK cells. In this compilation of my three main research projects, the interactions and binding capacity between the different MHC-I molecules and different members of the Ly49 receptor family are explored. In addition, the importance of the Ly49 receptors in NK cell-mediated cancer immunosurveillance is studied.

Acknowledgments

I would like to thank Dr. Andrew Makrigiannis for his guidance and support throughout the duration of my graduate studies. His constant reminder that hardwork will eventually pay off in the end has helped me stay focused on the end goal. I could not be happier with the research I've had the opportunity to be a part of. The opportunities which I've been afforded and the collaborations I've been a part of have helped me to grow as a researcher.

I'd like to also thank my past research supervisors, Drs. Reza Nokhbeh, Anne-Gaelle Rolland-Lagan and Thienny Mah, who I've had the opportunity to work with during my undergraduate studies. They've all helped to spark and shape my passion for research.

I would also like to thank my thesis advisory committee members Drs. Seung-Hwan Lee and Jonathon Lee, who have been with me since my Masters and subsequent transfer to the PhD program.

I would like to thank all the colleagues I've worked alongside, past and present. We've had many interesting, and at time random conversations, about science and life in general (ie. fig tree islands). I would especially like to thank Dr. Munir Rahim for the countless amounts of times he's read anything remotely scientific that I've written over the past five years. In addition, I'd like to thank Dr. Simon Belanger for training me when I first started in the lab and knew nothing about immunology or cell culture. Thank you to my friends in the Skerjanc, Nemer, Mah, and Lee labs, who were always available for useful discussions, rants about life, as well as being an invaluable resource for reagents and supplies when in a pinch.

Last but not least, I'd like to thank my family, Ba, Ma, Andrew, Mindy, Elaina and baby William, for their unconditional love, support and understanding. A special thank you to my husband Terry for being there for me throughout this process.

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List of Abbreviations

Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
ALAK	Adherent lymphokine activated killer
β₂m	β ₂ -microglobulin
B6	C57BL/6
BM	Bone marrow
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
DC	Dendritic cell
DNAM-1	DNAX-accessory molecule-1
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HA	Haemagglutinin
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KD	Knockdown
KIR	Killer cell immunoglobulin-like receptor

MCA	3-Methylcholanthrene
MCMV	Murine cytomegalovirus
MFI	mean fluorescence intensity
MHC-I	major histocompatibility complex class I
MICA/B	MHC class I polypeptide-related sequence A/B
NK cell	Natural killer cell
NKC	Natural killer complex
NKC^{KD}	Natural killer complex knockdown
PBS	Phosphate buffered saline
pDC	plasmacytoid dendritic cell
PE	R-phycoerythrin
PolyI:C	Polyinosinic:polycytidylic acid
Rae-1	Retinoic acid early inducible-1
SEM	Standard error mean
SHIP	SH2-containing inositol polyphosphate 5-phosphatase
SHP-1/2	SH2-containing protein tyrosine phosphatase-1/2
TAP	Transporter associated with antigen processing
TCR	T cell receptor
Tg	Transgene
TLR	Toll-like receptor
WT	Wildtype

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Chapter 1 : General Introduction

Preface

The following chapter consists of a general introduction to NK cells, Ly49 receptors, and their collective role in cancer. Portions of this chapter have been previously published as two separate review articles, with modifications as deemed appropriate:

Rahim MM, Tu MM, Mahmoud AB, Wight A, Abou-Samra E, Lima PD, and Makrigiannis AP. Ly49 receptors: innate and adaptive immune paradigms. *Frontiers in Immunology*. 2014; 5:145.

Tu MM, Mahmoud AB, Makrigiannis AP. Licensed and unlicensed NK cells: Differential roles in cancer and viral control. *Frontiers in Immunology*. 2016 May 2;7:166.

The specific contributions of each author for the published works are listed below.

For **Rahim MM, Tu MM, Mahmoud AB, Wight A, Abou-Samra E, Lima PD, and Makrigiannis AP. Ly49 receptors: innate and adaptive immune paradigms.**

Rahim MM: Wrote the “Introduction”, section on “Ly49 receptors”, section on “Ly49 expression and function on cells of the myeloid lineage”, and “Conclusion”. Constructed Figure 1 and Table 1. Edited other sections of the manuscript and responded to reviewers comments.

Tu MM: Wrote the section on “Ly49 expression and function on NK cells”. Constructed Figure 2.

Mahmoud AB: Contributed to parts of the “Introduction” and “Ly49 expression and function on NK cells” section. Constructed Figure 3.

Wight A: Wrote the section on section on “Ly49 expression and function on CD8⁺T cell subsets”

Abou-Samra E: Wrote the section on “Ly49 expression and function on NKT cells”

Lima PD: Wrote the section on “Ly49 expression and function on uterine NK cells”

Makrigiannis AP: Provided guidance and critical reading of the manuscript.

For Tu MM, Mahmoud AB, Makrigiannis AP. Licensed and unlicensed NK cells: Differential roles in cancer and viral control.

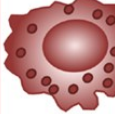
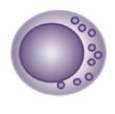
Tu MM: Wrote the “Introduction”, section on “MHC-I-mediated NK cell education/licensing”, section on “Cancer immunosurveillance by NK cells”, section on “Recognition of MHC-I-deficient tumor cells by licensed NK cells”, section on “Induced-self recognition of tumor cells by unlicensed NK cells”, and “Conclusion”. Tu MM co-wrote the section on “NK cell-mediated recognition of virus-infected cells. Constructed all the review figures, and responded to reviewers.

Mahmoud AB: Wrote the section “Is NK cell licensing always required for the recognition of virus-infected cells” and co-wrote the section on “NK cell-mediated recognition of virus-infected cells”. Provided input for Figure 3.

Makrigiannis AP: Provided guidance and critical reading of the manuscript.

Introduction

Major histocompatibility complex (MHC) recognition is central to both innate and adaptive immune recognition. The function of both the innate immune effectors, such as natural killer (NK) cells, and the adaptive immune effectors, such as T cells, depend upon the recognition of MHC-I molecules expressed on aberrant cells; however, the mode of recognition varies between the two lymphocyte subsets. T cell receptors (TCR) have specificity to the antigenic peptide bound to MHC-I and form contacts to the peptide as well as to the MHC molecule (Garboczi et al., 1996; Garcia et al., 1996). NK cell receptors, such as the Ly49 in mice, bind MHC-I molecules in a peptide-dependent but not peptide-specific manner (Hanke et al., 1999). An exception is the Ly49C, a member of the Ly49 receptor family (Table 1), whose binding to H-2K^b haplotype appears to be peptide-specific (Franksson et al., 1999; Hanke et al., 1999). The binding interface between Ly49 and MHC-I, revealed from crystal structures of Ly49A and H-2D^b molecules, is distinct and away from the peptide binding groove on MHC-I (Tormo et al., 1999). While signals downstream of TCR engagement specifically activate T cell functions, the Ly49 receptors can be activating or inhibitory in nature, and their expression is not limited to NK cells. In addition to NK cells, other leukocytes have also been shown to express inhibitory Ly49 receptors, such as the CD8⁺ T cells, CD3⁺ cells, intestinal epithelial lymphocytes (IELs), NKT cells, uterine NK cells (uNK) cells, and cells of the myeloid lineage (Denning et al., 2007; Gays et al., 2006; Kamogawa-Schifter et al., 2005; Maeda et al., 2001; Ortaldo et al., 1998; Toyama-Sorimachi et al., 2005; Yadi et al., 2008). Here, we review the expression of Ly49 receptors on different cells of the innate and adaptive immune system (Figure 1.1), and their contribution to immunity.

	NK	uNK	NKT	DC	pDC	Neutrophil	Macrophage	CD8 ⁺ T
Cell type								
Ly49 [®] repertoire	D [#] , H [#] , F, C/I, G, A	D [#] , H [#] , C/I, G, A	C/I, G, A	Q	Q	Q	Q*, B*	F, C/I, G, A
Functions	1) Missing-self response 2) Education/licencing 3) Tumor surveillance 4) Anti-viral	1) Granule biogenesis 2) VEGF-A production 3) Anti-viral	1) Regulates response to α -GalCer	n.d.	1) TLR-7 & 9 co-operation 2) IFN-I production 3) Pathogen recognition	1) Cellular polarization 2) Tissue invasion	1) Cytoskeletal rearrangement 2) Cellular polarization 3) Spreading	1) Regulates cell activation 2) Survival/proliferation

® Based on literature reports in C57BL/6 mice
 # Activating receptors
 * On distinct non-overlapping subpopulations

Figure 1.1: Schematic representation of cell types expressing Ly49 receptors

Receptor repertoire and proposed functions of Ly49 in different cellular subsets is shown based on literature reports in C57BL/6 mice. NK, natural killer; uNK, uterine natural killer; NKT, natural killer T; DC, dendritic cells; pDC, plasmacytoid dendritic cell; α -GalCer, α -galactosylceramide; VEGF-A, vascular endothelial growth factor A; TLR, Toll-like receptor; IFN-I, type I interferon; n.d., not determined.

Ly49 Receptors

The innate MHC-I receptors include the human killer cell immunoglobulin-like receptor (KIR), NKG2/CD94, and mouse Ly49 families of receptors. The genes that encode the mouse MHC-I receptors are clustered together in the natural killer gene complex (NKC) on mouse chromosome 6. The Ly49 receptors are homodimeric type II glycoproteins of the C-type lectin-like superfamily. They are structurally distinct but functionally similar to human KIR in terms of MHC-I recognition, and therefore provide a useful model system to study the role of this class of receptors in immune regulation. A list of activating and inhibitory Ly49 receptors in different mouse strains and KIR in humans is shown in Table 1.1. MHC-I receptors generally inhibit NK cell function when they are engaged by self-MHC ligands. Therefore, inhibitory Ly49 receptors are generally agreed to be important for the prevention of autoimmunity by suppressing NK cell activation. The acquisition of inhibitory Ly49 for self-MHC-I is also a key step in the “licensing” of developing NK cells to avoid a hyporesponsive state (Figure 1.2A) (Kim et al., 2005). In contrast, the activating Ly49 receptors recognize ligands that are expressed on abnormal or infected cells, and activate cytokine production and cellular cytotoxicity by NK cells. The integration of signals from the activating and inhibitory Ly49 receptors ultimately determines the functionality of NK cells.

Intracellular signaling

The inhibitory and activating members of Ly49 and KIR families are characterized by the presence or absence of immunoreceptor tyrosine-based inhibitory motif (ITIM) domains in their cytoplasmic tail (Olcese et al., 1996; Vély and Vivier, 1997; Vivier and Daëron, 1997). NK cell stimulation results in differential phosphorylation of Ly49 receptors. Mason and co-workers first

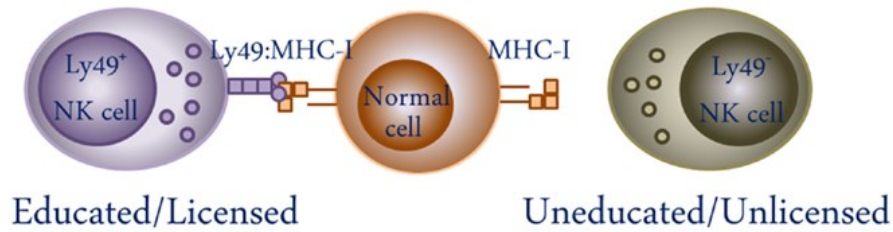
Table 1.1: Mouse Ly49 and human KIR receptors for MHC-I

Mouse^a				Human	Function
NOD	129	B6	BALB		
Ly49D	Ly49P	Ly49D	Ly49L	KIR2DL4	Activating
Ly49H	Ly49R	Ly49H		KIR2DS1	
Ly49M	Ly49U			KIR2DS2	
Ly49P ₁				KIR2DS3	
Ly49P ₃				KIR2DS4	
Ly49U				KIR2DS5	
Ly49W				KIR3DS1	
Ly49A	Ly49B ^b	Ly49A	Ly49A	KIR2DL1	Inhibitory
Ly49B ^b	Ly49E	Ly49B ^b	Ly49B ^b	KIR2DL2	
Ly49C	Ly49EC ₂	Ly49C	Ly49C	KIR2DL3	
Ly49E	Ly49G	Ly49E	Ly49E	KIR2DL5	
Ly49F	Ly49I ₁	Ly49F	Ly49G	KIR3DL1	
Ly49G ₂	Ly49O	Ly49G	Ly49I	KIR3DL2	
Ly49I	Ly49Q ₁	Ly49I	Ly49Q		
Ly49Q	Ly49S	Ly49J			
	Ly49T	Ly49Q			
	Ly49V				

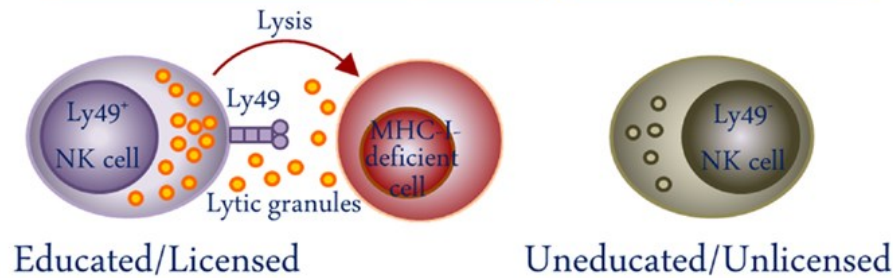
^aLy49 pseudogenes do not encode any functional product and are not included.

^bEncoded by a gene outside the Ly49 gene cluster.

A Education and licensing of developing NK cells



B Immunosurveillance (missing-self recognition)



C Immunosurveillance of MHC-I-sufficient tumors

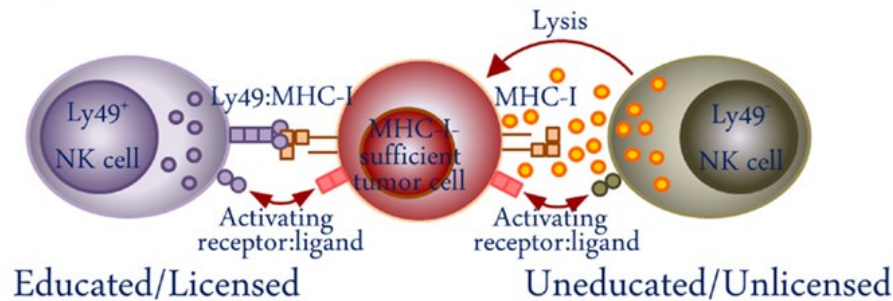


Figure 1.2: Schematic representation of the role of Ly49 receptors in NK cell development and function

(A) During NK cell development, interactions between the inhibitory Ly49 receptors and their self-MHC-I ligands on normal cells result in NK cell functional maturation (education/licensing). (B) Licensed Ly49^+ but not unlicensed Ly49^- NK cells recognize MHC-I-deficient cells and kill them through the release of lytic granules (missing-self recognition). (C) Tumor cells express ligands which are recognized by activating receptors on NK cells. However, MHC-I-expressing tumor cells can inhibit licensed NK cells through interactions with their inhibitory Ly49 receptors. Unlicensed NK cells will not be inhibited in this way because they lack Ly49 receptors.

reported that tyrosine phosphorylation was restricted to the inhibitory Ly49 molecules such as Ly49 A, C/I, and G2, while the activating Ly49D was not phosphorylated (Mason et al., 1997). The phosphorylated Ly49 molecules were shown to be associated with the src homology 2 (SH2) domain-containing protein phosphatase SHP-1 in this study (Mason et al., 1997). Phosphorylation of the tyrosine residue within the ITIM is responsible for the recruitment and activation of SH2 domain-containing protein tyrosine phosphatases (D'Ambrosio et al., 1995). Although SHP-1 appears to be the major phosphatase required for Ly49-mediated inhibition, it may not be the only inhibitory mediator since Ly49A function is diminished but not completely absent in mice deficient in SHP-1 (Nakamura et al., 1997). Although engagement of inhibitory receptors results in ITIM phosphorylation, it is greatly enhanced when it is cross-linked to an activating receptor complex (Binstadt et al., 1996; Bléry et al., 1997). This demonstrates an elegant regulatory mechanism whereby inhibitory receptor phosphorylation induced by engagement of activating receptors results in recruitment of protein tyrosine phosphatases, and the subsequent suppression of tyrosine phosphorylation-based signals downstream of the activating receptors. In contrast to the inhibitory receptors, the activating Ly49 receptors transduce signals through associated adaptor proteins, such as DAP12, which possess an immunoreceptor tyrosine-based activation motif (ITAM) (Gosselin et al., 1999; Makrigiannis et al., 1999; Smith et al., 1998). This interaction is mediated by a charged residue (arginine) in the transmembrane segment of Ly49, as was shown for Ly49D (Mason et al., 1998). Cross-linking of activating Ly49 leads to phosphorylation of tyrosine residues within the ITAM of DAP12 as well as phosphorylation of other proteins, including members of the Src-family of tyrosine kinases, the mediators of downstream signaling events (McVicar et al., 1998).

Gene Diversity

One of the striking features of Ly49 receptors is the polymorphic and polygenic nature of the *Ly49* gene cluster among the inbred mouse strains (Makrigiannis et al., 2002, 2005; Proteau et al., 2004; Yokoyama et al., 1990). This results in heterogeneity in both the type and level of Ly49 molecules expressed in different mouse strains (Table 1.1) (Ortaldo et al., 1999). Among the four inbred mouse strains whose *Ly49* gene haplotypes have been extensively characterized, the BALB/c mouse strain possess the smallest haplotype with 8 genes (Anderson et al., 2005), and the NOD/ShiLtJ strain possess the largest haplotype with 21 discernable genes (Belanger et al., 2008). The C57BL/6 haplotype, which was the first to be characterized, and the 129 mouse strain haplotype possess 15 and 19 genes, respectively (Brown et al., 1997; Depatie et al., 2000; Makrigiannis et al., 2002, 2005; McQueen et al., 1998). In all the characterized *Ly49* haplotypes, there appears to be a limited degree of conservation in the form of “framework” genes which delineate the regions of variable numbers of strain-specific genes. The framework gene pairs in mice are *Ly49q-e*, *Ly49i-g*, and *Ly49c-a*. *Ly49* haplotype diversity has evolved as a result of multiple duplication and deletion events. Examples of strain-specific Ly49 gene inactivation are evident in different strain of mice mostly mediated by stop codons within the coding region (Makrigiannis and Anderson, 2001; McQueen et al., 1999; Silver et al., 2001). Such diversity is possibly driven by selective pressure due to pathogenic challenge, since NK cells appear to be critical for the control of viral infections (Biron et al., 1989; Orange, 2002). In mice, the activating Ly49H receptor directly interacts with the murine cytomegalovirus (MCMV) *m157* gene product on NK cells and confers resistance to MCMV in the C57BL/6 mouse strain which possesses the *Ly49h* gene (Figure 1.3). Conversely, 129 and BALB/c strains

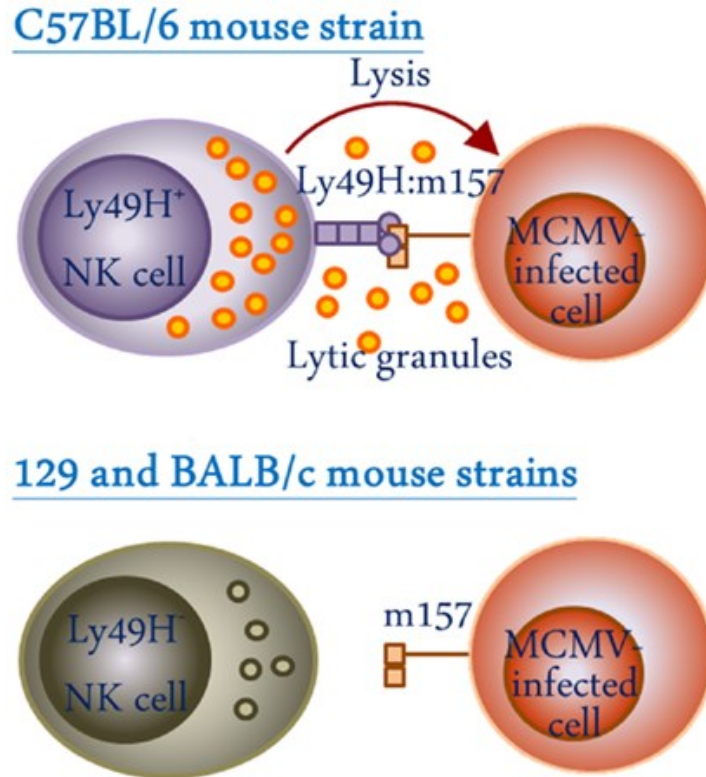


Figure 1.3: Schematic representation of the role of the activating Ly49H receptors in recognition of MCMV-infected cells

NK cells from C57BL/6 but not 129 or BALB/c mouse strains express Ly49H and are capable of recognizing m157 viral protein on the surface of MCMV-infected cells, thereby, conferring resistance against MCMV infection.

lack the gene for activating Ly49H and hence are highly susceptible to MCMV infection (Arase et al., 2002; Brown et al., 2001; Daniels et al., 2001; Lee et al., 2001; Scalzo et al., 1990; Smith et al., 2002).

Ly49 Expression and Function on NK cells

Ly49 receptors are best known for their role in the regulation of NK cell functions. Both the activating and inhibitory Ly49 receptors are expressed by NK cells (Figure 1). Murine NK cells express up to six members of the Ly49 receptor family in an overlapping fashion, such that the average NK cell expresses two to three Ly49 receptors (Kubota et al., 1999). Ly49 expression begins early during NK cell development in the bone marrow (BM). An *in vivo* differentiation study demonstrated that there are five stages of NK cell development in murine BM, as assessed by surface expression of NK cell markers (Kim et al., 2002). In the first three stages of development, NK cells sequentially attain expression of CD122 (stage I; CD122⁺ NK1.1⁻ DX5⁻ Ly49⁻), then NK1.1, DX5, and NKG2A (stage II; CD122⁺ NK1.1⁺ DX5^{lo} CD94/NKG2A⁺ Ly49⁻), and finally Ly49 at stage III. Afterward, immature NK cells undergo extensive cell division and expansion in stage IV followed by functional maturation at stage V of their development (Kim et al., 2002). Ly49E is the only member of Ly49 family that is expressed on fetal NK cells and its expression is lost early after birth (Van Beneden et al., 2001). Expression of other Ly49 family members are detected on developing NK cells during the first 2–3 weeks after birth and reach optimal levels within 6–8 weeks after birth (Dorfman and Raulet, 1998; Van Beneden et al., 2001).

NK Cell Education and Target Cell Recognition

Ly49 receptors play an important role in NK cell education and recognition of target cells. The mechanism behind NK cell recognition of target cells was discovered by Kärre and colleagues, and accordingly named the “missing-self hypothesis” (Figure 1.2B) (Kärre et al., 1986). According to the hypothesis, NK cells survey MHC-I expression on cells with which they come into contact (Kärre et al., 1986). Abnormal or infected cells often down-regulate expression of MHC-I on their surface in order to avoid detection and killing by cytotoxic T cells; however, this down-regulation is recognized by, and functions as a kill signal for NK cells (Kärre et al., 1986). In this seminal work, the RBL-5 lymphoma cell line was mutagenized to derive two new cell lineages, an MHC-I-expressing RMA and MHC-I-deficient RMA-S. It was observed that the MHC-I-deficient RMA-S cells, following injection into mice, were rapidly eliminated while the MHC-I-expressing RMA cells were able to develop into tumors (Kärre et al., 1986). This “missing-self” hypothesis was further supported by the finding that cells isolated from mice lacking expression of β_2m , the light chain component of MHC-I that is necessary for its surface expression, were readily recognized and killed by NK cells, while re-introduction of a β_2m transgene restored the resistance to NK cell killing (Liao et al., 1991). In addition to the increased susceptibility of cells isolated from MHC-I-deficient mice to NK cell killing, it was also noted that NK cells from $\beta_2m^{-/-}$ mice exhibited a diminished ability to kill traditional NK cell targets, when compared to wild-type (WT) mice (Liao et al., 1991). This led to the proposal that MHC-I interactions during NK cell development are needed for induction of NK cytolytic activity (Figure 2A). This hypothesis was later substantiated by the discovery that NK cells must undergo an MHC-I-dependent “licensing” process in order to be functional, in which a self-specific Ly49 receptor interacts with self-MHC-I (Kim et al., 2005). Similarly, we have demonstrated that the NK cells from Ly49-deficient mice are unlicensed and

show impaired missing-self response against MHC-I-deficient target cells (Bélanger et al., 2012). This education requirement has also been found in human NK cells with the interaction of MHC-I and KIR (Anfossi et al., 2006; Kim et al., 2008). The licensed NK cells are functionally active while at the same time self-tolerant due to recognition of MHC-I through their inhibitory Ly49 receptors. In contrast, the unlicensed NK cells – which lack self-MHC-specific inhibitory receptors – are tolerant due to their hyporesponsive nature.

The mechanics of the education process are still unknown, with various contested models. While education was originally thought to occur during NK cell development in the BM (Kim et al., 2005), adoptive transfer studies utilizing splenic NK cells from MHC-I deficient mice into WT hosts were able to restore NK functionality, thus showing that education does not occur only during development of immature NK cells, but is actually a dynamic and reversible process (Elliott et al., 2010; Joncker et al., 2010). The arming and disarming model are the most widely accepted models thus far. Based on the arming model, NK cells which lack expression of an inhibitory self-MHC-I-specific Ly49 receptor are unable to fully mature into functional NK cells (become “armed”) and thus remain in their hyporesponsive state (Raulet and Vance, 2006). In contrast, the disarming model proposes that NK cells which lack expression of an inhibitory self-MHC-I-specific Ly49 receptor, and thus are chronically stimulated due to lack of inhibitory signaling, become anergic and hyporesponsive to target cells (Raulet and Vance, 2006).

The type of interaction between the Ly49 receptor and the MHC-I molecule during education has also been shown to be important and somewhat controversial: it is unknown whether the Ly49 is interacting in *cis* with MHC-I on the NK cell surface itself or in *trans* with the MHC-I expressed on another cell. *Trans*-mediated licensing is supported by various groups, using either adoptive transfer from MHC-I-deficient mice in WT recipients or doxycycline-inducible MHC-I

expression in MHC-I-deficient $\beta_2m^{-/-}$ mice to observe interactions between MHC-I-deficient NK cells in an MHC-I-sufficient environment (Ebihara et al., 2013; Elliott et al., 2010; Joncker et al., 2010). On the other hand, using a modified Ly49A receptor, which is only capable of binding in *trans* with ligands on other cell surfaces but not in *cis*, Chalifour and colleagues found that NK cells from these mice did not appear to be fully educated (Chalifour et al., 2009). As well, Bessoles and colleagues' recent work also suggests a *cis* interaction contribution to NK functionality, though the precise role is not clear (Bessoles et al., 2013).

NK cell-mediated immunosurveillance

Major histocompatibility complex-I-dependent “missing-self” recognition by NK cells has great implications on the immunosurveillance of abnormal cells which have lost surface expression of MHC-I molecules. Lost or aberrant surface expression of MHC-I in human tumors is classified into seven phenotypes as follows: (1) total HLA loss, (2) HLA haplotype loss, (3) HLA locus loss, (4) HLA allelic loss, (5) compound phenotypes, (6) unresponsiveness to interferons (IFNs), and (7) gain of MHC-Ib (García-Lora et al., 2003). Altered MHC-I expression is highly prevalent in human tumors and various alterations have been associated with different cancer types: HLA haplotype loss is the most common alteration and has been described in laryngeal (Maleno et al., 2002), non-Hodgkin's lymphoma (Drénou et al., 2004), and pancreatic cancer (Ryschich et al., 2004). Loss of MHC-I expression along with up-regulation of ligands for activating NK cell receptors on tumor cells results in their recognition and elimination by NK cells. We have shown that Ly49-deficient mice are unable to recognize and eliminate MHC-I-deficient target cells (Bélangier et al., 2012). On the other hand, the 5E6 antibody (to block the inhibitory Ly49I/C signaling) and 1-7F9 antibody (to block KIR signaling) have been shown to be useful in the treatment of leukemia and multiple myeloma in mice and humans, respectively; therefore,

manipulation of human KIR signaling has been proposed as a potential cancer therapeutic (Benson et al., 2012; Koh et al., 2001; Romagné et al., 2009; Vey et al., 2012). Finally, there is also evidence that a member of the Ly49 receptor family, Ly49A, recognizes the non-classical MHC-I molecule H2-M3 and in part mediates NK cell licensing (Andrews et al., 2012). Ly49A⁺ NK cells from WT mice exhibit enhanced responsiveness compared to H2-M3-deficient mice. H2-M3-deficient mice display defective tumor control with increased B16F10 pulmonary metastatic burden, and increased incidence of MCA-induced fibrosarcoma (Andrews et al., 2012; Xu et al., 2006).

More recently, a paradigm shift was suggested following the study by Tarek and colleagues, wherein it was found that unlicensed NK cells are better at killing neuroblastoma tumors (Tarek et al., 2012). Unlike the traditionally low levels of MHC-I expression on tumors, neuroblastoma cells express high levels of MHC-I, which can inhibit licensed NK cells. On the other hand, the unlicensed NK cells are not inhibited by MHC-I on neuroblastoma cells (Figure 1.2C). It was found that patients lacking HLA ligands for their KIRs showed improved prognosis and survival when treated with 3F8, an antibody targeting the disialoganglioside surface antigen, in contrast to the educated NK cells which were selectively inhibited by HLA ligands expressed on the tumor cells (Tarek et al., 2012). In such case, the inhibitory signals through KIR-dependent recognition of HLA on the target cell dampen the beneficial effect of educated NK cells. Blocking this KIR interaction with MHC-I could prove therapeutically beneficial in such circumstances.

The importance of the immune system in tumor control is highlighted by the increased cancer risk in immune-compromised individuals. Those with human immunodeficiency virus (HIV) infection, including individuals who have progressed to acquired immunodeficiency syndrome (AIDS), are at notably greater risk of developing lung cancer independent of smoking

(Kirk et al., 2007). Immunosuppressed renal transplant patients have increased incidence of skin cancer over the general population (Moloney et al., 2006). Those having undergone heart transplants are particularly at increased risk for non-Hodgkin's lymphoma, oral, and lung cancers (Jiang et al., 2010). Moreover, in human cross-sectional studies, the presence of tumor infiltrating lymphocytes is a strong predictor of positive patient outcome (Clemente et al., 1996), indicating a correlation between the immune system and cancer protection or recovery.

In support of the importance of NK cells in cancer immunity, NK-compromised *beige* mice – a model for human Chediak–Higashi syndrome – exhibit defective cytotoxic activity against tumor cells, and are more susceptible to spontaneous fatal tumor development, possibly due to ineffective immunosurveillance (Haliotis et al., 1985; Roder and Duwe, 1979). Chediak–Higashi syndrome is caused by a homozygous or compound heterozygous mutation in the lysosomal trafficking regulator gene. Affected individuals present with a host of immunodeficiency disorders such as granular anomalies in their lymphocytes, defective chemotactic and bactericidal activity of their neutrophils, defective NK cell function, and defective peptide loading and antigen presentation (Efrati and Jonas, 1958; Kritzler et al., 1964; Roder et al., 1980; Windhorst et al., 1966). Antibody-mediated depletion of NK cells prior to tumor cell injection in various mouse strains results in prolonged tumor survival, as well as an increased number of artificial lung metastases and spontaneous metastases (Gorelik et al., 1982). In humans, NK cells comprise up to 15% of the blood lymphocytes (Lanier et al., 1986). In a clinical setting, low NK cell activity in cancer-diagnosed individuals has been associated with poor prognosis, and those with advanced stage cancer often possess minimally cytotoxic NK cells (Pross and Lotzová, 1993). High cytotoxic activity of peripheral blood NK cells is correlated with up to 10% reduced incidence of

cancer (Imai et al., 2000). As well, in a clinical case of childhood-onset Hodgkin's lymphoma, this individual was observed to have non-functional NK cells (Komiyama et al., 1987).

Recognition of MHC-I-Deficient Tumor Cells by Licensed NK Cells

Tumors have developed multiple mechanisms for evading host immune recognition. One well-documented escape mechanism, the downregulation of MHC-I expression, is effective against T cells, but renders the tumor more susceptible to NK cells. Reduced expression levels of MHC-I has been documented in bladder, breast, cervical, colorectal, and ovarian human cancers (Cathro et al., 2010; Goepel et al., 1991; Han et al., 2008; McDougall et al., 1990; Mehta et al., 2008; Vitale et al., 1998).

The classic tumor model, in which missing-self was first discovered retrospectively, also helps to highlight the importance of the MHC-I status of the target cell (Kärre et al., 1986). Mutagenesis of RBL-5, a Rauscher virus-induced leukemia, led to the derivation of the MHC-I-deficient RMA-S and MHC-I-expressing RMA cell lines (Kärre et al., 1986). The difference in MHC-I expression levels of these two cell lines leads to differential recognition by NK cells (Kärre et al., 1986). The RMA-S induced flank tumors are much better controlled compared to the accelerated growth of the RMA tumors (Kärre et al., 1986). With the use of *B2m*^{-/-} mutant mice, it has been shown that a MHC-I deficiency renders the NK cells defective at killing traditionally well recognized MHC-I-deficient NK tumor cell line targets (Liao et al., 1991).

Our group has shown that mice lacking Ly49-mediated NK licensing also exhibit reduced activity against MHC-I-deficient tumor cells both *in vitro* and *in vivo* (Figure 1.4) (Bélanger et al., 2012). In the *in vivo* rejection assays spanning up to 18 h, these mice exhibited reduced capacity

at eliminating the MHC-I-deficient variants of RMA and C1498 compared to wild-type mice (Bélanger et al., 2012).

Induced-Self Recognition of Tumor Cells by Unlicensed NK Cells

While the self-specific inhibitory Ly49 receptors have been shown to be the mediators of NK cell licensing required for effective missing-self recognition (Koh et al., 2001), blockade of these receptors also helps to elicit a stronger NK cell response. Antibody-mediated blockade of self-specific Ly49 receptors improved *B2m*^{-/-} bone marrow allograft success similar to results seen with complete NK cell depletion; suggesting the importance of the inhibitory self-specific Ly49C/I subset in the effector functions of NK cells (Sun et al., 2012). This study shows successful grafting of MHC-I-deficient bone marrow cells in WT mice when Ly49C/I⁺ NK cells are depleted, thus providing evidence for missing self-recognition of MHC-I-deficient bone marrow cells by licensed NK cells.

Treatment of NK cells with monoclonal blocking antibody to Ly49C/I led to inhibited *in vitro* growth of MHC-I-expressing C1498, a murine leukemia cell line, and EL4, a T cell lymphoma cell line (Koh et al., 2001). *In vivo* antibody blockade prior to leukemia induction with C1498 led to increased survival (Koh et al., 2001). Considering everything we know about NK cell education and the importance of the Ly49C/I subset in licensing, in this case, the inhibitory nature of these receptors takes precedence over their educating role. However, one thing of note is the MHC-I status of the tumor cell lines utilized, with both lines expressing moderate to high levels of MHC-I, suggesting that missing-self recognition by licensed NK cell subsets is not a key requirement in this case. Self MHC-licensed NK cells are much less efficient

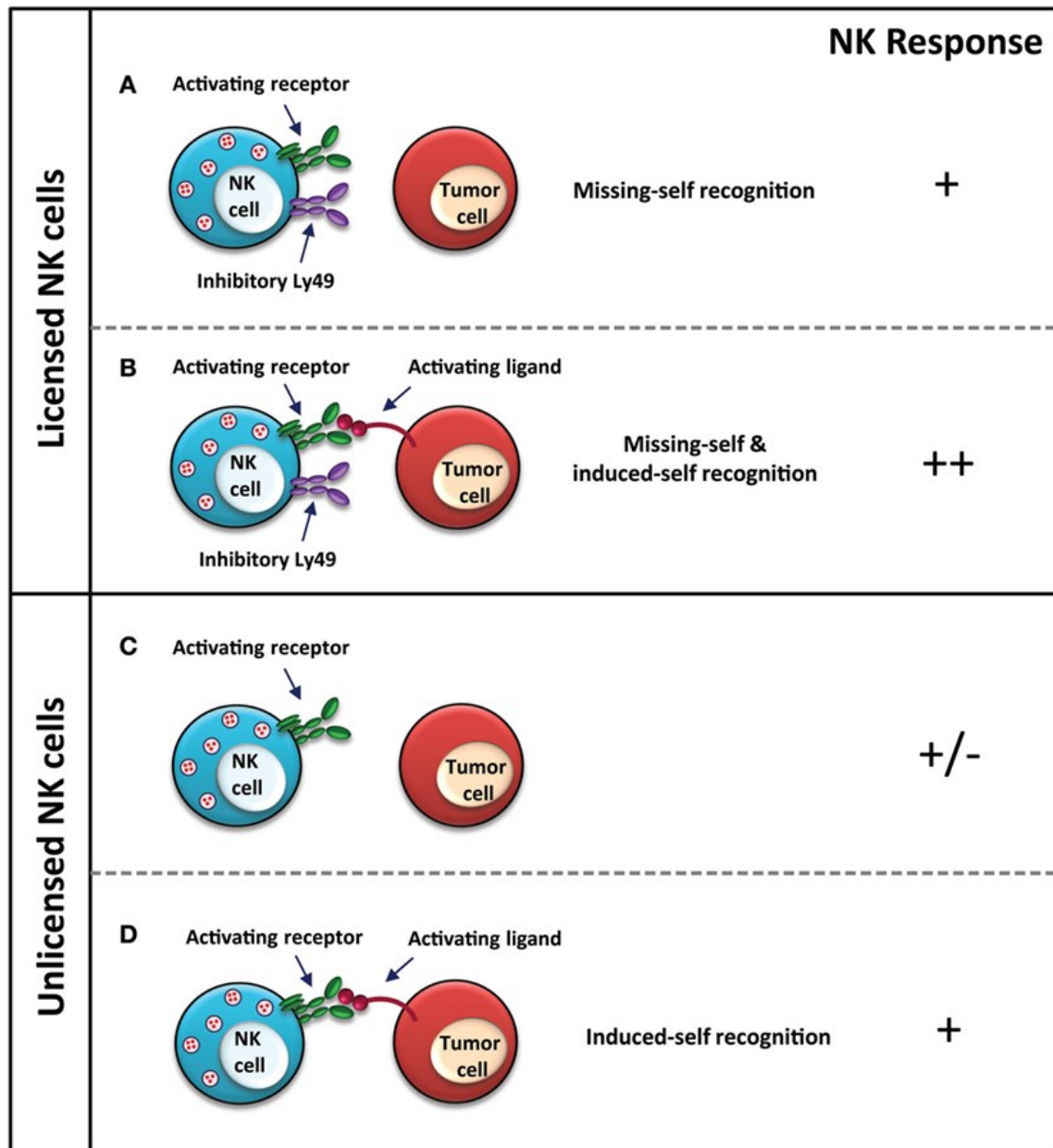


Figure 1.4: NK cell response to MHC-I-deficient tumors

Licensed NK cell recognition MHC-I-deficient tumors through ‘missing-self’ due to a lack of MHC-I expression on the tumor cell (A), as well as through ‘induced-self’ via stress ligands which are recognized by activating receptors on NK cells (B). Unlicensed NK cells are unable to recognize MHC-I-deficient tumors through ‘missing-self’ (C), however, are still functional upon activating receptor-ligand interaction for ‘induced-self’ recognition (D). NK cell response ‘-’ represents no activation, and ‘+’ represents activation. In the case of 1C, +/- represents that the response of the NK cell, whether it is activated or not, also depends upon other immune cells, receptor-ligand interactions and cytokines in the tumor microenvironment which are not depicted in the figure for brevity.

than unlicensed NK cells at responding to the MHC class I-expressing target RMA cells (Orr et al., 2010). The requirement for licensed NK cells is not as imperative in the event that the tumor cells express MHC-I, since these cells would not traditionally be recognized through missing-self. Various cancer cells, which maintain expression of MHC-I on their cell surface, while escaping immune recognition through a missing-self response can also dampen NK cell activity through engagement of inhibitory KIRs (Benson et al., 2012; Romagné et al., 2009; Vey et al., 2012). Antibody-mediated blockade of all KIR2D receptors elicits a heightened NK cell response with respect to cell cytotoxicity and has proven its efficacy in stage 1 clinical trials against acute myeloid leukemia and multiple myeloma (Benson et al., 2012, 2015; Vey et al., 2012).

In some instances, it appears that the unlicensed NK cells are more efficient at eliminating MHC-I-expressing aberrant cells (Figure 1.5). Unlicensed human NK cells, which lack inhibitory self-KIR expression, are more effective at killing neuroblastoma cells through antibody-dependent cell-mediated cytotoxicity (ADCC), following treatment with an antibody, which targets the disialoganglioside surface antigen GD2 on tumor cells (Tarek et al., 2012). The activated NK cells recognize MHC-I on the cell surface as a health marker, thus sparing the MHC-I-expressing neuroblastoma cells and concurrently selecting for the MHC-I-expressing subset. MHC-I-expressing tumor cells can inhibit licensed NK cells through the engagement of inhibitory KIRs. Unlicensed NK cells, on the other hand, are not inhibited and are better mediators of neuroblastoma cell killing *via* ADCC, which is particularly relevant in the absence of tumor-expressed NK activating ligands (Tarek et al., 2012). Therefore, unlicensed NK cells appear to be the better mediators of an anti-tumor response when the tumor cells express ligands for self-specific inhibitory NK cell receptors.

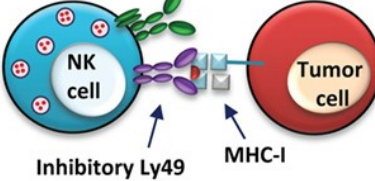
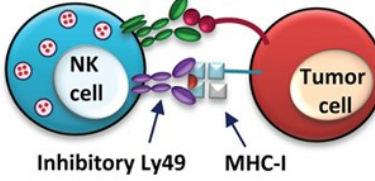
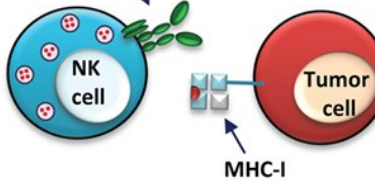
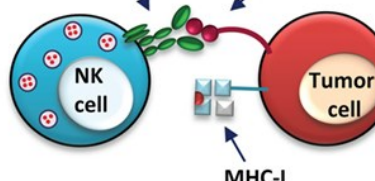
		NK Response	
Licensed NK cells	A Activating receptor  Inhibitory Ly49 MHC-I Immunevasion —		
	B Activating receptor Activating ligand  Inhibitory Ly49 MHC-I Induced-self recognition +/-		
Unlicensed NK cells	C Activating receptor  MHC-I —		
	D Activating receptor Activating ligand  MHC-I Induced-self recognition ++		

Figure 1.5: NK control of MHC-I-sufficient tumors

Licensed NK cells do not elicit a response against MHC-I-expressing tumor cells, since the presence of MHC-I is recognized as a health marker. Tumor cells have evolved various immune evasion mechanisms, such as in this case, to make them appear as a healthy 'normal' cell to NK cells by presenting MHC-I, however, this may render them susceptible still to cytotoxic T cells (A). The response to both activating and inhibitory signals, such as expression of both MHC-I and an activating stress ligand is dependent on a balance of activating and inhibitory signals which will determine the response (B). Unlicensed NK cells do not respond strongly to tumor cells which express MHC-I (C); however, a stronger response is elicited if the tumor cell expresses stress ligands recognized by the activating receptor (D). In the case of unlicensed NK cells, the response is stronger since the activating signal is not dampened by inhibitory signals via inhibitory Ly49:MHC-I interaction as in the licensed NK cells. NK cell response '-' represents no activation, and '+' represents activation.

Natural killer cells can also kill certain virus-infected and tumor cells despite their expression of MHC-I, as explained by the “induced-self” model (Leiden et al., 1989; Litwin et al., 1993; Nishimura et al., 1988). The licensed status of the NK cell, in this case, does not wholly dictate its response. Several studies have shown that unlicensed NK cells can recognize aberrant cells through the recognition of activating ligands, similar to licensed NK cells (Figures 1.4 and 1.5). The NKG2D activating receptor plays a major role in the control of both lymphoid and non-lymphoid cancers; loss of this receptor leads to increased susceptibility of oncogene-driven cancer development (Guerra et al., 2008). Expression of Rae1, the ligand for the activating NKG2D receptor, on RMA cells elicits a strong *in vivo* rejection response by unlicensed NK cells in Ly49-deficient mice (Bélanger et al., 2012). *In vitro* killing of splenocytes from Rae1 ϵ transgenic mice is comparable between licensed and unlicensed NK cells, indicating no effect of licensing in this model (Bélanger et al., 2012). Additionally, *in vitro* and *in vivo* killing of Rae1 β -expressing RMA-S cells is comparable between NK cells from wild-type mice and unlicensed NK cells from *B2m*^{-/-} and Ly49-deficient mice, suggesting that other signals, such as those from activating receptors, are able to compensate for the hyporesponsiveness of unlicensed NK cells to the loss of MHC-I expression (Bélanger et al., 2012).

NK Cell-Mediated Recognition of Virus-Infected Cells

The Ly49 receptors on NK cells also play a role in immunity against virus infections. The importance of NK cells in innate defense against viral infections was highlighted by studies which have shown severe and recurrent infections by otherwise benign viruses in individuals lacking NK cell functions (Biron et al., 1989; Orange, 2002). Viruses have developed several strategies to evade recognition of NK cells. This may occur by expression of viral proteins on infected cells that resemble host MHC-I molecules and inhibit NK cells through interaction with the inhibitory

receptors, or by down-regulation of activating ligands on the surface of infected cells (Rajagopalan and Long, 2005). The arms race between the viruses and NK cells has resulted in the selection of receptors for their ability to specifically recognize virus-infected cells. For instance, the activating Ly49H receptor on NK cells has evolved to recognize the glycoprotein m157, a MCMV-encoded structural homolog of MHC-I, on the surface of infected cells and imparts resistance in the C57BL/6 mouse strain (Figure 1.3) (Arase et al., 2002). Ly49H-deficient C57BL/6 mice and other mouse strains (129 and BALB/c), which lack the gene encoding for Ly49H, are highly susceptible to MCMV infection (Arase et al., 2002; Lee et al., 2001; Smith et al., 2002). The mutant MCMV strain that lacks m157 glycoprotein is significantly more virulent in C57BL/6 mice (Bubić et al., 2004), indicating an important role for Ly49H in host defense against MCMV. Similarly, the activating Ly49P receptor protects MA/My mice from lethal MCMV infection through the recognition of MCMV-encoded m04 proteins in association with H-2D^kMHC-I haplotype (Xie et al., 2007). Mutant MCMV strains that lack m04 are lethal in MA/My mice since this mouse also lacks the activating Ly49H (Kielczewska et al., 2009). Interestingly, Orr and colleagues have demonstrated that Ly49H⁺ NK cells that do not express inhibitory Ly49 receptors are more efficient in controlling MCMV infection than Ly49H⁺ NK cells that express inhibitory Ly49 receptors, possibly due to the lack of inhibitory signals in the former (Orr et al., 2010). Therefore, blocking inhibitory receptors on NK cells may have beneficial therapeutic effects in certain viral infections, as has been shown in cancers of humans and mice (Benson et al., 2012; Koh et al., 2001; Romagné et al., 2009; Vey et al., 2012).

Ly49 Expression and Function on Uterine NK Cells

Uterine NK cells (uNK) are resident lymphocytes of the uterus. In the non-pregnant mouse uterus, uNK cells are few and express CD122, CD49b (DX5), NKp46, CD11b, CD27, and CD69; a phenotype similar to peripheral blood and spleen NK cells (Mallidi et al., 2009; Yadi et al., 2008). These cells also express Ly49G2 (Croy et al., 2003; Kiso et al., 1992). During pregnancy, a massive influx of NK cells expressing Ly49 receptors occurs in the mouse decidualized uterus (Chiossone et al., 2014; Paffaro et al., 2003; Peel, 1989; Yadi et al., 2008). In the decidua, two subsets of uNK cells are identified based on the expression of *N*-acetyl-L-galactosamine, which can bind the *Dolichos biflorus agglutinin* (DBA) lectin: DBA⁺ and DBA⁻ subsets (Paffaro et al., 2003; Yadi et al., 2008). Both subsets are known to express Ly49 receptors (Figure 1) (Yadi et al., 2008). DBA⁻ uNK cells originate from NK progenitors cells (Lin⁻CD122⁺NK1.1⁻) and like splenic NK cells, they are the source of decidual IFN- γ . However, their frequency remains constant throughout the pregnancy (Chen et al., 2012; Chiossone et al., 2014; Yadi et al., 2008). DBA⁺ uNK cells are absent in the non-pregnant uterus, expand dramatically up to mid-gestation (gestational day 8.5–10.5), and decrease again in late pregnancy (Lima et al., 2012; Paffaro et al., 2003; Yadi et al., 2008). The DBA⁺ uNK cells have a unique receptor repertoire which includes Ly49 receptors (Yadi et al., 2008). Early in pregnancy, they perform potent vasculo-angiogenic activity demonstrating that they are active NK cells (Ashkar et al., 2000; Chen et al., 2012; Degaki et al., 2012; Hofmann et al., 2014; Lima et al., 2012; Tayade et al., 2007). The origin of DBA⁺ uNK cells is unclear, but uterus transplant experiments suggest that DBA⁺ uNK cells are recruited from the circulation (Chantakru et al., 2002).

The Ly49 receptor repertoires of DBA⁺ and DBA⁻ uNK cells differ. Ly49C/I and D are expressed at low levels and Ly49G2 is highly expressed on DBA⁺ compared to the DBA⁻ uNK cells, while Ly49A and H are expressed at similar levels (Yadi et al., 2008). They seem to play an

important role during mouse pregnancy. Antigenic disparity between parental H-2K genes (BALB/C: *d haplotype* and B6: *b haplotype*) affects the trophoblast-induced transformation of uterine vasculature (Madeja et al., 2011). In the B6 mouse strain, the trophoblast giant cells express high levels of H-2K^b and H-2D^b, the ligands for various Ly49 receptors (Johansson et al., 2009; Madeja et al., 2011; Makrigiannis et al., 2001). Our observation of pregnancy defects in the Ly49-knockdown mouse model and the works described above, suggest an important role of Ly49 receptors on uNK cells in mouse pregnancy.

In addition to their role in normal pregnancy, uNK cells can act as effector cells against infectious agents at the maternal–fetal interface. Human cytomegalovirus (HCMV) is the most common cause of intra-uterine and congenital virus infection (Cannon, 2009). Human uNK cells have been shown to display cytotoxic effector function upon recognition of HCMV-infected cells (Siewiera et al., 2013). During mouse pregnancy, MCMV infection results in vascular dysfunction of mesenteric and uterine arteries (Gombos et al., 2009). The activating Ly49H receptor is responsible for recognition and elimination of MCMV-infected cells (Arase et al., 2002). In addition, uNK cells express several vaso-active factors to regulate vaso-constriction and dilation (Hatta et al., 2011), thereby making them potential modulators during normal and infectious pregnancy complications.

Ly49 Expression and Function on NKT Cells

NKT cells are defined as CD1d-restricted T cells that express an invariant T cell antigen receptor, variable (V) and joining (J) V α 14J α 18 in mice, and V α 24J α 18 in humans, combined with a limited TCR β chain repertoire (V β 8.2, V β 7, or V β 2 in mice and V β 11 in humans). These TCR

$\alpha\beta$ -chain combinations result in NKT cells with specificity for glycolipid antigens presented by the MHC-I-related CD1d surface protein (Borg et al., 2007; Pellicci et al., 2009).

Murine TCR $\alpha\beta$ ⁺ NK1.1⁺ NKT cells are known to express other NK cell-associated molecules including CD122, CD16, DX5, CD94/NKG2, and the Ly49 family of receptors. Thus far, only inhibitory Ly49 receptors have been shown to be expressed on NKT cells (Figure 1) (Sköld et al., 2003). One reason for this could be that, forced expression of Ly49D, an activating receptor, on T cells by transgenesis results in impaired thymocyte maturation (Merck et al., 2011). It has been shown that Ly49 expression inversely correlates with their response to α -galactosylceramide (α -GalCer), a potent stimulator of CD1d-restricted NKT cells. MHC-I-dependent α -GalCer presentation by dendritic cells (DC) stimulates Ly49⁻ but not Ly49⁺ NKT cells (Maeda et al., 2001). On the other hand, MHC-I-deficient DCs effectively present α -GalCer to Ly49⁺ splenic NKT cells. When Ly49:MHC-I interactions are blocked using anti-Ly49 A, C/I, and G monoclonal antibodies (mAb), Ly49⁺ NKT cells were efficiently stimulated with α -GalCer-pulsed DCs, demonstrating inhibition of TCR-mediated activation signals by inhibitory Ly49:MHC-I interactions on NKT cells (Sköld et al., 2003). Therefore, Ly49 receptors appear to have a similar role in regulating NKT cell responses as has been described for NK cells.

Expression of Ly49 and response to α -GalCer vary greatly among NKT cells from different anatomical sites. Almost 85% of splenic CD1d-independent NKT cells from β 2m-deficient mice express Ly49A, C, G, or I, whereas only 50% of splenic NKT cells from WT mice express these Ly49 receptors (Maeda et al., 2001). In the mouse thymus, over 75% of NKT cells stain with CD1d tetramers (Matsuda et al., 2000). Similarly, Oberg and colleagues have reported that almost 70% of thymic NKT cells express Ly49A, C, G, or I (Maeda et al., 2001). Despite the high percentage of CD1d-restricted NKT cells in the thymus, thymic NKT cells are weakly stimulated with α -

GalCer-pulsed DCs as compared with splenic NKT cells. The inability of most thymic NKT cells to respond to α -GalCer does not seem to be due to inhibition via Ly49:MHC-I interaction, because α -GalCer-pulsed MHC-I-deficient DCs also fail to stimulate thymic NKT cells (Yokoyama, 1995). It seems likely that CD1d-restricted Ly49⁺ thymic NKT cells may be functionally immature and may constitute the precursors for mature CD1d-restricted NKT cells. Whereas thymic NKT cells are thought to be precursors of splenic CD1d-restricted NKT cells, the origin of NKT cells in BM is still unclear. Phenotypically, they are different from thymic NKT cells (Zeng et al., 1999), and are thought to be extra-thymically derived (Makino et al., 1993). Almost 85% of BM NKT cells express Ly49A, C, G, or I, however, only 30% of them bind CD1d tetramers (Matsuda et al., 2000). Like their thymic counterparts, BM NKT cells do not respond to α -GalCer-pulsed MHC-I-sufficient and -deficient DCs. It remains to be determined whether all CD1d-restricted BM NKT cells are Ly49⁺, and whether they are immature NKT cells belonging to a separate NKT cell lineage.

Ly49 Expression and Function on Cells of the Myeloid Lineage

While most members of Ly49 family of receptors are exclusively expressed on NK, NKT, and T cell subsets, Ly49Q and B have a unique pattern of expression and are found on distinct subsets of myeloid lineage cells (Gays et al., 2006; Toyama-Sorimachi et al., 2004, 2005). Ly49B is expressed on multiple distinct subpopulations of myeloid cells defined by CD11b and Gr-1 expression, with morphological characteristics of granulocytes and monocyte/macrophages (Gays et al., 2006). Ly49B expression was generally low in these cells and was up-regulated when cells were treated with lipopolysaccharide (LPS), IFN- α , and IFN- γ . Cells expressing Ly49B were reported to be more numerous in the Peyer's patches and the lamina propria of the gut, implicating

a role of Ly49B in gut immunobiology (Gays et al., 2006). Interestingly, Ly49B and Q were reported to be expressed on non-overlapping myeloid subpopulations among freshly isolated spleen and BM cells (Gays et al., 2006). Ly49Q is an inhibitory receptor and has been shown to associate with SHP-1/2 upon cross-linking (Toyama-Sorimachi et al., 2004). Its expression was detected on Gr-1⁺ cells in mouse fetal liver and adult BM and spleen. Expression was found to be higher on immature myeloid precursors and IFN- γ -treated macrophages (Toyama-Sorimachi et al., 2004). Ly49Q cross-linking on macrophage cell lines induces formation of cell polarity and spreading through cell cytoskeletal rearrangement (Figure 1.1) (Toyama-Sorimachi et al., 2004). Similarly, Ly49Q appears to be essential for cellular polarization and invasion of extravascular tissues by neutrophils during an inflammatory response (Figure 1.1) (Sasawatari et al., 2010). In addition, Ly49Q expression is induced on osteoclasts which differentiate from the monocyte/macrophage lineage cells by receptor activator of nuclear factor- κ B ligand (RANKL) treatment, and plays a role in osteoclastogenesis (Hayashi et al., 2010).

Another myeloid lineage cell type that expresses high levels of Ly49Q is the plasmacytoid dendritic cell (pDC) (Figure 1.1). Virtually all peripheral and the majority of BM pDCs express Ly49Q, which also correlates with the sequential development and activation of these cells (Kamogawa-Schifter et al., 2005; Omatsu et al., 2005; Toyama-Sorimachi et al., 2005). pDCs are specialized in direct virus recognition and secretion of large amounts of IFN-I early during infection (Siegal et al., 1999; Zucchini et al., 2008). Using a gene knock-out mouse model, we have shown that Ly49Q, despite being an inhibitory receptor, positively regulates Toll-like receptor (TLR)-mediated IFN-I production by pDCs (Rahim et al., 2013; Tai et al., 2008). In mice lacking Ly49Q, both TLR7 and TLR9-mediated IFN-I production by pDCs is attenuated (Tai et al., 2008). Although the precise mechanism of this positive regulation is not known, it is Ly49Q

ITIM-dependent but may not involve interaction with the SHP-1/2 phosphatases (Rahim et al., 2013). One of the possible mechanisms could be a cooperative activation of interferon regulatory factors (IRF) in conjunction with TLR-mediated downstream signals, leading to enhanced IFN-I production. Alternatively, Ly49Q may aid in the uptake of microbial macromolecules, such as unmethylated CpG oligodeoxynucleotides (ODN), and their trafficking to endosomal compartments where TLRs are located. We have demonstrated a specific down-regulation of Ly49Q from the pDC surface when treated with TLR9 agonist CpG ODN (Rahim et al., 2013). This is similar to the down-regulation and co-internalization of human KIR3DL2 with CpG ODN in NK cells (Sivori et al., 2010). A direct *in vitro* binding of CpG ODN to KIR3DL2 was also shown in this study. Co-localization of Ly49Q with CpG ODN in TLR9 containing endosome/lysosome compartments has previously been demonstrated, however it remains to be determined if Ly49Q is the sensor and distributor of CpG ODN to these compartments (Yoshizaki et al., 2009).

Ly49 Expression and Function on CD8⁺ T Cell Subsets

Although Ly49 receptors are best known as innate immune receptors, their expression is not limited to the cells of the innate immune system which we have discussed above (Figure 1.1). Here, we will shift our attention to the Ly49-expressing T cells from the CD8 lineage, cells of the adaptive immune system. This population, along with the analogous KIR-expressing CD8⁺ T cells in humans, has only recently garnered much attention.

CD8 $\alpha\alpha$ + intestinal intraepithelial lymphocytes (IEL)

Ly49-expressing CD8⁺ T cells can be further classified based on whether they express a CD8 $\alpha\alpha$ or CD8 $\alpha\beta$ co-receptor. T cells expressing CD8 $\alpha\alpha$ and Ly49 receptors were identified

among the IELs (Denning et al., 2007). Microarray analysis, confirmed by flow cytometry, comparing these cells to traditional intestinal CD8 $\alpha\beta$ T cells showed a marked increase in surface Ly49 expression, with CD8 $\alpha\alpha$ IELs possessing an Ly49 repertoire similar to – but distinct from – the NKT cells discussed above. Notably, although these IELs, like NKT cells, express predominantly inhibitory Ly49 receptors, the CD8 $\alpha\alpha$ T cells have also been shown to up-regulate Ly49D and H mRNA levels and display an increase in the activating DAP12 adaptor molecule, suggesting they may have some degree of activating Ly49 expression (Denning et al., 2007). Also, their Ly49 expression profile is remarkable based on its high levels of Ly49E and F, both of which are very rare in NK cell populations. Indeed, the bulk of CD8 $\alpha\alpha$ IELs are Ly49E and/or F positive, with the proportion of Ly49E/F double-positive cells roughly corresponding to the product of the proportions of single-positive cells (Denning et al., 2007). What role these cells may play in gut homeostasis is still unclear, although one report suggests that a subset of Ly49-expressing CD8 $\alpha\alpha$ IELs recognize the non-classical MHC-I molecule Qa-1 (HLA-E in humans) through their TCR and play an immune-regulatory role (Fanchiang et al., 2012).

CD8 $\alpha\beta$ + T cells

In both humans and mice, the CD8 $\alpha\beta$ Ly49-expressing cells are mainly found among the CD122-expressing, memory-phenotype CD8⁺ T cells: CD25⁻Ly6C⁺CD44⁺ in mice, and CD28⁻CD45RA⁻CD45R0⁺ in humans (Ugolini et al., 2001). Despite being memory-phenotype and expanding with the age of the animal, these cells are present from an early age, suggesting that they are not true memory cells but a naturally occurring subpopulation of CD8⁺ T cells (Coles and Raulet, 2000). As with NKT cells, they appear to express only inhibitory NK receptors. Additionally, like the CD8 $\alpha\alpha$ IELs, these CD8 $\alpha\beta$ T cells have non-NK-like Ly49 repertoires, in this case dominated by Ly49F expression in B6 mice (Coles and Raulet, 2000).

Attempts to discern the purpose of inhibitory Ly49 or KIR expression on these cells have thus far identified two non-exclusive candidate functions. First, and most intuitive, expression and subsequent engagement of an inhibitory MHC-I receptor down-regulates killing activities from the T cell. Transgenic expression of the inhibitory Ly49A or KIR2DL3 on T cells inhibits their ability to kill MHC-mismatched target cells, provided that those targets express a ligand for the inhibitory NK receptor (Cambiaggi et al., 1997; Zajac et al., 1999). As this role for Ly49 or KIR expression is shared between T and NK cells, it is probable that it is for the same purpose: both CD8⁺ T cells and NK cells are directly lytic to self-cells while mounting an anti-viral or anti-tumor response, and so must have a method for healthy cells to be recognized and spared to avoid rampant autoimmunity (Zajac et al., 1999). Especially in the case of regulatory T cells (discussed further below), Ly49 expression may allow for a population of CD8⁺ T cells with highly self-reactive TCRs that are regulated by inhibitory Ly49 receptors, analogous to NK immune surveillance. Such a model also suggests that T cells express only inhibitory Ly49 receptors because their TCR expression is performing a similar role as the activating receptor on the NK cell.

Interestingly, however, these inhibitory NK receptors appear to have a second function on T cells, conferring enhanced expansion and survival to the Ly49 or KIR positive cell. Although to date there is no study directly showing that Ly49 expression on mouse T cells confers enhanced IL-15 sensitivity, there are lines of evidence to suggest this. First, there is a marked correlation between Ly49 expression and IL-15 sensitivity on mouse CD8⁺ T cells (Judge et al., 2002). Using a simple CFSE proliferation assay, this report showed that, although many CD122-expressing T cells are IL-15 sensitive, those that also expressed Ly49 expanded most vigorously after IL-15 stimulation. Second, transgenic expression of a KIR on murine T cells causes those T cells, but not WT or KIR-expressing B cells, to possess a marked proliferation advantage when transferred

into mice transgenic for the KIR ligand, but not when transferred into WT mice (Ugolini et al., 2001). Taken together, these reports strongly suggest that although Ly49/KIR expression may reduce a T cell's cytotoxicity, it also enhances that cell's longevity.

CD8⁺ T cells with regulatory functions

Such a paradigm, where Ly49⁺ T cells are long-lived, broadly active, and/or highly self-reactive cells regulated by inhibitory Ly49 receptors, agrees conceptually with recent work from the Cantor lab and others demonstrating that Ly49-expressing CD8 $\alpha\beta$ ⁺ T cells include or entirely comprise a regulatory population implicated in preventing or controlling such autoimmune diseases as systemic lupus erythematosus (SLE), multiple sclerosis (MS), and rheumatoid arthritis (RA) (Hu et al., 2004; Kim et al., 2011; Leavenworth et al., 2013). Much like the proposed regulatory CD8 $\alpha\alpha$ T cells above, these CD8 $\alpha\beta$ ⁺T_{reg} cells are characterized by a TCR restricted to the non-classical MHC molecule Qa-1 (HLA-E in humans) and reacting to a peptide derived from the HSP60 leader sequence (Jiang et al., 1995). They act as indirect regulators of antibody production, by targeting the Qa-1-expressing follicular helper T cells (T_{FH}) and lysing them in a perforin-dependent manner (Kim et al., 2010). Employing a strategy not unlike the NK cell use of inhibitory and activating Ly49, these CD8⁺ T_{reg} cells express NKG2A, an inhibitory NK receptor that recognizes Qa-1 presenting its dominant peptide, Qdm, which normally prevents their killing of T_{FH} cells. However, in cases of overactive T_{FH} giving rise to excessive antibody production by plasma cells, a shift toward more HSP60sp being presented by Qa-1, allows the CD8 T_{reg} cell's TCR to compete with NKG2A and transmit an activating signal to the CD8⁺ T_{reg} cell. In a recent report, it was found that disrupting the ability for Qa-1 to interact with the CD8⁺ T_{reg} cell's TCR – but not disrupting the NKG2A interaction – caused a massive expansion in T_{FH} and plasma cell numbers, splenic germinal center area, and serum and kidney antibody deposition, analogous to

that seen in SLE-susceptible mice (Kim et al., 2010). Furthermore, adoptive transfer of these CD8⁺ T_{reg} cells into Rag^{-/-} mice with a reconstituted CD4⁺CD25⁻ T cell and IgM⁺ B cell compartment results in a suppression of antibody production upon immunization with NP, which appears to be mediated by the Ly49⁺, but not Ly49⁻ CD8⁺ T_{reg} cells (Kim et al., 2010). While no studies have yet shown a direct role for Ly49 in this regulation, the dual functions discussed above fit well with this model of regulation. On the one hand, the longevity and antigen-independent, IL-15-driven activation and proliferation of these T cells allows for their continuous presence and immunosurveillance within the organism in question; at the same time, the limiting effect of Ly49 and NKG2A, coupled with the scarcity of this CD8⁺ T_{reg} population, ensures that enough antibody is allowed to be produced to protect the host from infection.

Chapter 2 : Optimized tetramer analysis reveals Ly49 promiscuity for MHC ligands

Preface

The following chapter consists of original research looking at the interactions between different Ly49 receptors and their MHC-I ligand. This chapter has been previously published as a research article:

McFall E*, Tu MM*, Al-Khattabi N, Tai L-H, St-Laurent AS, Tzankova V, Hall CW, Belanger S, Troke AD, Wight A, Mahmoud AB, Zein HS, Rahim MM, Carlyle JR, and Makrigiannis AP. Optimized tetramer analysis reveals Ly49 promiscuity for MHC ligands. *Journal of Immunology*. 2013; 191(11):5722-9. *equal contribution

The specific contributions of each author to this paper are listed below:

McFall E: Performed the original tetramer binding experiments without acid treatment. Transfected some of the cell lines used. Created the tables.

Tu MM: Performed the tetramer binding experiments, with and without acid treatment. Contributed to the writing/editing of the manuscript

Tai LH: Performed the Ly49Q experiments in Figure 1.

Al-Khattabi, St-Laurent AS, Tzankova V, Hall CW, Belanger S, Troke AD, and Rahim MM: Performed the cloning and transfections of the cell lines used. Rahim MM also contributed to the editing of the manuscript.

Wight A, Mahmoud AB, and Zein HS: Performed western blot experiments which were ultimately removed from the final manuscript and so not included in this chapter. Wight A also made modifications to the tables during revision.

Carlyle JR: A collaborator at the University of Toronto who provided reagents needed

Makrigiannis AP: Supervised experimental design and execution. Contributed to the writing/editing of the manuscript

Abstract

Murine Ly49 receptors, which are expressed mainly on NK and NKT cells, interact with MHC class I (MHC-I) molecules with varying specificity. Differing reports of Ly49/MHC binding affinities may be affected by multiple factors, including *cis* versus *trans* competition and species origin of the MHC-I L chain (β_2 -microglobulin). To determine the contribution of each of these factors, Ly49G, Ly49I, Ly49O, Ly49V, and Ly49Q receptors from the 129 mouse strain were expressed individually on human 293T cells or the mouse cell lines MHC-I-deficient C1498, H-2^b-expressing MC57G, and H-2^k-expressing L929. The capacity to bind to H-2D^b- and H-2K^b-soluble MHC-I tetramers containing either human or murine β_2 -microglobulin L chains was tested for all five Ly49 receptors in all four cell lines. We found that most of these five inhibitory Ly49 receptors show binding for one or both self-MHC-I molecules in soluble tetramer binding assays when three conditions are fulfilled: 1) lack of competing *cis* interactions, 2) tetramer L chain is of mouse origin, and 3) Ly49 is expressed in mouse and not human cell lines. Furthermore, Ly49Q, the single known MHC-I receptor on plasmacytoid dendritic cells, was shown to bind H-2D^b in addition to H-2K^b when the above conditions were met, suggesting that Ly49Q functions as a pan-MHC-Ia receptor on plasmacytoid dendritic cells. In this study, we have optimized the parameters for soluble tetramer binding analyses to enhance future Ly49 ligand identification and to better evaluate specific contributions by different Ly49/MHC-I pairs to NK cell education and function.

Introduction

Natural killer cells are lymphocytes that play a major role in the innate immune system as illustrated by individuals with NK cell deficiency, who suffer from otherwise benign viral infections (Orange, 2006). NK cells travel throughout the body via the blood and lymphatics and kill abnormal cells, including cancerous cells and pathogen-infected cells. NK cell killing is regulated via receptor binding to constitutively expressed or induced surface proteins on host cells (Lanier, 2005). Unlike T and B cells, NK cells do not undergo gene rearrangement during development to acquire receptor diversity and specificity. These innate responses are regulated by a large array of surface receptors, some of which are stochastically expressed (Raulet et al., 2001). NK cells have different types of receptors that can be either activating or inhibitory. Ligand binding by activating receptors will lead to killing of target cells through the release of cytotoxic granules containing granzymes and perforin, whereas inhibitory receptors prevent granule release, leading to host cell survival. Cytokine secretion is similarly regulated by such receptors. Because multiple receptors with opposing function are expressed simultaneously, the action of NK cells depends on the balance between these activating and inhibiting signals. These receptors include CD94/NKG2, NKR-P1, PIR, killer cell Ig-like receptor (KIR), Ly49, and others. Certain receptors are particular to specific species such as KIRs found in humans and Ly49, the functional analog found in mice. KIRs are type I transmembrane glycoproteins composed of Ig-like domains encoded by genes located in the leukocyte receptor complex.

In contrast to KIR, Ly49 are type II transmembrane glycoproteins and are expressed as homodimers linked by four disulfide bonds. *Ly49* genes are composed of seven exons and encode the following domains: exon 1 is noncoding, exon 2 forms the cytoplasmic domain, exons 3 and 4

form the transmembrane domain and α -helix stalk region, respectively, whereas exons 5, 6, and 7 form the C-type lectin-like extracellular domain (NK recognition domain). The NK recognition domain is composed of two α -helices and two antiparallel β -sheets. It is the main region of the Ly49 receptor that interacts with ligand; however, the extracellular stalk may also be involved in binding (Lian et al., 1998; Mason et al., 2003). The stalk in some Ly49 has conserved glycosylation sites, which when glycosylated lead to steric hindrance and decreased binding to MHC-I (Mason et al., 2003).

Ly49 are expressed mainly on NK cells, NKT cells, and a small percentage of activated CD8⁺T cells. Additionally, two Ly49, Ly49B and Ly49Q, are expressed on myeloid-derived cells, including macrophages, neutrophils, and DCs (Gays et al., 2006; Toyama-Sorimachi et al., 2004). Initial cDNA screening of inbred mouse strains revealed extreme genetic diversity and polymorphism in different mouse strains (Makrigiannis and Anderson, 2000), which was later revealed to be a result of genomic plasticity within the Ly49 region resulting in cluster sizes ranging from 8 to 22 Ly49 genes (Anderson et al., 2005; Belanger et al., 2008). Most Ly49 receptors identified to date are inhibitory and contain a cytoplasmic ITIM domain encoded in exon 2 that becomes phosphorylated during ligand binding. Src homology region 2 domain-containing phosphatase 1 is recruited, activated, and ultimately dephosphorylates tyrosine residues in kinases, leading to signal termination (McVicar and Burshtyn, 2001). Dampening of NK cell function ultimately results in target cell survival. Paradoxically, NK cell licensing and functional potential depend on the expression of self-MHC-I receptors during development (Fernandez et al., 2005; Kim et al., 2005). In agreement with these education models, the absence of Ly49 expression by NK cells in gene-targeted mice results in lack of self-MHC-I education and the loss of the ability to kill MHC-I-deficient cells (Bélanger et al., 2012).

Crystallography studies revealed that Ly49A interacts with the side of the MHC-I molecule, H-2D^d, at the α_1 and α_2 domains and also associates with the β_2 -microglobulin (β_2m) L chain, but it binds beneath the peptide-binding platform itself (Matsumoto et al., 2001; Tormo et al., 1999). Because NK cells also express MHC-I molecules it is possible for Ly49 to interact with MHC-I on the same cell, as has been shown for Ly49A and H-2D^d (Doucey et al., 2004). This is referred to as *cis* interaction, whereas binding to MHC-I on another cell is a *trans* interaction. *Cis* and *trans* interactions have two distinct receptor conformations made possible due to the flexible stalk; the stalk is able to bend into a back-fold conformation allowing for *trans* binding and straightens into an extended conformation for *cis* interactions (Back et al., 2009). *Cis* interactions are stable and sequester Ly49A so that most receptors are not available for MHC-I binding in *trans* (Back et al., 2007). The end result is that the NK cell is more sensitive to small changes in MHC-I levels on target cells and ultimately lowers the activation threshold (Doucey et al., 2004). Finally, using a Ly49A mutant with an inflexible stalk domain, it was shown that *cis* binding is essential for the ability of Ly49A to educate NK cells (Chalifour et al., 2009).

The objective of this study was to determine the full potential of binding between Ly49 from 129 strain inbred mice and self-MHC-I. The putative functional inhibitory Ly49 in the 129 strain mice includes Ly49E, Ly49EC₂, Ly49G, Ly49I₁, Ly49O, Ly49S, Ly49T, Ly49Q₁, and Ly49V. No mAbs have been produced for Ly49EC₂ and Ly49S, although the open reading frames are intact and the proteins are likely expressed, and *Ly49ec₁*, *Ly49i₂*, *Ly49q₂*, and *Ly49q₃* are pseudogenes in 129 strain mice due to early stop codons and/or missing exons (Makrigiannis et al., 2005). Previously, the Ly49 from 129 strain mice were tested for binding to seven different MHC-I molecules using 293T as the host cell type expressing the Ly49 proteins and soluble mouse MHC-I tetramers containing human β_2m (Makrigiannis et al., 2001). Binding was observed for

Ly49G:D^d, Ly49I:K^d, Ly49O:D^d/L^d, and Ly49V:D^b/D^d/D^k/L^d (Makrigiannis et al., 2001). Similarly, using lacZ-based reporter cell assays, as well as soluble MHC-I tetramers, it was previously reported that the plasmacytoid dendritic cell (pDC)–expressed Ly49Q displays strong H-2K^b binding, but apparently no binding to H-2D^b or MHC-I alleles expressed in cells from mice possessing H-2^{a,q,k,d} MHC haplotypes (Tai et al., 2007). However, since these initial studies there have been new advances in our knowledge of Ly49/MHC-I interactions in the B6 inbred mouse strain, including the role of *cis* versus *trans* competitive binding and loss of binding observed when the whole MHC-I tetramer is not mouse derived (Michaëlsson et al., 2001).

In light of new knowledge concerning B6-derived Ly49/MHC-I interactions, two questions have been raised. First, are 129-derived Ly49/MHC-I interactions also affected when the MHC-I tetramer is composed of a human β_2m L chain compared with a mouse β_2m ? Second, what is the contribution of *cis* interactions to 129-derived Ly49/MHC-I binding? In the present study five inhibitory receptors (Ly49G, Ly49I, Ly49O, Ly49Q, and Ly49V) derived from 129 strain inbred mice were tested for binding to the self–MHC-I molecules in 129 strain mice (H-2D^b and H-2K^b) using MHC-I tetramers containing either human or mouse β_2m . Additionally, Ly49 were expressed in four different cell lines varying in their expression of MHC-I, as well as species origin, to resolve the above questions.

Materials and Methods

Mice

H2D^{b-/-} and *H2K^{b-/-}* mice on a B6 background were purchased from Taconic Farms (Albany, NY). All breeding and manipulations performed on animals were in accordance with university guidelines and approved by the University of Ottawa Animal Ethics Committee.

Cells

BWZ.36 cells were obtained from Dr. N. Shastri (University of California, Berkeley, CA). Construction of the CD3 ζ /NKR-P1B/Ly49Q chimeric receptor and BWZ assay was described previously (Tai et al., 2007). L929 was developed from s.c. areolar and adipose tissue fibroblasts of a C3H/An mouse and is MHC-I⁺ for the H-2^k haplotype. YB2/0 (rat hybridoma) and YB.D^d, YB.D^b, YB.D^k, YB.K^k, and YB.L^d stable transfectants were provided by Dr. Stephen Anderson (SAIC-Frederick, Frederick, MD). YB.K^b/D^d was a gift of Dr. Kevin Kane (University of Alberta, Edmonton, AB, Canada). The coding region of H-2K^b and H-2D^b was amplified and cloned into the pEF6-TOPO vector (Invitrogen) and then used to make stable transfectants of L929 via lipofectamine (Invitrogen). MC57G cells are MHC-I⁺ (H-2^b) and are derived from B6 fibroblasts. MC57G was obtained from Dr. W.-K. Suh (Institut de Recherches Cliniques de Montréal, Montreal, QC, Canada) and 293T cells (human embryonic kidney epithelial) were obtained from Dr. M.-A. Langlois (University of Ottawa). DCs were isolated from collagenase (Roche Diagnostics, Laval, QC, Canada)-treated splenocytes using anti-CD11c-conjugated microbeads (Miltenyi Biotec, Auburn, CA). MHC-I-deficient C1498 cells are a derivative of the C1498 acute myeloid leukemia cells of B6 origin and were previously produced in our laboratory (Bélanger et al., 2012).

Acid treatment

Cells were acid-treated as previously described with minor modifications (Doucey et al., 2004). Cells were washed twice with PBS and resuspended at room temperature in citrate buffer (0.133 M citric acid and 0.066 M Na₂HPO₄ [pH 3.3]) at a density of 2×10^6 cells/ml. Acid treatment was stopped after 4 min with 40 ml 5% heat-inactivated FBS in PBS. Cells were washed with PBS once more and subsequently stained with tetramers as described below. Cell viability was not adversely affected by acid treatment as assessed by trypan blue exclusion and forward and side scatter analysis.

Abs and soluble MHC-I tetramers

mAb staining was carried out using FITC-labeled 4D11 (binds to Ly49G in 129 strain mice) ([eBioscience](#)), FITC-labeled 4E5 (Ly49O and Ly49V) (BD Pharmingen), PE-labeled 14B11 (Ly49I) ([eBioscience](#)), and purified or biotinylated NS-34 (Ly49Q), which was a gift from Dr. Noriko Toyama-Sorimachi (International Medical Center, Tokyo, Japan). Secondary mAbs used for flow cytometry included PE-labeled goat anti-rat IgG or PE-labeled streptavidin. All Ab testing was carried out with 0.25 µg of the given Ab. Streptavidin-PE–conjugated murine class I MHC tetramers H-2D^b and H-2K^b were provided by the National Institutes of Health Tetramer Core Facility at Emory University (Emory University Vaccine Center, Atlanta, GA). Tetramers were refolded in the presence of murine β₂m L chain and a peptide previously shown to form a stable tetrameric structure, and are as follows: H-2D^b, gp_{33–41}(KAVYNFATC) of lymphocytic choriomeningitis virus; H-2K^b, OVA_{257–264} (SIINFEKL) of chicken OVA. PE-conjugated D^b and K^b tetramers containing human β₂m L chain were purchased from Beckman Coulter (Fullerton, CA). Staining reactions included 20% normal rat/mouse serum (Sigma-Aldrich, Oakville, ON,

Canada) or 10 $\mu\text{g/ml}$ 2.4G2 (anti-CD16/32; BD Pharmingen) mAb for blocking nonspecific binding. No differences in staining were seen when either 2.4G2, rat, or mouse serum was used, as judged by the mean fluorescence intensity (MFI) of the staining (data not shown). Cells ($n = 250,000$) were counted for each cell line and incubated at 4°C with 2.4G2. Staining was then carried out with the mAb or tetramers listed above for 20 min at 4°C or 37°C , respectively. Washing (2 ml FACS buffer [0.5% BSA and 0.2% sodium azide in PBS]) and centrifugation at $500 \times g$ for 5 min was performed at the beginning, between mAb stainings, and at the end of the experiment. Ten thousand events were collected (excluding dead cells) during flow cytometry, and experiments were repeated a minimum of three times. Flow cytometry was performed on a CyAN-ADP using Summit software (Beckman Coulter). Data were analyzed with Kaluza software.

Results

BWZ.Ly49Q reporter cells are stimulated by cells expressing the $\alpha 1\alpha 2$ domains of H-2K^b, but not H-2D^b

We have previously shown via BWZ reporter cell assays and soluble tetramer analyses that H-2K^b is a strong ligand for Ly49Q (Tai et al., 2007), and via gene-knockout mice that this interaction is key for the production of IFN- α by pDCs (Tai et al., 2008). In particular, BWZ.Ly49Q but not BWZ.36 parental reporter cells were stimulated by cells of the H-2^b haplotype, as well as immobilized recombinant H-2K^b. However, BWZ.Ly49Q reporter cells did not respond to cells derived from non-H-2^b mice, *B2m*^{-/-}, *H-2K^b*^{-/-}*H-2D^b*^{-/-} mice, nor to recombinant H-2D^b (Tai et al., 2007). To further test the selective binding of Ly49Q we decided to “rescue” a nonstimulating tumor cell, specifically L929 fibroblasts, which bear H-2^k MHC-I molecules, by transfection of H-2K^b or H-2D^b. As previously reported, L929 tumor cells are unable to stimulate lacZ production by BWZ.Ly49Q^{BALB} or BWZ.Ly49Q^{B6} reporter cells (Tai et al., 2007). However, stable expression of H-2K^b, but not H-2D^b, in L929 was able to stimulate both types of BWZ.Ly49Q reporter cells, but not parental BWZ.36 parental cells, in line with previous findings (Fig. 2.1A). Two different clones of each transfectant were tested and showed similar results. PMA plus ionomycin treatment of all reporter cell lines showed that each was functional.

Ligand-bearing DCs were previously shown to be good stimulators of the T cell-derived BWZ cells (Tai et al., 2007). In agreement with L929 transfectants, we found that DCs from *H-2D^b*^{-/-} mice were able to stimulate BWZ.Ly49Q reporter cells, but *H-2K^b*^{-/-} DCs were lacking in this respect (Fig. 2.1B). Finally, to test a larger panel of MHC-I transfectants and to localize

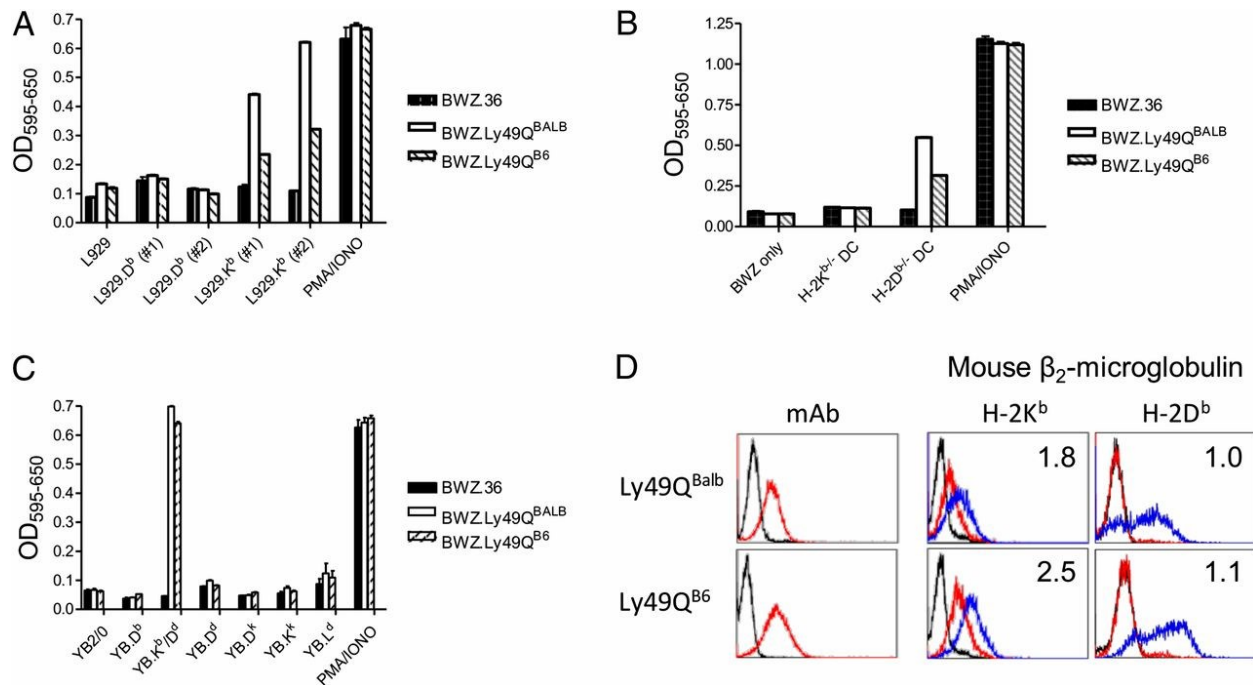


Figure 2.1: Ly49Q-CD3 ζ fusion receptor-bearing BWZ cells are stimulated by cells expressing the $\alpha_1\alpha_2$ domain of H-2K^b, but not H-2D^b

(A) BWZ.36 parental cells or transductants expressing a chimeric protein (introduced by a retrovirus containing IRES-GFP) with the extracellular domain of either B6 or BALB/c Ly49Q proteins were incubated with parental L929 mouse fibroblasts or two separate stable transfectant clones expressing either H-2K^b or H-2D^b. (B) CD11c⁺ DCs isolated from the indicated gene-knockout mouse strains were coincubated with BWZ reporter cells. (C) Parental and Ly49Q-CD3 ζ fusion receptor-transduced BWZ reporter cells were incubated with parental rat YB2/0 cell line or stable transfectants expressing the indicated mouse H-2 molecules. The YB.K^b/D^d cell line expresses an H-2 fusion molecule composed of the $\alpha_1\alpha_2$ domains of H-2K^b and the α_3 /transmembrane/cytosolic domains of H-2D^d. After overnight incubation, lacZ production was revealed by addition of chlorophenol-red- β -D-galactopyranoside substrate and OD measurements at the indicated wavelengths. Maximum lacZ production capability by BWZ cells is shown by PMA/ionomycin treatment. (D) BWZ.36 parental cells and transductants expressing either B6 or BALB/c Ly49Q proteins were briefly treated with mild acid to disrupt class I MHC/peptide interactions and then stained with specific PE-conjugated mAb or PE-conjugated MHC-I tetramers (H-2K^b or H-2D^b) containing mouse β_2 m L chain. After mAb or tetramer staining, cells were analyzed by flow cytometry. BWZ.36 parental staining levels are shown in black; specific staining of stable Ly49 transfectants before and after acid treatment are shown in red and blue, respectively. Average MFI fold increase in the non-acid-treated transfectants over the parentals is indicated. Data are representative of three independent experiments.

the domains necessary for Ly49Q binding, a panel of mouse MHC-I stable transfectants of the rat hybridoma YB2/0 were used as stimulator cells. H-2D^b, H-2D^d, H-2D^k, H-2K^k, and H-2L^d were not able to stimulate lacZ production by Ly49Q reporter cells; however, a fusion MHC-I composed of the $\alpha_1\alpha_2$ domains of H-2K^b and the α_3 , transmembrane, and cytosolic domains of H-2D^d efficiently stimulated both types of BWZ.Ly49Q reporter cells (Fig. 2.1C). These results reinforce our previous findings that in this assay Ly49Q appears to be exquisitely specific for a single type of MHC-I, but also extend our findings and show that $\alpha_1\alpha_2$, but not α_3 , domains are sufficient for Ly49Q binding.

Two troubling matters were perceived: 1) it is not intuitive that pDCs would express a receptor that is vital for their function, but that only binds a ligand found in some mouse strains, and 2) the reporter cells themselves express H-2K^k and H-2D^k molecules that may interfere with the assay via *cis* competition, as has been shown for Ly49Q and other Ly49 (Doucey et al., 2004; Tai et al., 2007), or this may cause selection of reporter cells that only respond to very strong receptor/ligand binding, assuming that H-2K^k/D^k/D^b binding is less strong than Ly49Q binding to H-2K^b. We made several attempts to produce MHC⁻ variants of BWZ.36 cells to test this hypothesis by chemical mutagenesis followed by anti-MHC-I staining and cell sorting, but each time the resulting MHC-I⁻ BWZ.36 variants did not have functional lacZ responses to PMA/ionomycin. We next tried to resolve these questions using soluble MHC-I tetramers.

Ly49Q binds to H-2Db tetramers containing mouse β_2m in the absence of *cis* interactions

We previously reported that H-2K^b tetramers refolded with human β_2m L chain bound to mouse pDCs and that this binding could be blocked with anti-Ly49Q mAb (Tai et al., 2007). Furthermore, tetramer binding was significantly increased on pDCs from *H-2K^b^{-/-}H-2D^b^{-/-}* mice,

suggesting that Ly49Q on pDCs can bind to H-2K^b in both *cis* and *trans* (Tai et al., 2007). Thus, it may be possible that *cis* interactions with MHC-I inhibit *trans* binding of Ly49Q to MHC-I, as reported for other Ly49 (Scarpellino et al., 2007), and possibly explaining the inability of Ly49Q to bind H-2D^b in BWZ reporter cell assays. To investigate this possibility, we treated BWZ cells expressing Ly49Q with mild acid to eliminate possible *cis* interactions and tested binding of H-2K^b and H-2D^b tetramers containing mouse β_2m . In accordance with the reporter assay data, H-2K^b but not H-2D^b tetramer bound to Ly49Q. However, upon mild acid treatment to disrupt *cis* interactions, H-2D^b tetramer was also able to bind to Ly49Q, suggesting that both H-2K^b and H-2D^b are able to bind Ly49Q in the absence of any *cis* interactions (Fig. 2.1D). As well, we generated Ly49Q¹²⁹ stable transfectants of an MHC-I⁻ variant of the mouse B cell line, C1498, and then stained them with soluble H-2K^b and H-2D^b tetramers containing human β_2m . The lack of MHC-I expression in this C1498 derivative will not allow for *cis* interactions. As previously reported, H-2D^b/human β_2m tetramers failed to interact with Ly49Q¹²⁹ even in the absence of possible *cis* interactions, whereas H-2K^b/human β_2m tetramers bound strongly to C1498.Ly49Q cells compared with parental C1498 cells (Fig. 2A, *middle panel*). There was no binding of either tetramer to the parental C1498 line. It has been previously reported that the species origin of the β_2m L chain affects Ly49/MHC-I binding (Michaëlsson et al., 2001; Scarpellino et al., 2007). To determine whether this was a factor in Ly49Q/MHC-I binding, the experiment was repeated but with tetramers containing mouse β_2m . This time, both H-2K^b/mouse β_2m and H-2D^b/mouse β_2m tetramers showed positive staining of C1498.Ly49Q¹²⁹ cells (Fig. 2.2A, *right panel*), and, additionally, H-2K^b tetramer binding was increased. As expected, owing to the absence of *cis* interactions in the MHC-I-deficient variant of C1498, acid treatment did not affect tetramer staining levels as judged by the overlapping

Cells: mouse C1498-MHC-I-deficient

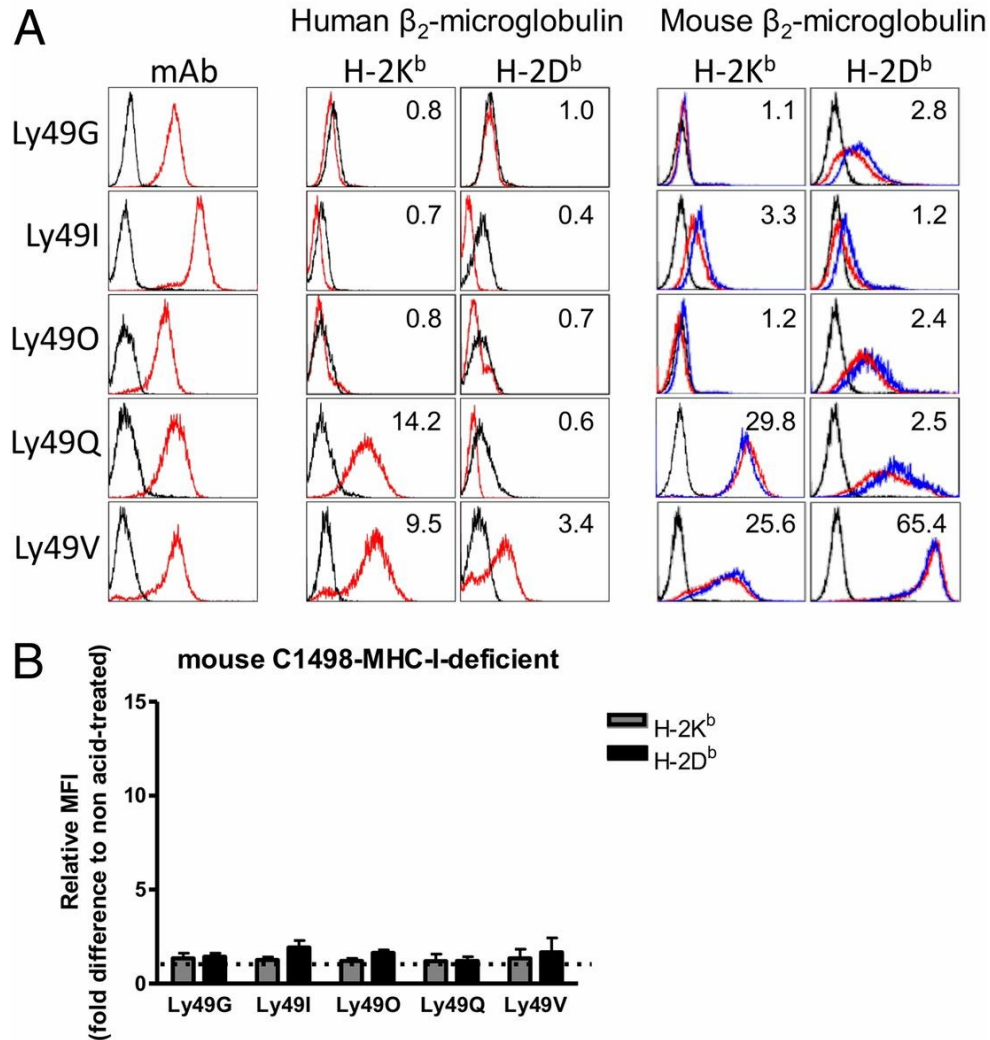


Figure 2.2: Enhanced Ly49 binding to MHC-I molecules is observed in the absence of *cis* interactions using tetramers containing mouse β_2 m L chain

An MHC-I-deficient derivative of the C1498 cell line was stably transfected with the indicated Ly49 from 129 strain mice, treated with mild acid to disrupt class I MHC/peptide interactions, and then stained with specific PE- or FITC-conjugated mAb or PE-conjugated MHC-I tetramers (H-2K^b or H-2D^b) containing human (*middle panel*) or mouse (*right panel*) β_2 m L chain. After mAb or tetramer staining, cells were analyzed by flow cytometry. **(A)** C1498 parental staining levels are shown as black histograms, and specific staining of stable Ly49 transfectants before and after acid treatment are shown as red and blue histograms, respectively. Average MFI fold increase in the non-acid-treated transfectants over the parentals is indicated. **(B)** Quantification of binding of MHC-I tetramers (H-2K^b or H-2D^b) containing mouse β_2 m L chain to acid-treated C1498-MHC-I-deficient cells transfected with the indicated Ly49. Relative MFI indicates the fold increase in binding following acid treatment compared with no treatment (dotted line). Means $\pm$ SEM of two to three independent experiments are shown.

histograms for nontreated and acid-treated cells (Fig. 2.2A, *right panel*), with negligible quantified differences in MFI of acid-treated relative to non-acid-treated (Fig. 2.2B). Overall, these results show that under optimized conditions, Ly49Q can bind to MHC-I molecules other than H-2K^b.

We previously tested the MHC-I specificity of the 129 strain repertoire of Ly49 molecules using tetramers containing human β_2m (Makrigiannis et al., 2001). To determine whether some interactions were previously missed because of this factor, a simultaneous comparison was performed of the ability of human versus mouse β_2m -containing H-2K^b and H-2D^b tetramers to bind to MHC-I-deficient C1498 cells stably transfected with Ly49G¹²⁹, Ly49I¹²⁹, Ly49O¹²⁹, and Ly49V¹²⁹. Expression levels of the Ly49 molecules are shown with specific mAbs (Fig. 2A). Positive staining with human β_2m -containing tetramers was only observed for Ly49Q (H-2K^b) and Ly49V (H-2K^b and H-2D^b), as previously reported (Makrigiannis et al., 2001; Tai et al., 2007). In contrast, a larger number of interactions were identified with tetramers containing mouse β_2m : Ly49G (H-2D^b), Ly49I (H-2K^b), Ly49O (H-2D^b), Ly49Q (H-2K^b and H-2D^b), and Ly49V (H-2K^b and H-2D^b). Furthermore, the binding of MHC-I/mouse β_2m tetramers to Ly49V, similar to Ly49Q, was greater than that seen with MHC-I/human β_2m tetramers, and weak but reproducible binding of Ly49O:K^b and Ly49I:D^b was observed. The binding ability of MHC-I tetramers containing either human or mouse β_2m to Ly49-transfected MHC-I-deficient C1498 cells is summarized in Table 2.1. These results confirm and extend prior reports that the presence of human β_2m in mouse MHC-I molecules inhibits binding by Ly49. In particular, these results demonstrate that pDCs have the ability to bind to MHC-I alleles other than H-2K^b.

Table 2.1: Enhanced Ly49 binding with MHC-I tetramers containing mouse β_2m

	C1498-MHC-Deficient ^a			
	Human β_2m^b		Mouse β_2m	
	K ^b	D ^b	K ^b	D ^b
Ly49G	— ^c	—	—/+	+
Ly49I	—	—	++	—/+
Ly49O	—	—	—/+	+
Ly49Q	+++	—	+++	+
Ly49V	+++	++	+++	+++

^a Summary of binding of soluble MHC-I tetramers to MHC-I-deficient C1498 cell lines stably transfected with five different Ly49.

^b Species origin of the L chain in soluble PE-conjugated MHC-I tetramers.

^c The fold increase of observed tetramer binding (MFI) in transfected cells relative to untransfected parental C1498 cells. MFI ratios are indicated as follows: —, ≤ 1 ; —/+, 1.1–1.5; +, 1.6–3; ++, 3.1–5; +++, >5.

***Cis* interactions with H-2^b and H-2^k MHC-I molecules inhibit Ly49 *trans* binding to soluble MHC-I tetramers**

Testing the MHC-I tetramer binding capacity of Ly49 expressed on an MHC-I-deficient cell line in Fig. 2.2 represented a “best case scenario,” as it has been previously reported that *cis*Ly49/MHC-I interactions inhibit *trans* MHC-I binding by Ly49A^{B6}, Ly49C^{B6}, and Ly49I^{B6} (Scarpellino et al., 2007). To determine whether *cis* interactions can also inhibit MHC-I binding by Ly49 from 129 strain mice, the tetramer binding experiments shown in Fig. 2.2 were repeated in the MHC-I-sufficient MC57G fibrosarcoma cell line isolated from B6 mice, which expresses H-2K^b and H-2D^b (data not shown). Overall, the binding of MHC-I tetramers to MC57G-expressing Ly49 was absent or very low compared with MHC-I-deficient C1498 cells (Fig. 3A). Tetramers containing human β_2m only showed weak binding for Ly49Q (H-2K^b) and Ly49V (H-2K^b) (Fig. 2.3A, *middle panel*). In comparison, tetramers containing mouse β_2m displayed only slightly stronger binding of the same Ly49 and, additionally, Ly49V binding of H-2D^b could be detected (Fig. 2.3A, *right panel*). These results are summarized in Table 2.2. This observed reduction in binding of the MHC-I tetramers in MC57G was confirmed to be due to Ly49/MHC-I *cis* interactions because acid treatment of the Ly49 transgenic MC57G cells resulted in increased binding (Fig. 2.3A, *middle panel*, 3B). Notably, major increases were seen for Ly49G (H-2D^b), Ly49O (H-2D^b), Ly49Q (H-2D^b), and Ly49V (H-2K^b and H-2D^b). Therefore, the ability of 129 strain Ly49 to bind MHC-I (containing either human or mouse β_2m) in *trans* is inhibited by *cis* interactions with self-MHC-I.

The inability of BWZ.Ly49Q reporter cells to be stimulated by H-2D^b is suggested to be due to inhibition of binding via *cis* interactions with H-2K^k and/or H-2D^k (Fig. 2.1D). To test this hypothesis, the tetramer binding analysis was repeated with the C3H.He-derived (H-2^k) L929

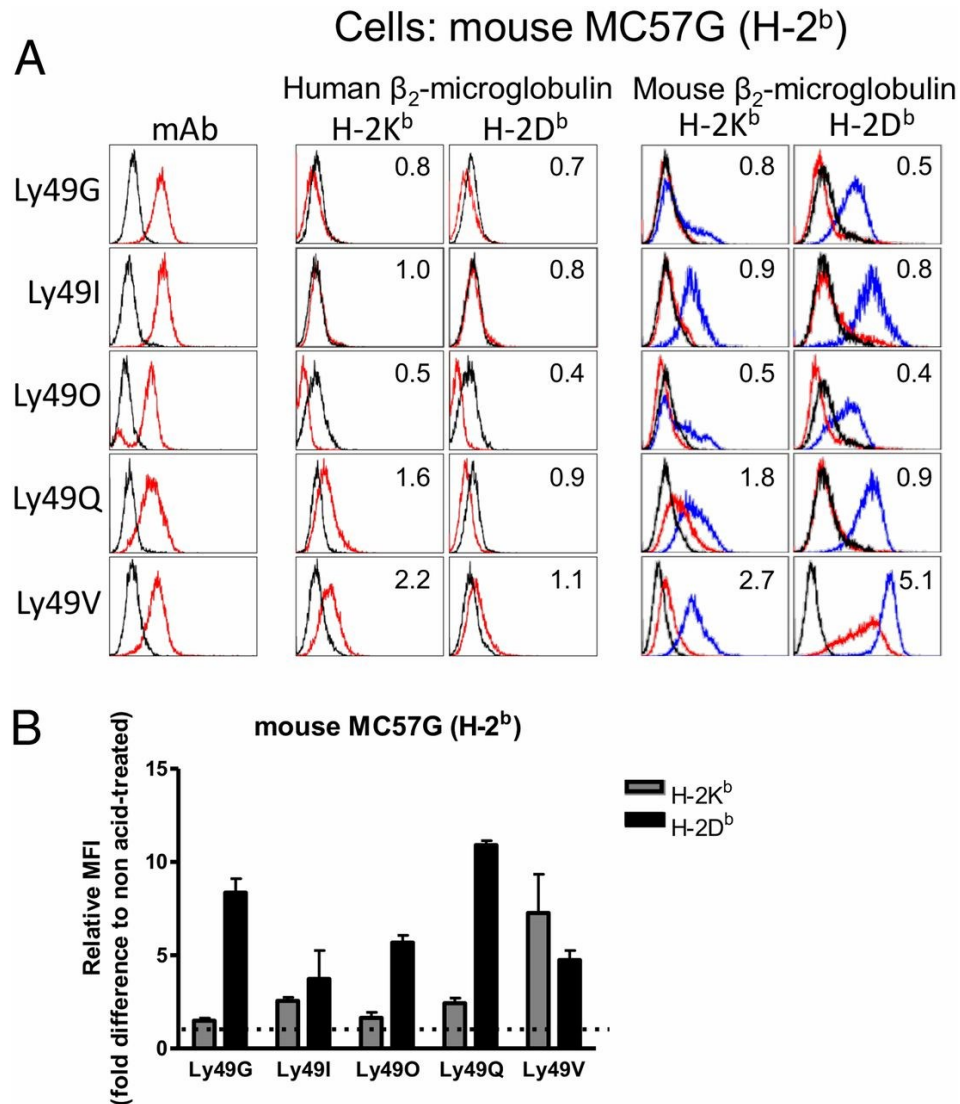


Figure 2.3: MHC-I coexpression in an H-2^b cell line drastically reduces soluble MHC-I tetramer binding to Ly49 molecules

(A) The MHC-I-sufficient MC57G cell line was stably transfected with the indicated Ly49 from 129 strain mice, treated with mild acid to disrupt class I MHC/peptide interactions, and then stained with specific PE- or FITC-conjugated mAb or PE-conjugated MHC-I tetramers (H-2K^b or H-2D^b) containing human (*middle panel*) or mouse (*right panel*) β_2 m L chain. After mAb or tetramer staining, cells were analyzed by flow cytometry. MC57G parental staining levels are shown in black; specific staining of stable Ly49 transfectants before and after acid treatment are shown in red and blue, respectively. Average MFI fold increase in the non-acid-treated transfectants over the parents is indicated. (B) Quantification of binding of MHC-I tetramers (H-2K^b or H-2D^b) containing mouse β_2 m L chain to acid-treated MC57G transfected with the indicated Ly49. Relative MFI indicates the fold increase in binding following acid treatment compared with no treatment (dotted line). Means $\pm$ SEM of two to three independent experiments are shown.

Table 2.2: Endogenous MHC-I expression results in weaker binding of Ly49 to MHC-I tetramers

	C1498-MHC-Deficient ^a				MC57G (H-2 ^b)				L929 (H-2 ^k)			
	Human β_2m^b		Mouse β_2m		Human β_2m		Mouse β_2m		Human β_2m		Mouse β_2m	
	K ^b	D ^b	K ^b	D ^b	K ^b	D ^b	K ^b	D ^b	K ^b	D ^b	K ^b	D ^b
Ly49G	— ^c	—	—/+	+	—	—	—	—	—	—	—	—/+
Ly49I	—	—	++	—/+	—	—	—	—	—	—	+++	—/+
Ly49O	—	—	—/+	+	—	—	—	—	—	—	—	—/+
Ly49Q	+++	—	+++	+	+	—	+	—	+	—/+	++	—/+
Ly49V	+++	++	+++	+++	+	—/+	+	+++	++	+	+	+++

^a Summary of binding of soluble MHC-I tetramers to MHC-I-deficient C1498 and MHC-I-sufficient MC57G and L929 cell lines stably transfected with five different Ly49

^b Species origin of the L chain in soluble PE-conjugated MHC-I tetramers

^c The fold increase of tetramer binding in transfected cells is shown. MFI ratios are indicated as follows: —, ≤ 1 ; —/+, 1.1–1.5; +, 1.6–3; ++, 3.1–5; +++, >5

fibroblast cell line. As observed with MC57G, tetramers containing human β_2m bound to Ly49Q (H-2K^b) and to Ly49V (H-2K^b and H-2D^b), in agreement with BWZ reporter cell results (Fig. 2.4A, *middle panel*). Tetramers containing mouse β_2m showed increased strength of these bindings plus the detectable binding of H-2K^b to Ly49I and a slight affinity in H-2D^b for all Ly49 (Fig. 2.4A, *right panel*). However, similar to MC57G, stable Ly49 expression on L929 showed less affinity for soluble MHC-I compared with the same Ly49 expressed on the MHC-I-deficient C1498 cell line (Fig. 2.2). MHC-I tetramer binding to Ly49 expressed on L929 cells is summarized in Table 2.2. Acid treatment of Ly49-expressing L929 resulted in increased binding of soluble tetramers to Ly49G (H-2K^b), Ly49I (H-2D^b), Ly49O (H-2K^b and H-2D^b), Ly49Q (H-2K^b and H-2D^b), and Ly49V (H-2K^b and H-2D^b) (Fig. 2.4A, *right panel*, 2.4B). These results suggest that H-2K^k and/or H-2D^k *cis* binding to Ly49Q inhibits H-2D^b and reduces H-2K^b binding in *trans*. This *cis*-mediated inhibition is not Ly49Q-specific and is seen to affect *trans* binding of soluble MHC-I tetramers by other Ly49 as well.

Ly49 expressed on human 293T cells do not show optimal binding of soluble MHC-I tetramers due to cross-species *cis* interactions with human MHC-I

Many previous studies, including our own, have used easily transfectable primate cell lines such as human kidney epithelial 293T or green monkey COS7 cells to study Ly49/MHC-I binding (Hanke et al., 1999; Makrigiannis et al., 2001; Scarpellino et al., 2007). Because 293T cells lack mouse MHC-I, we predicted that soluble MHC-I tetramer binding to Ly49 expressed on these cells would be efficient, as there are no competing *cis* interactions, and that binding efficiency would be similar to that seen with MHC-I-deficient C1498 Ly49 stable transfectants.

However, soluble MHC-I tetramer staining of human 293T cells was very similar to that observed with MHC-I-sufficient MC57G or L929 cell lines. Specifically, tetramers bound

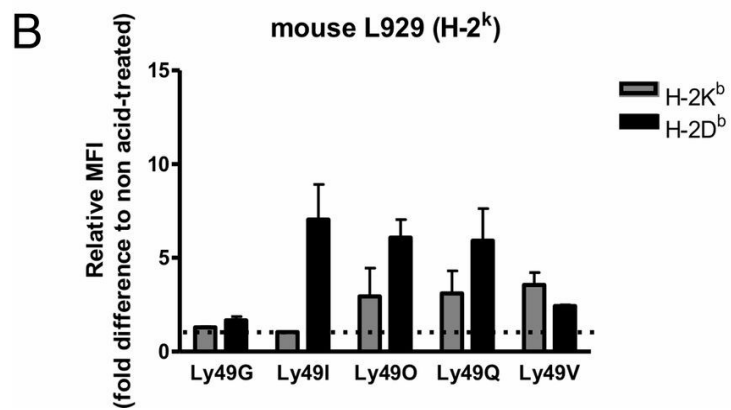
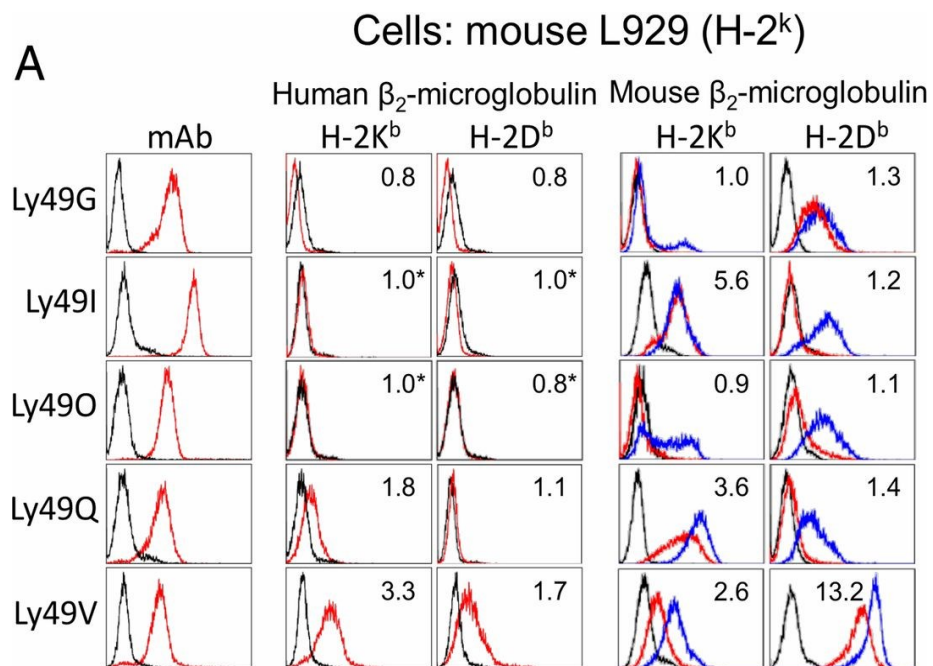


Figure 2.4: MHC-I coexpression in an H-2^k cell line also reduces soluble MHC-I tetramer binding to Ly49 molecules

(A) MHC-I-sufficient L929 cells were stably transfected with the indicated Ly49 from 129 strain mice, treated with mild acid to disrupt class I MHC/peptide interactions, and then stained with specific PE- or FITC-conjugated mAb or PE-conjugated MHC-I tetramers (H-2K^b or H-2D^b) containing human (*middle panel*) or mouse (*right panel*) β_2m L chain. After mAb or tetramer staining, cells were analyzed by flow cytometry. L929 parental staining levels are shown as a black histogram and specific staining of stable Ly49 transfectants before and after acid treatment are shown as red and blue histograms, respectively. Average MFI fold increase of non-acid-treated transfectants over parentals is indicated. In cases marked by an asterisk, fold increase was calculated either as the ratio of tetramer staining in the transfectants to the parentals or as the ratio of specific tetramer staining to control PE-conjugated streptavidin staining. (B) Quantification of binding of MHC-I tetramers (H-2K^b or H-2D^b) containing mouse β_2m L chain to acid-treated L929 transfected with the indicated Ly49. Relative MFI indicates the fold increase in binding following acid treatment compared with no treatment (dotted line). Means $\pm$ SEM of two to three independent experiments are shown.

strongly to Ly49I (H-2K^b), Ly49Q (H-2K^b), and Ly49V (H-2K^b and H-2D^b) (Fig. 2.5A). The binding of Ly49O and Ly49Q to H-2D^b observed using C1498 cells was greatly reduced in transfected 293T cells. The comparison of MHC-I tetramer binding ability to Ly49 expressed by 293T versus C1498 cells is summarized in Table 2.3.

The surprising non-optimal binding of Ly49 expressed on human 293T cells with soluble MHC-I tetramers suggests that there may be interference preventing binding of the tetramer to the Ly49 receptor expressed on the surface. Although 293T cells do not express mouse MHC-I due to their origin, it was of interest to determine whether cross-species *cis* interactions between human MHC-I and mouse Ly49 could be the cause of this observed low binding of MHC-I tetramer. Acid treatment of the Ly49-transfected 293T cells resulted in notably increased tetramer binding for Ly49G (H-2D^b), Ly49I (H-2D^b), Ly49O (H-2K^b and H-2D^b), Ly49Q (H-2D^b), and Ly49V (H-2K^b) (Fig. 2.5A, *right panel*, 2.5B). These results strongly suggest that human MHC-I and mouse Ly49 are able to bind.

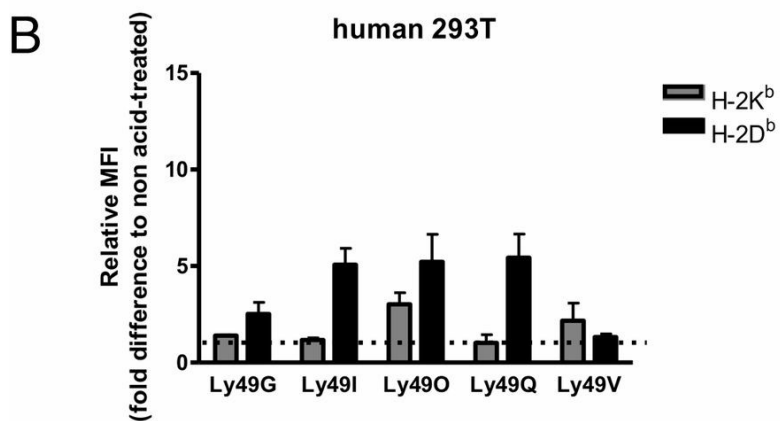
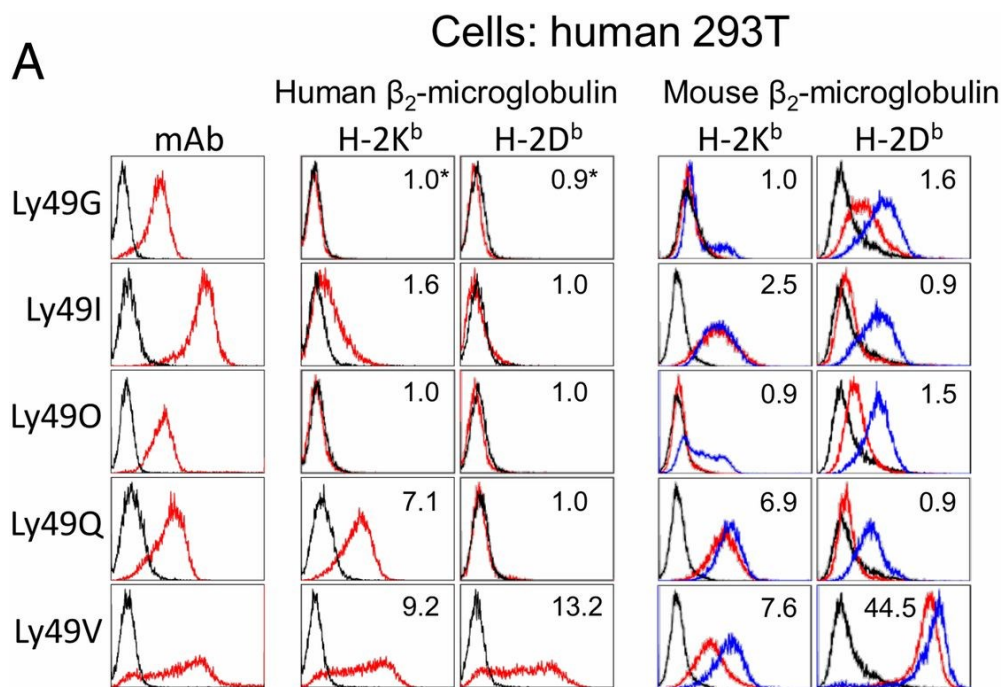


Figure 2.5: Lack of mouse MHC-I coexpression in human cells still results in low MHC-I tetramer binding by Ly49

(A) The human 293T cell line was stably transfected with the indicated Ly49 from 129 strain mice, treated with mild acid to disrupt class I MHC/peptide interactions, and then stained with specific PE- or FITC-conjugated mAb or PE-conjugated MHC-I tetramers (H-2K^b or H-2D^b) containing human (*middle panel*) or mouse (*right panel*) β_2m L chain. After mAb or tetramer staining, cells were analyzed by flow cytometry. 293T parental staining levels are shown as black histograms, and specific staining of stable Ly49 transfectants before and after acid treatment are shown as red and blue histograms, respectively. Average MFI fold increase of non-acid-treated transfectants over parentals is indicated. In cases marked by an asterisk, fold increase was calculated either as the ratio of tetramer staining in the transfectants to the parentals or as the ratio of specific tetramer staining to control PE-conjugated streptavidin staining. (B) Quantification of binding of MHC-I tetramers (H-2K^b or H-2D^b) containing mouse β_2m L chain to acid-treated MC57G transfected with the indicated Ly49. Relative MFI indicates the fold increase in binding following acid treatment compared with no treatment (dotted line). Means $\pm$ SEM of two to three independent experiments are shown.

Table 2.3: Increased MHC-I tetramer binding by Ly49 expressed on mouse cell lines

	293T ^a				C1498-MHC-Deficient			
	Human β_2m^b		Mouse β_2m		Human β_2m		Mouse β_2m	
	K ^b	D ^b	K ^b	D ^b	K ^b	D ^b	K ^b	D ^b
Ly49G	— ^c	—	—	+/-	—	—	-/+	+
Ly49I	+	—	+	—	—	—	++	-/+
Ly49O	—	—	—	-/+	—	—	-/+	+
Ly49Q	+++	—	+++	—	+++	—	+++	+
Ly49V	+++	+++	+++	+++	+++	++	+++	+++

^a Summary of binding of soluble MHC-I tetramers to human 293T and mouse MHC-I-deficient C1498 cell lines stably transfected with five different Ly49

^b Species origin of the L chain in soluble PE-conjugated MHC-I tetramers

^c The fold increase of tetramer binding in transfected cells is shown. MFI ratios are indicated as follows: —, ≤ 1 ; +/-, 1.1–1.5; +, 1.6–3; ++, 3.1–5; +++, > 5

Discussion

We previously reported that Ly49Q binds to H-2K^b, but not H-2D^b, in BWZ reporter cell experiments (Tai et al., 2007). In the present study, we show that Ly49Q does in fact bind to H-2D^b and that the likely reason for the lack of interaction in the BWZ assay was the expression of H-2K^k and/or H-2D^k on the BWZ cells themselves causing *cis* interactions with the chimeric Ly49Q leading to competition for *trans* binding to H-2D^b. In BWZ reporter cell and tetramer assays H-2K^b was able to overcome *cis* interactions, but H-2D^b was not, suggesting it has a weaker affinity than H-2K^b for Ly49Q. Furthermore, tetramer binding analyses showed H-2D^b binding to Ly49Q only when the tetramers were refolded with mouse β_2m L chain; a lack of competing *cis* interactions alone was not enough to allow H-2D^b/human β_2m to bind to Ly49Q. Because *cis* competition and species origin of the L chain were not taken into account when the 129 strain Ly49/MHC-I binding characteristics were first studied (Makrigiannis et al., 2001), tetramer analyses were repeated in the present study using stable transfectants of MHC-I-deficient and MHC-I-sufficient mouse and human cell lines and novel interactions were uncovered.

Specifically, we found that in addition to Ly49Q, both Ly49G and Ly49O bound to H-2D^b/mouse β_2m tetramers and that Ly49I was able to bind H-2K^b/mouse β_2m tetramers. Most or all of these interactions were lost when *cis* MHC-I interactions from either H-2^b or H-2^k haplotypes were introduced via Ly49 expression in MC57G or L929 cell lines, respectively, or when MHC-I tetramers containing human β_2m were used to stain Ly49 transfectants. Our present observations are similar to previous studies showing Ly49A binds to H-2D^b/mouse β_2m tetramers (Michaëlsson et al., 2000), but no interaction of Ly49A was observed with H-2D^b/human β_2m tetramers (Hanke

et al., 1999). In line with these observations, Ly49D binding to H-2D^d is only observed when the tetramer contains mouse L chain (Mason et al., 2003).

We have reported that NK cells expressing Ly49I¹²⁹ and Ly49O/V¹²⁹ on an H-2^b background show evidence of licensing or self-MHC-I education in that they make more IFN- γ than the corresponding negative subset upon activation via NKG2D or Nkp46 stimulatory receptors (Patel et al., 2010). Our present study confirms the possibility of a licensing effect of H-2^b for Ly49I¹²⁹ and Ly49O/V¹²⁹, as H-2K^b or H-2D^b tetramers bound to these Ly49 under optimal conditions. We were unable to detect any difference in IFN- γ production upon stimulation of Ly49G¹²⁹-positive versus -negative NK cells, although we show in the present study that Ly49G¹²⁹ can bind to H-2D^b. It is possible that the lack of licensing of Ly49G¹²⁹ seen previously is due to H-2D^b being a weak self-educator as shown in splenocyte rejection assays using *H-2K^b^{-/-}*, *H-2D^b^{-/-}*, or *H-2K^b^{-/-}H-2D^b^{-/-}* mice as donors (Bélanger et al., 2012; Patel et al., 2010). Furthermore, this hypothesis agrees with the observations that Ly49A can bind to H-2D^b when the L chain is of mouse origin (Michaëlsson et al., 2001), but that Ly49A⁺ NK cell subsets in H-2^b background mice produce little if any IFN- γ upon stimulation, compared with Ly49A⁺ subsets in H-2^d background mice (Jonsson et al., 2010); H-2D^d is a strong ligand for Ly49A (Karlhofer et al., 1992).

Additionally, when human β_2m - and mouse β_2m -containing tetramers both showed binding to the same Ly49, the mouse β_2m -containing tetramers showed higher binding as measured by increased MFI. In no case was it observed that tetramers made with human β_2m L chain showed a specificity that was not also observed with MHC-I/mouse β_2m tetramers. The β_2m sequence is 70% homologous between humans and mice and therefore relatively conserved through evolution. The peptide, H chain, and L chain are held together solely by noncovalent bonds, and for this

reason MHC-I tetramers are unstable. However, tetramers produced with human β_2m possess enhanced stability compared with those made with mouse β_2m . Mouse MHC-I/human β_2m tetramers are used for T cell clonal expansion assays, as the L chain does not significantly affect TCR binding to the MHC-I groove/peptide complex.

The soluble tetramer assay, although greatly improved in the present study, is still prone to false-negative results for the following reasons. First, non-mouse peptides were used to produce the tetramers used in the present study. However, it has been long known that peptide sequence greatly affects Ly49 affinity for MHC-I (Hanke et al., 1999; Michaëlsson et al., 2000). Chicken OVA peptide (H-2K^b) and lymphocytic choriomeningitis virus gp33 peptide (H-2D^b) were chosen for the present study for the sake of comparison with previous studies and for their ability to stabilize tetramers. Ly49 mediate their function in mice by binding MHC-I molecules presenting endogenous peptides. Second, tetramer H chains are produced in bacteria and so will lack posttranslational glycosylation and perhaps other modifications. Glycosylation of MHC-I, specifically on the α_2 domain of H-2D^d, is necessary for optimal binding by Ly49A and Ly49C (Lian et al., 1998). Receptor downmodulation on NK cells from mice expressing single MHC-Ia molecules, as previously reported (Johansson et al., 2005), may still be the best way to determine Ly49/MHC-I specificity for the above reasons. This assay shows that Ly49A surface expression on NK cells from H-2D^b single MHC-I transgenic mice is significantly lower than NK cells from *B2m*^{-/-} mice (Johansson et al., 2005), in agreement with mouse β_2m tetramer studies (Michaëlsson et al., 2000, 2001). However, it is possible that binding affinity and surface downmodulation do not always go hand in hand. For example, Ly49C was clearly downmodulated by the presence of H-2K^b, but Ly49I was not despite reports of Ly49I interacting with soluble H-

2K^b tetramers containing either mouse or human β_2m (Hanke et al., 1999; Johansson et al., 2005; Scarpellino et al., 2007).

The data presented in the present study suggest that the lesser MHC-I tetramer binding of Ly49 transiently transfected into 293T cells in our 2001 study was due in large part to binding of mouse Ly49 to human MHC-I molecules in *cis*. Ly49-expressing 293T cells stained with mouse β_2m -containing tetramers still showed a low level of tetramer specificity, especially as compared with C1498-MHC-I-deficient cells expressing the same Ly49. This was unexpected because 293T cells are human-derived and so *cis* interactions, which can inhibit tetramer binding, were not thought to take place. Following acid treatment, however, MHC-I tetramer binding to Ly49-transfected 293T cells notably increased, suggesting possible cross-species *cis* interaction between human MHC-I and mouse Ly49 on 293T cells, thus leading to the observed low MHC-I tetramer binding. Other groups have also shown the ability of receptor/ligand interactions across different species, particularly between humans and mice. The MHC-I-related neonatal Fc receptor, FcRn, whose role is to regulate the serum half-life of IgG and albumin, exhibits cross-reactive species binding based on ELISA and surface plasmon resonance results. Mouse FcRn is able to bind human IgG1 and human serum albumin, as well as IgG from other species, including guinea pig, rat, bovine, and sheep (Andersen et al., 2010; Ober et al., 2001). Furthermore, species cross-reactivity has been reported for human and mouse ligand/receptor interactions from the TNF superfamily based on flow cytometry analysis (Bossen et al., 2006).

Interestingly, the greatest increases in binding after acid treatment were observed with H-2D^b tetramer binding for human 293T cells (Fig. 2.5B) as well as the mouse-derived cells MC57G (Fig. 2.3B) and L929 (Fig. 2.4C). The low H-2D^b tetramer binding prior to acid treatment suggests that *cis* interactions on the cell surface were a greater hindrance toward H-2D^b tetramer binding

than to that of H-2K^b. This may be due to the previously reported ability of H-2D^b to bind a greater number of 129-derived Ly49 receptors, specifically Ly49V, G2, and O, than that of H-2K^b to only Ly49I (Makrigiannis et al., 2001). The diversified binding capacity of H-2D^b may lead to H-2D^b being more likely to be involved in *cis* interactions with the various Ly49 receptors transfected in the respective human and mouse cell lines.

In summary, the data presented in the present study suggest caution in the use of non-mouse cell lines for the identification of ligands for mouse Ly49, as they may lead to false-negative results. Furthermore, we show that the affinity for different MHC-I molecules by 129-derived Ly49 is greater than previously appreciated owing to *cis* interactions with native MHC-I. Finally, we confirm that MHC-I tetramers show the greatest affinity for 129-Ly49 when they have been produced with mouse-derived L chain.

Acknowledgements

We thank Drs. Lionel Filion and Gina Graziani (University of Ottawa) for helpful discussions.

Chapter 3 : Ly49 family receptors are required for cancer immunosurveillance mediated by natural killer cells

Preface

The following chapter consists of original research looking at the role of the Ly49 receptors on NK cells in the control of tumors. This chapter has been previously published as a research article:

Tu MM, Mahmoud AB, Wight A, Mottashed A, Bélanger S, Rahim MM, Abou-Samra E, and Makrigiannis AP. Ly49 family receptors are required for cancer immunosurveillance mediated by natural killer cells. *Cancer Research*. 2014; 74(14):3684-94.

The specific contributions of each author to this paper are listed below along with the author contribution section published in the paper:

Tu MM: Designed, performed and analyzed data from all experiments. Wrote manuscript, constructed all the figures, and responded to reviewers comments.

Mahmoud AB: Assisted with tumor measurements for the MCA-induced tumors

Wight A: Performed transfection of the double MHC-I-deficient B16F10. Provided critical reading of the manuscript

Mottashed A: Summer student who assisted with the harvesting of the E μ -myc tumors for flow cytometry analysis

Belanger S: Created the RAE1 expressing cell lines and MHC-I deficient RMA-S. Provided critical reading of the manuscript

Rahim MM: Assisted with experimental design and analysis. Provided critical reading of the manuscript.

Abou-Samra E: Performed immunohistochemistry of tumors. This was ultimately not included in the final version of the manuscript and thus not included in this chapter.

Makrigiannis AP: Supervised experimental design, execution and analysis. Provided critical reading of the manuscript.

Cancer Research Author Contribution Statement:

Conception and design: M.M. Tu, A.P. Makrigiannis

Development of methodology: M.M. Tu, A.P. Makrigiannis

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.M. Tu, A.B. Mahmoud, A. Wight, A. Mottashed, E. Abou-Samra

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.M. Tu, A.B. Mahmoud, A. Mottashed, M.M.A. Rahim

Interpretation of data: A.P. Makrigiannis

Writing, review, and/or revision of the manuscript: M.M. Tu, A. Wight, S. Belanger, A.P. Makrigiannis

Study supervision: A.P. Makrigiannis

Monitored NKCK^D.Eμ-myc^{Tg} and WT.Eμ-myc^{Tg} mice B-cell lymphoma progression and conducted experiments associated with these mice: A. Mottashed

Abstract

According to the missing-self hypothesis, natural killer (NK) cells survey for target cells which lack class I major histocompatibility complex (MHC-I) molecules. The Ly49 receptor family recognizes loss of MHC-I and is critical for educating NK cells, conferring the ability to eliminate transformed or infected cells. In this study, we evaluated their requirement in innate immune surveillance of cancer cells using genetically-manipulated mice with attenuated expression of Ly49 receptors (NKC^{KD}), in several models of carcinoma and metastasis. We found that NKC^{KD} mice exhibited uncontrolled tumor growth and metastases. Expression of two MHC-I alleles, H-2K^b and H-2D^b, was decreased in tumors from NKC^{KD} mice in support of the likelihood of NK-mediated tumor immunoediting. These tumor cells exhibit directed alterations to their cell surface expression in response to the genetically-altered immune environment in order to evade host recognition. Immunoediting in NKC^{KD} mice was restricted to MHC-I molecules, which are ligands for Ly49 receptors, while expression of Rae-1 and Mult1, ligands for another NK cell receptor, NKG2D, were unaffected. Restoring NK cell education in NKC^{KD} mice with a transgene for the inhibitory self-MHC-I receptor Ly49I restored suppression of cancer onset and growth. Interestingly, immune surveillance mediated by activating Ly49 receptors remained intact in NKC^{KD} mice, as demonstrated by the ability to stimulate the NKG2D receptor with tumor cells or splenocytes expressing Rae-1. Together, our results genetically establish the integral role of Ly49 in NK cell-mediated control of carcinogenesis through MHC-I-dependent missing-self recognition.

Introduction

Natural killer (NK) cells were originally identified by their unique killing of tumor cells without prior sensitization, which differed from the defined functions of T and B lymphocytes (Herberman et al., 1975; Kiessling et al., 1975a). Rare individuals with reduced NK cell numbers, cytotoxicity, and/or cytokine production are characteristically more susceptible to certain viral infections, highlighting the necessity of NK cells in immunity (Orange, 2002). Low NK cell activity in cancer-diagnosed individuals is associated with poor prognosis, and those with advanced stage cancer often possess minimally cytotoxic NK cells (Pross and Lotzová, 1993).

NK cells recognize tumor targets by the action of various activating and inhibitory receptors on their surface (Lanier, 2005). Members of the Ly49 receptor family, the murine functional homologue of the human killer-cell Ig-like receptor (KIR) family, can be either activating or inhibitory, and interact with class I major histocompatibility complex (MHC-I) molecules (Yokoyama et al., 1990). NK cells detect aberrant cells with reduced surface expression of MHC-I through the Ly49 receptors: successful engagement of MHC-I transmits an inhibitory signal, while an absence of MHC-I and/or the presence of activating ligands induces killing (Kärre et al., 1986; Leiden et al., 1989; Litwin et al., 1993). Accordingly, target cells can express ligands that bind to a variety of activating and inhibitory receptors on NK cells, and the interplay between inhibitory and activating signals determines the NK cell response (Lanier, 2005). NK cell functionality depends on earlier exposure to MHC-I, as proposed by the education hypothesis, in which a self-specific Ly49 receptor must interact with self-MHC-I in order for the NK cell to become functional (Fernandez et al., 2005; Kim et al., 2005).

Cancer immunoediting describes how the immune system possesses a dual role in protecting the host, as well as in shaping the tumor environment (Dunn et al., 2002). Tumors evade immune recognition through various mechanisms, such as alteration of the tumor microenvironment or changes in MHC-I expression. Reduced or complete loss of MHC-I expression has been seen in a wide array of human cancers including colorectal (Goepel et al., 1991; McDougall et al., 1990), breast (Goepel et al., 1991; Vitale et al., 1998), bladder (Cathro et al., 2010), ovarian (Han et al., 2008) and cervical carcinoma (Mehta et al., 2008), suggesting the presence of immunoediting.

Our lab has generated a mutant mouse strain in which expression levels of the Ly49 receptors – as well as the NKG2, CD94, and KLRI receptors – in the mouse natural killer gene complex (NKC) are down-regulated (Bélanger et al., 2012). NKC^{KD} mice provide a genetically-based approach to study the importance of Ly49 receptors in cancer immunosurveillance. As previously described (Bélanger et al., 2012), NKC^{KD} mice exhibit silenced Ly49 expression on approximately 80% of NK cells but with normal cell numbers and proportions, as well as unaffected expression levels of cell surface markers encoded outside the NKC. In this study, we show that Ly49 plays a major role in NK cell cancer immunosurveillance.

Materials and Methods

Mice

C57BL/6, $B2m^{-/-}$, and $E\mu$ -*myc* transgenic [strain: B6.Cg-Tg(IghMyc)22Bri/J] mice were purchased from The Jackson Laboratory. $Ly49I^{Tg}$ mice were kindly provided by Dr. Michael Bennett (University of Texas, Dallas) and backcrossed to the C57BL/6 background for at least six generations. B6.Ly49¹²⁹, B6.NKC^{KD} (Klra15^{tm1.1Apm}) and NK^{KD}-Ly49I^{Tg} mice were previously described (Bélanger et al., 2012; Liu et al., 2000; Patel et al., 2010). Due to linkage disequilibrium, B6.NKC^{KD} mice possess a 129-derived NKC. Therefore, the B6.Ly49¹²⁹ congenic mouse strain, which harbors a 129-derived NKC on the B6 background, serves as the genetically correct WT control. For brevity, B6.NKC^{KD} and B6.Ly49¹²⁹ are referred to as NK^{KD} and WT.

$E\mu$ -*myc* transgenic mice were crossed with WT and NK^{KD} to produce WT. $E\mu$ -*myc*^{Tg} and NK^{KD}. $E\mu$ -*myc*^{Tg} mice, respectively. Genotyping for the $E\mu$ -*myc* transgene was performed by PCR using primers: forward 5'CTGGGTCACTCACATTTAAC and reverse 5'GTATATCAGTCACCGCAGGT to attain a product size of approximately 500bp. All *in vivo* experiments utilized mice between 6 and 9 weeks of age. Breeding and manipulations performed on animals were in accordance with and approved by the University of Ottawa Animal Ethics Committee.

Cells, Antibodies, and Flow Cytometry

B16F10.LacZ was kindly provided by Dr. Rebecca Auer (Ottawa Hospital Research Institute, Ottawa, Ontario). All cell lines have been tested and authenticated. MHC-I staining of all tumors cells are conducted every 6 to 12 months. All cell lines were verified to be mycoplasma-free and showed appropriate pathologic morphology. B16F10.LacZ were stably transfected with

H-2K^b-pEF6 and/or H-2D^b-pEF6 expression vectors using Lipofectamine 2000 (Invitrogen), as per manufacturer's guidelines, and selected in 4.5 µg/mL or 9 µg/mL blasticidin. Antibody staining was performed using anti-H-2K^b (AF6-88.5.5.3) (eBioscience), anti-H-2D^b (KH95) (BD Biosciences), anti-MULT1 (5D10) (eBioscience), and anti-pan Rae-1 (186107) (R&D Systems). Cells were acquired on a CyAN-ADP flow cytometer with Summit 4.3 software (Beckman Coulter), and analyzed using Kaluza 1.2 software (Beckman Coulter).

Flank tumor model

WT, NKC^{KD}, and NKC^{KD}.Ly49I^{Tg} mice were challenged on the right flank by subcutaneous injection of 5x10⁵ or 1x10⁶ RMA, RMA-S, B16F10.LacZ, B16F10.H-2K^b H-2D^b, or B lymphoma cells in PBS. Mice were monitored daily for tumor development. Date of tumor appearance was recorded from when the tumor is first palpable. Tumor size was determined using an electronic caliper (Marathon) to measure the length and width. Mice were euthanized upon tumor length or width exceeding 12 mm.

Experimental metastasis model

WT, NKC^{KD}, and NKC^{KD}.Ly49I^{Tg} mice were intravenously challenged with 1x10⁵ or 2x10⁵ B16F10.LacZ cells in PBS. For experiments with poly(I:C) stimulation, 100 µg of poly(I:C) in PBS were injected intravenously 24 hours prior to tumor injection. Animals were euthanized 5 or 14 days following tumor inoculation and lungs were stained with X-gal (Bioshop) as described previously (Kirstein et al., 2009). Representative tumor burden was determined on the largest lung (left) lobe, by the number of surface visible metastases. For histology, fresh-frozen lungs embedded in OCT were sectioned at 8 µm thickness and H&E stained.

Carcinogen-induced model

WT and NKC^{KD} mice were injected subcutaneously into the hind flank with 100 µg methylcholanthrene dissolved in corn oil. Mice were monitored weekly for tumor development and scored as tumor positive upon tumor width exceeding 5 mm and exhibiting progressive growth. Tumor size was determined using an electronic caliper (Marathon) to measure length and width. Sarcoma growth rate (mm²/day) was calculated from tumor area with respect to number of days post-MCA injection.

Spontaneous lymphoma model

Eµ-*myc*^{Tg} mice were monitored daily, and euthanized upon exhibiting progressively swollen lymph nodes upon palpation or respiratory distress. The spleen and lymph nodes were dissociated to attain a single cell suspension. Tumor cells were incubated with Fc block (anti-CD16/CD32), then stained with anti-IgM (eB121-15F9) (eBioscience), anti-B220 (RA3-6B2) (eBioscience), anti-H-2K^b (AF6-88.5.5.3) (eBioscience), anti-H-2D^b (KH95) (BD Biosciences), anti-Mult1 (5D10) (eBioscience), and anti-pan Rae-1 (186107) (R&D Systems). Mean fluorescence intensity for H-2K^b, H-2D^b, Rae-1 and Mult1 expression in the spleen and lymph nodes of Eµ-*myc*^{Tg} mice was standardized to levels in WT control mice.

***In vitro* and *in vivo* NK cell assays**

Adherent lymphokine (IL-2)–activated killer (ALAK) cells were grown in culture and used as effector cells in [⁵¹Cr]–release cytotoxicity assays, as previously described (Makrigiannis et al., 2004). Intracellular staining for IFN-γ was performed as previously described (Bélanger et al., 2012). NKp46⁺TCRβ[−] cells were analyzed for IFN-γ by flow cytometry. Splenocyte and tumor cell

rejection assays were performed as previously described (Bélanger et al., 2012; Patel et al., 2010). Alternatively, MHC-I-expressing, MHC-I-deficient, and MHC-I-deficient Rae-1 β -expressing tumor cells were differentially labelled with 0.5 μ M, 3 μ M and 8 μ M CFSE, and combined at a 1:1:1 ratio for a total of 2×10^6 cells. Peritoneal cells were harvested 16 hours following injection and analyzed by flow cytometry for the presence of CFSE-labelled tumor cells.

Statistical analysis

Statistical comparisons were performed on Kaplan-Meier plots depicting tumor onset using the log-rank test with Prism (GraphPad Software). For the remainder, statistical significance was determined by a two-tailed *t* test with cutoff P value of 0.05. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

Results

Accelerated tumor cell-induced flank tumor growth in NKC^{KD} mice

It was of interest to determine whether the lack of MHC-I immunosurveillance by NK cells in NKC^{KD} mice affects long-term tumor control. Onset of MHC-I-deficient RMA-S induced flank tumor formation was detected at least 3 days earlier in NKC^{KD} mice than WT mice (Fig. 1A and C). Tumor incidence was more prevalent in the NKC^{KD} group, with 92% (dose of 5×10^5 cells/mouse) and 100% (dose of 1×10^6 cells/mouse) developing tumors after 30 days, compared to 65% and 85%, respectively, in the WT group (Fig. 3.1A and C). Tumor size at comparable dates was larger in NKC^{KD} mice (Fig. 3.1B and D); the overall growth rate of the tumors in NKC^{KD} mice is accelerated. Tumor development and growth in NKC^{KD} mice is comparable to that of $B2m^{-/-}$ mice (Fig. 3.1C and D), which also possess hyporesponsive NK cells.

B16F10 are highly aggressive murine melanoma cells which lack MHC-I expression (Fidler, 1975; Seliger et al., 2001). Subcutaneous injection of 1×10^6 B16F10.LacZ cells promoted solid tumor formation in both NKC^{KD} and WT mice. At day 6, 7% of WT mice developed tumors in contrast to 53% of NKC^{KD} mice (Supplementary Fig. S3.1A). Tumor size at comparable dates was larger in NKC^{KD} mice (Supplementary Fig. S3.1B). This defect in NKC^{KD} tumor control is MHC-I dependent, as flank tumor challenge outcomes with MHC-I-expressing RMA, and transfected B16F10 expressing both MHC-I molecules H-2K^b and H-2D^b were similar between NKC^{KD} and WT mice (Fig. 3.1E and F, and Supplementary Fig. S3.1C and D).

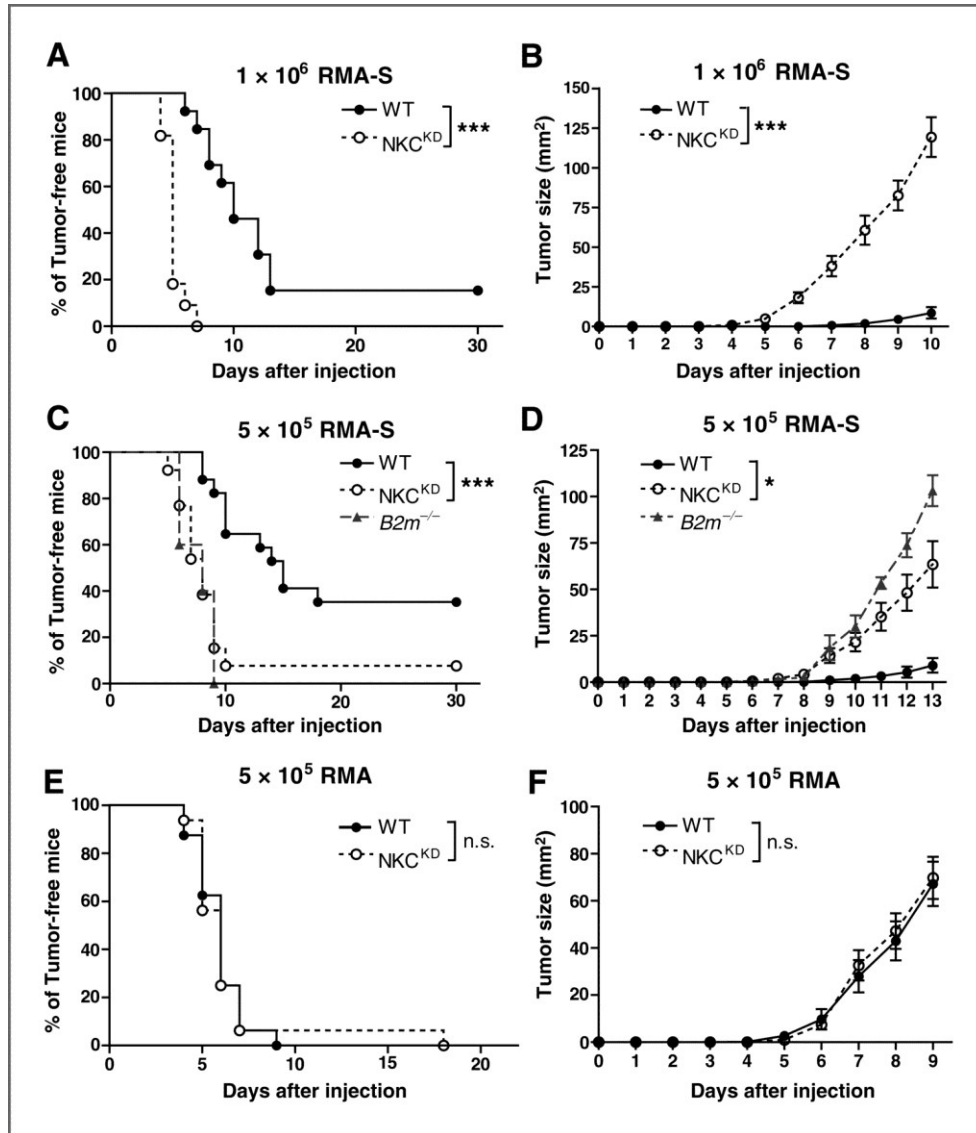


Figure 3.1: Accelerated tumor cell-induced flank tumor growth in NKC^{KD} mice

Formation of solid flank tumors following subcutaneous injection of (A and B) 1×10^6 MHC-I-deficient RMA-S. WT $n=13$. NKC^{KD} $n=11$. (C and D) 5×10^5 RMA-S. WT $n=17$. NKC^{KD} $n=13$. $B2m^{-/-}$ $n=5$. (E and F) 5×10^5 MHC-I-expressing RMA. WT $n=16$. NKC^{KD} $n=16$. (A, C and E) Date of tumor appearance and (B, D and F) mean tumor size $\pm$ SEM. Data are pooled from three independent experiments.

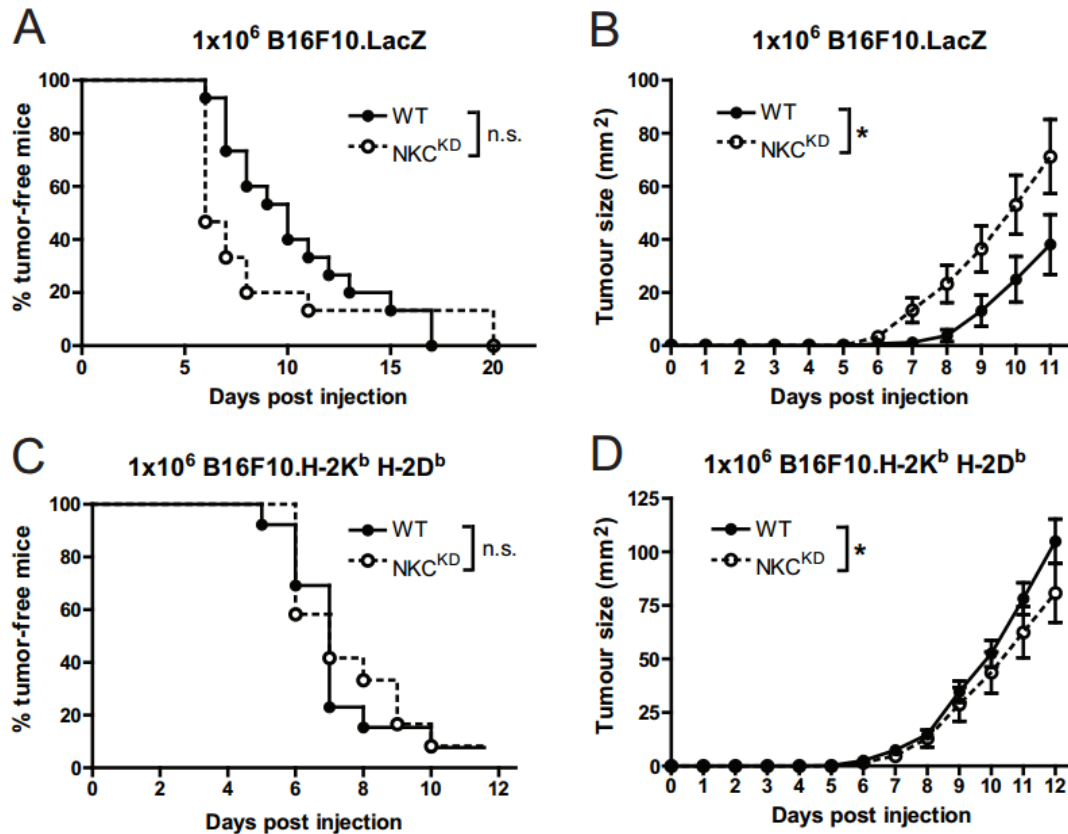


Figure S3.1. Defective control of B16F10-derived flank tumors in NKC^{KD} is MHC-I-dependent

Formation of solid flank tumors following subcutaneous injection of tumor cells. (A and B) 1×10^6 MHC-I-deficient B16F10.LacZ. WT $n=15$. NKC^{KD} $n=15$. (C and D) 1×10^6 B16F10 transfected to express the MHC-I molecules H-2K^b and H-2D^b. WT $n=13$. NKC^{KD} $n=12$. (A and C) Date of tumor appearance and (B and D) mean tumor size $\pm$ SEM. Data are pooled from two to three independent experiments.

Accelerated melanoma cell-induced metastatic growth in NKC^{KD} mice

The B16F10.LacZ cells were also used in an experimental pulmonary metastases model to determine the ability of NKC^{KD} mice to control metastatic growth. The number of metastases was greater in NKC^{KD} mice compared to WT mice regardless of incubation time and cell dose (Fig. 3.2A and B). Histologically, NKC^{KD} mice exhibited increased metastatic nodules in the lung parenchyma, and loss of morpho-functional structures such as open and wide pulmonary alveoli, instead having alveoli obstructed with hyperemic areas (Fig. 3.2C). Leukocyte infiltration can also be noted more frequently in NKC^{KD} mouse lungs (Fig. 3.2C). These data suggest an inability of NKC^{KD} mice to eliminate B16F10.LacZ tumor cells before they are able to seed in the lungs.

To demonstrate that the observed hyporesponsiveness is not due to a cytotoxic defect, the NK cells in WT and NKC^{KD} mice were directly activated by poly(I:C) injection. The lungs of untreated WT mice contained a mean of 45 metastases compared to a mean of 11 in the poly(I:C)-treated group (Fig. 3.2D). Comparatively, for NKC^{KD} mice, metastases were also significantly reduced from a mean of 118 to 39 with poly(I:C) stimulation (Fig. 3.2D). The efficacy of poly(I:C) treatment in both WT and NKC^{KD} mice suggests that NK cells from NKC^{KD} mice are still responsive if activated in a Ly49-independent manner.

Defective recognition of melanoma cell-induced experimental pulmonary metastases is MHC-I-dependent

To determine whether defective rejection of the parental, MHC-I-negative B16F10.LacZ lung metastases by NKC^{KD} mice is due to defective missing-self recognition, the cells were stably transfected to express two MHC-I alleles, H-2K^b and H-2D^b, either individually or

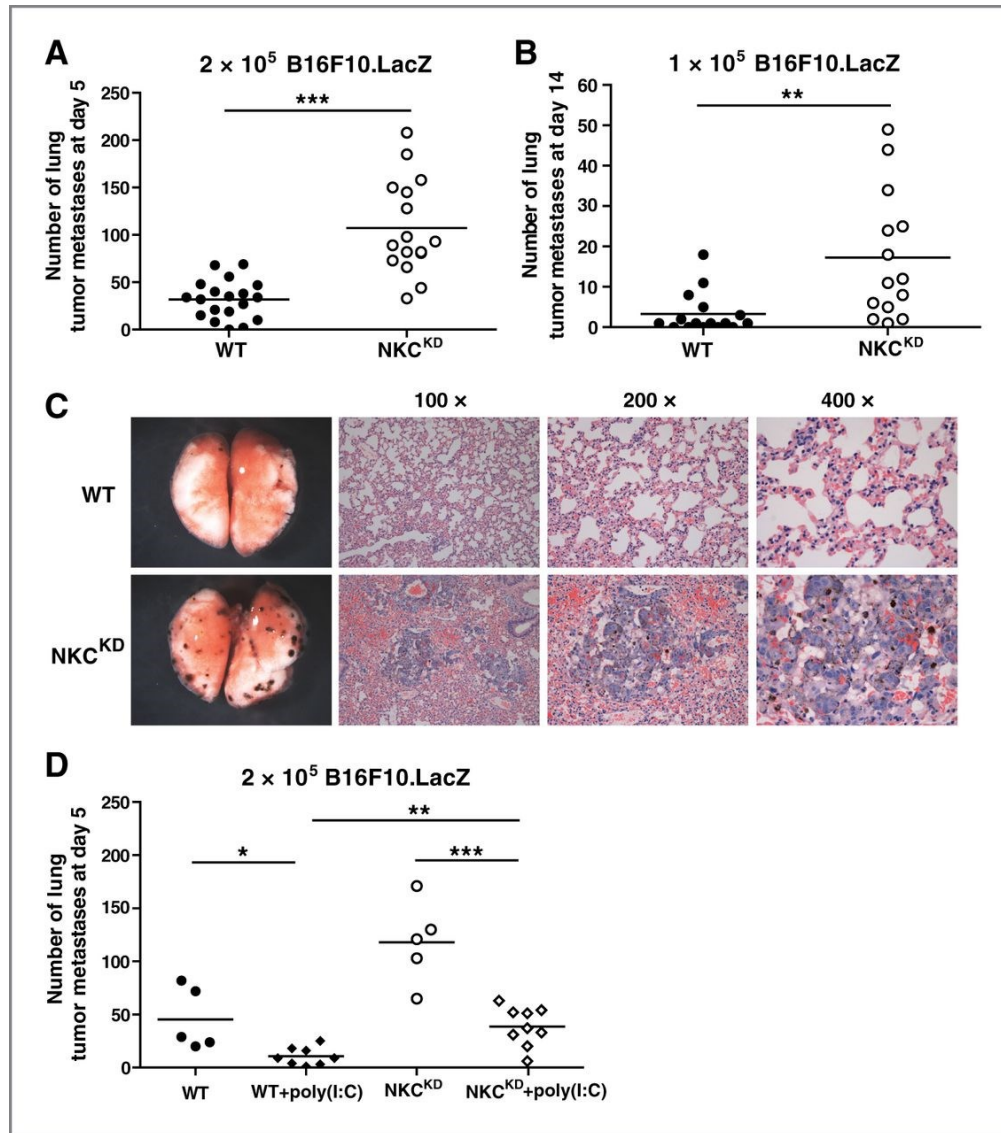


Figure 3.2: Accelerated melanoma cell-induced metastatic growth in NKC^{KD} mice

Pulmonary metastases induced by intravenous injection of MHC-I-deficient B16F10.LacZ melanoma cell (A) 2×10^5 cells injected and harvested 5 days later, and (B) 1×10^5 cells injected and harvested 14 days later. Data are pooled from three independent experiments. (C) Representative whole lung image and cross-section H&E staining of lungs harvested 14 days following intravenous injection of 1×10^5 B16F10.LacZ cells. (D) Stimulation with 100 μ g poly(I:C) prior to injection of 2×10^5 B16F10.LacZ cells. Number of metastases quantified on largest lung lobe after 5 days. Data are pooled from two independent experiments. Each symbol represents a single mouse. Horizontal line represents mean.

together (Fig. 3.3A). Transfection of the cells did not affect expression of the NKG2D ligands, Rae-1 and Mult1 (Fig. 3.3B). Expression of a single MHC-I allele was insufficient to significantly protect these tumors from WT NK control compared to the NKC^{KD} (Fig. 3.3C and 3E). However, expression of both H-2K^b and H-2D^b (Fig. 3.3G) resulted in similar metastatic numbers in the lungs of WT and NKC^{KD} mice, suggesting a loss of NK-mediated control due to MHC-I inhibition. These results suggest that NK cell recognition and elimination of B16F10.LacZ tumor cells is MHC-I-dependent, since upon expression of both H-2K^b and H-2D^b, metastatic numbers in WT and NKC^{KD} mice are comparable. Similarly to the parental B16F10, poly(I:C) stimulation prior to tumor challenge greatly reduced the number of metastases in both WT and NKC^{KD} compared to untreated (Fig. 3.3D, F and H). These results support that the hyporesponsive nature of NK cells from NKC^{KD} mice is not due to a more generalized defect since stimulation restores function.

Inhibitory self-MHC-I-specific Ly49I restores NK cell cancer immunosurveillance in NKC^{KD} mice

To test the hypothesis that NK cells from NKC^{KD} mice are uneducated and hyporesponsive, and therefore unable to control tumor and metastatic growth, a transgene for Ly49I was introduced into NKC^{KD} mice. Introduction of the Ly49I^{Tg} significantly delayed RMA-S-induced flank tumor onset in NKC^{KD} mice, approaching WT levels (Fig. 3.4A). WT and $\text{NKC}^{\text{KD}}\text{-Ly49I}^{\text{Tg}}$ mice presented with similar late period tumor growth, in contrast with NKC^{KD} mice (Fig. 3.4B). Similar results were also seen with the experimental lung metastases, wherein the number of metastases was reduced in $\text{NKC}^{\text{KD}}\text{-Ly49I}^{\text{Tg}}$ mice (mean of 24) compared to NKC^{KD} (mean of 89) (Fig. 3.4C). These observed differences in the numbers of metastases

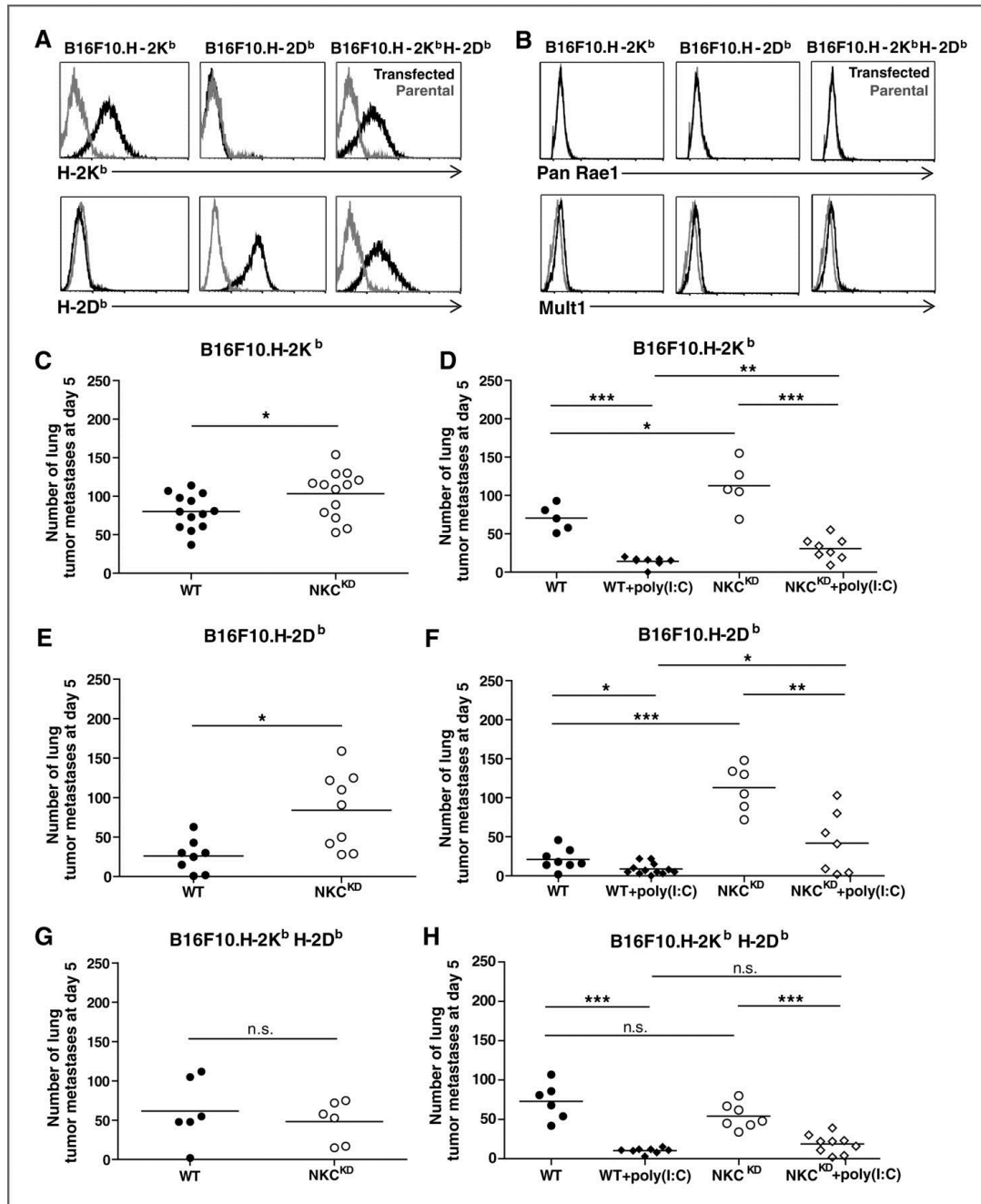


Figure 3.3: Defective recognition of melanoma cell-induced experimental pulmonary metastases is MHC-I-dependent

Pulmonary metastases following intravenous injection of MHC-I-deficient B16F10.LacZ transfected with H-2K^b, H-2D^b or both. Flow cytometry results showing (A) H-2K^b and H-2D^b, and (B) Rae-1 and Mult1 expression in transfected cells compared to parental B16F10.LacZ. Quantification of metastases 5 days following injection of 2×10^5 B16F10.LacZ cells expressing (C-D) H-2K^b, (E-F) H-2D^b or (G-H) H-2K^b and H-2D^b with (D, F and H) poly(I:C) stimulation of recipient mice prior to tumor injection. Horizontal lines represent mean. Each symbol represents a single mouse. Data are pooled from two to three independent experiments.

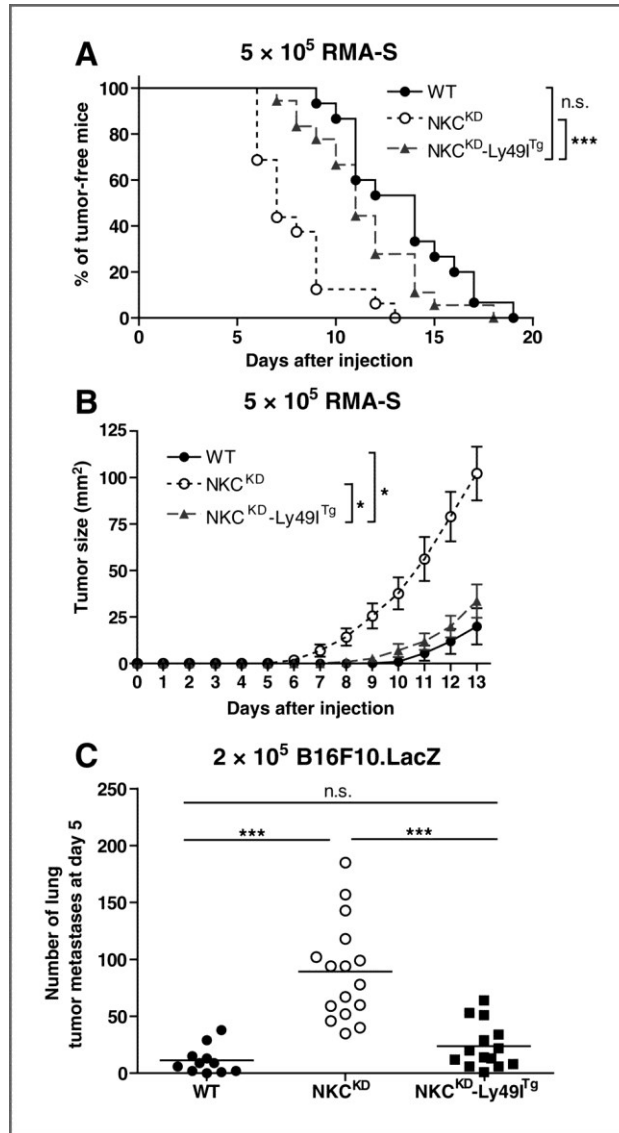


Figure 3.4: Inhibitory self-MHC-I-specific Ly49I restores NK cell function in NKC^{KD} mice

Formation of solid flank tumors following subcutaneous injection of MHC-I-deficient RMA-S. (A) Date of tumor appearance and (B) mean tumor size $\pm$ SEM. WT $n=15$. NKC^{KD} $n=16$. NKC^{KD} -Ly49ITg $n=18$. (C) Quantification of pulmonary metastases induced by 2×10^5 B16F10.LacZ melanoma cells harvested 5 days later. Each symbol represents a single mouse. Horizontal line represents mean. Data are pooled from three independent experiments.

suggest that Ly49I-mediated education is necessary to engender an NK cell response against tumors.

Accelerated onset and growth of MCA-induced sarcoma in NKC^{KD} mice

Previous studies show a role for NK cells in controlling methylcholanthrene (MCA)-induced tumors (Crowe et al., 2002; Smyth et al., 2000, 2001). It was of interest to determine whether NKC^{KD} mice with hyporesponsive NK cells would be more susceptible to MCA-induced tumors. NKC^{KD} mice exhibited earlier sarcoma onset than WT; by day 85, all NKC^{KD} mice had developed tumors, compared to approximately 50% of WT mice (Fig. 3.5A). Tumor growth was accelerated in NKC^{KD} mice (Fig. 3.5B), with a significantly greater relative growth rate in NKC^{KD} (mean of 1.97) than WT mice (mean of 0.65) (Fig. 3.5C). This suggests that control of MCA-induced tumors is impaired in NKC^{KD} mice, and that the defect is not restricted to MHC-I-deficient tumor cell recognition.

Earlier onset of B cell lymphoma and evidence for MHC-I-directed tumor immunoediting in NKC^{KD} mice

E μ -*myc* transgenic mice possess the *myc* oncogene coupled to the immunoglobulin μ enhancer, resulting in spontaneous B cell lymphoma development, a lethal malignancy starting at 6 weeks of age (Adams et al., 1985). Considering the pathological importance of this cancer, especially with parallels to human Burkitt's lymphoma, NKC^{KD}.E μ -*myc*^{Tg} and WT.E μ -*myc*^{Tg} mice were generated to study the effect of silenced Ly49 expression. NKC^{KD} mice developed lymphomas significantly earlier than WT mice, and exhibited reduced tumor control; following 100 days of age, approximately 60% of WT mice were lymphoma positive, compared to

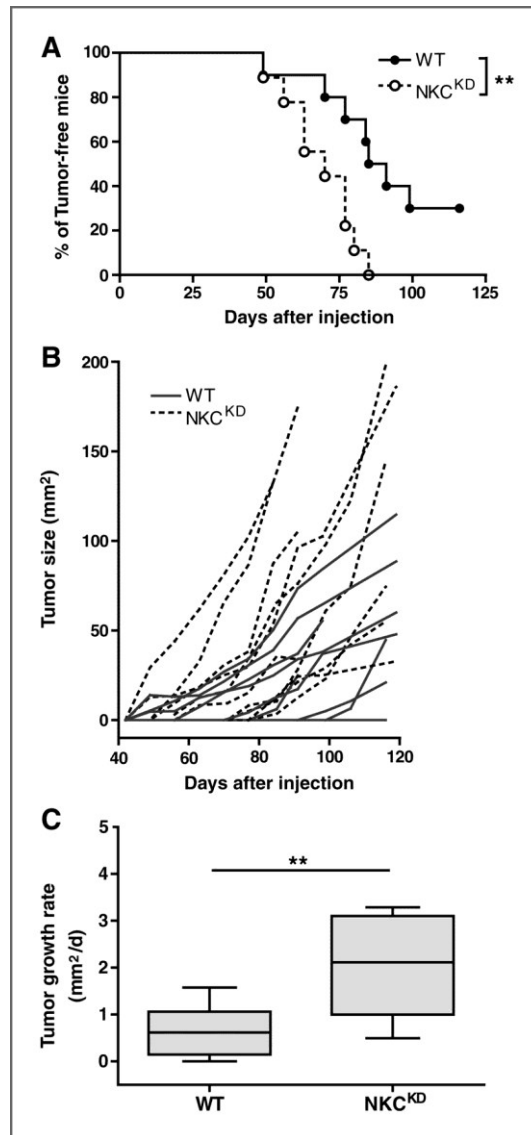


Figure 3.5: Accelerated onset and growth of MCA-induced sarcoma in NKC^{KD} mice

Sarcoma development following subcutaneous injection of 100 μ g MCA into the hind flank of WT ($n=10$) and NKC^{KD} ($n=10$) mice. Mice were observed weekly for tumor development. (A) Date of tumor appearance. (B) Individual tumor growth was measured weekly with each line representing tumor growth in a single mouse. (C) Sarcoma growth rate calculated from tumor size with respect to time. Upper and lower limits of the box represent 25th and 75th percentiles with whiskers indicating minimum and maximum values. Data are pooled from two independent experiments.

approximately 90% of NKC^{KD} mice (Fig. 3.6A). While both groups possessed mice exhibiting delayed lymphoma onset, this lapse was more prominent in WT mice.

Since NKC^{KD} mice lack MHC-I-educated NK cells, it was of interest to determine whether this would lead to cancer immunoediting. To test this hypothesis, immature (IgM⁻) and mature (IgM⁺) B lymphoma cells from the spleen and lymph nodes were analyzed for expression of the MHC-I molecules, H-2K^b and H-2D^b, as well as the NKG2D ligands, Rae-1 and Mult1. NKC^{KD}.Eμ-*myc*^{Tg} mice exhibited reduced MHC-I expression in both the mature and immature B cell populations compared to WT mice with and without the Eμ-*myc*^{Tg} (Fig. 3.6B-H). In contrast, expression of NKG2D ligands were negligible or very low, with no statistically significant differences between WT.Eμ-*myc*^{Tg} and NKC^{KD}.Eμ-*myc*^{Tg} (Supplementary Fig. S3.2A-G). This highlights the significance of the observed downregulation of MHC-I in NKC^{KD}.Eμ-*myc*^{Tg}.

B lymphoma cells were isolated from a NKC^{KD}.Eμ-*myc*^{Tg} mouse which exhibited reduced MHC-I expression and low level expression of NKG2D ligands (Fig. 3.6I). This decrease in MHC-I expression rendered the tumor cells more susceptible to rejection by WT mice compared to NKC^{KD} mice (Fig. 3.6J). Furthermore, WT mice were able to better control the growth of B lymphoma cell-induced flank tumors (Fig. 3.6K). Overall, our observation of decreased MHC-I expression in NKC^{KD}.Eμ-*myc*^{Tg}, but not NKG2D ligand expression, suggests that silenced Ly49 expression in NKC^{KD} mice leads to MHC-I-directed tumor immunoediting.

Signaling through NKG2D is unaffected in NKC^{KD} mice

To study the effects of activating ligand expression on MHC-I-expressing and –deficient target cells in NKC^{KD} mice, the rejection of splenocytes from B6 or *B2m*^{-/-} mice transgenically expressing Rae-1 was studied. Activation through NKG2D is intact in NKC^{KD} mice since

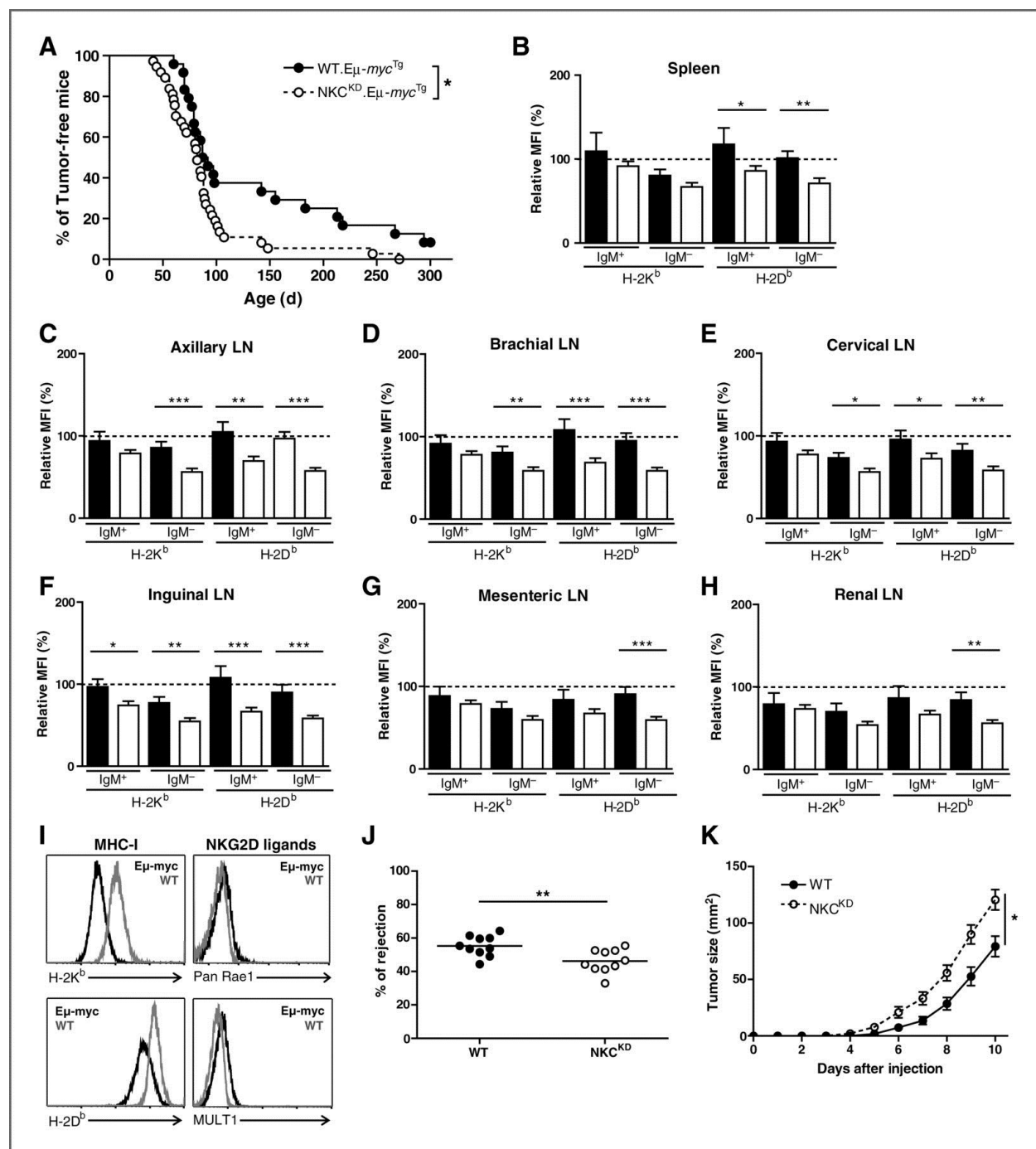


Figure 3.6: Earlier onset of B cell lymphoma and MHC-I-directed tumor immunoediting in NKC^{KD} mice

(A) B cell lymphoma progression in $\text{NKC}^{\text{KD}}.\text{E}\mu\text{-myc}^{\text{Tg}}$ ($n=35$) and $\text{WT.E}\mu\text{-myc}^{\text{Tg}}$ ($n=24$) mice. (B-H) *Ex vivo* analysis of spleen and lymph node cell suspensions isolated from $\text{NKC}^{\text{KD}}.\text{E}\mu\text{-myc}^{\text{Tg}}$ ($n=30$) and $\text{WT.E}\mu\text{-myc}^{\text{Tg}}$ ($n=21$) mice. B cells (B220^+) were analyzed separately as immature (IgM^-) or mature (IgM^+) for expression of H-2K^{b} and H-2D^{b} by flow cytometry. MFI was standardized to that of B cells from non- $\text{E}\mu\text{-myc}$ transgenic mice (dotted line). (I) MHC-I and NKG2D ligand expression in B lymphoma cells cultured from a $\text{NKC}^{\text{KD}}.\text{E}\mu\text{-myc}^{\text{Tg}}$ (black histogram) compared to B cells from a non- $\text{E}\mu\text{-myc}^{\text{Tg}}$ mouse (grey histogram). (J) *In vivo* rejection of B lymphoma cells originating from an $\text{NKC}^{\text{KD}}.\text{E}\mu\text{-myc}^{\text{Tg}}$ relative to the MHC-I expressing RMA. Each symbol represents a single mouse. Horizontal line represents mean. Data are pooled from two independent experiments. (K) Tumor development following subcutaneous injection of 5×10^5 B lymphoma cells from an $\text{NKC}^{\text{KD}}.\text{E}\mu\text{-myc}^{\text{Tg}}$ injected into the hind flank of WT ($n=19$) and NKC^{KD} ($n=18$) mice. Mean $\pm$ SEM. Data are pooled from four independent experiments.

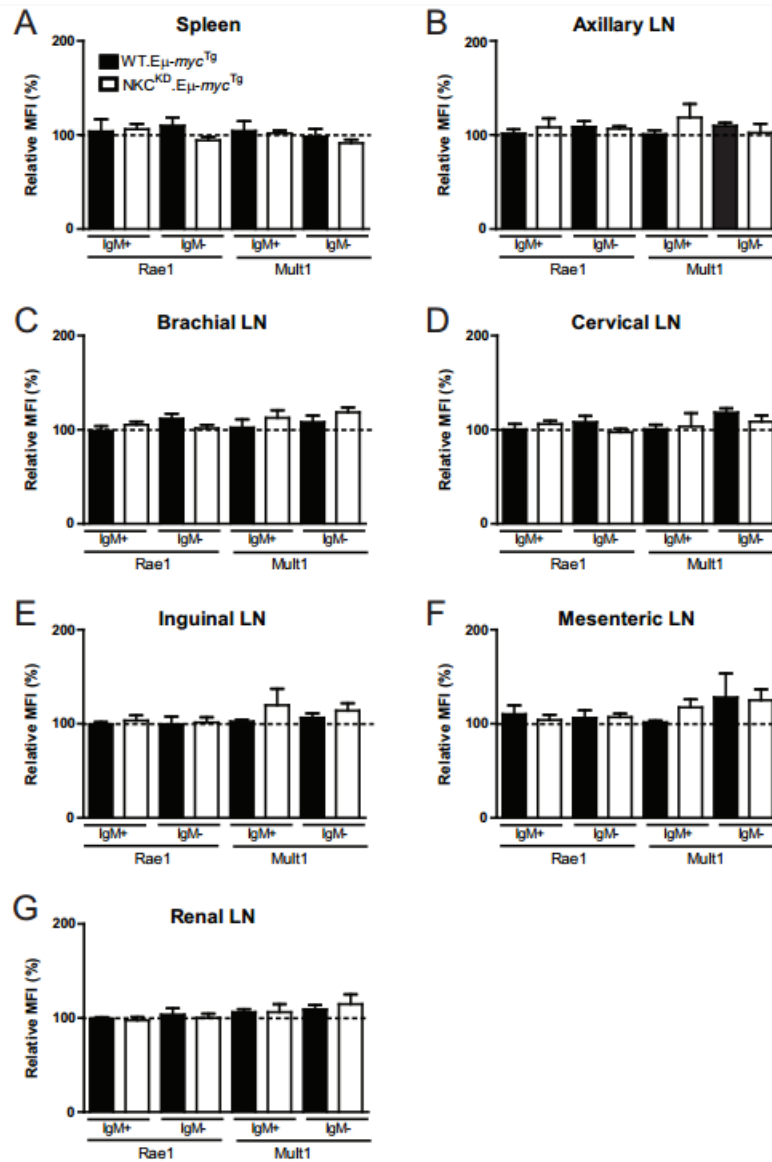


Figure S3.2: Lack of NKG2D ligand expression on B lymphoma cells from WT and NKC^{KD} E μ -myc^{Tg} mice

Ex vivo analysis of the indicated spleen and lymph node cell suspensions isolated from NKC^{KD}.E μ -myc^{Tg} ($n=6$) and WT.E μ -myc^{Tg} ($n=6$) mice. B cells (B220⁺) were analyzed separately as immature (IgM⁻) or mature (IgM⁺) for expression of the NKG2D ligands, Rae-1 and Mult1, by flow cytometry. MFI was standardized as a percentage to expression levels that of B cells from WT non-E μ -myc^{Tg} mice (dotted line).

rejection of splenocytes from Rae-1 ϵ^{Tg} mice were similar between WT and NKC^{KD} (Fig. 3.7A). While there is lower rejection of *B2m*^{-/-} Rae-1 ϵ^{Tg} splenocytes by NKC^{KD} mice than WT mice, this can be attributed to the loss of MHC-I expression on these cells, as seen in the rejection of *B2m*^{-/-} (Fig. 3.7B). Similarly, *in vivo* rejection of RMA and RMA-S ectopically expressing Rae-1 β by WT and NKC^{KD} mice showed parallel results. Rejection of both Rae-1 β^{high} and Rae-1 β^{low} cells were similar (Fig. 3.7C-E), suggesting that NKG2D signaling is fully intact in NKC^{KD} mice. While rejection of RMA-S-Rae-1 β was lower in NKC^{KD} mice (Fig. 3.7F), this is attributed to the MHC-I-deficiency of these cells, and is evidenced by the differential rejection of RMA-S by WT and NKC^{KD} mice. These results suggest that while NKC^{KD} mice exhibit defective missing-self recognition, signaling through NKG2D, independent of MHC-I expression, is able to induce NK cell killing.

Decreased cytotoxicity but not IFN- γ production in NKC^{KD} mice

It was of interest to determine whether loss of Ly49 expression affects cytotoxicity or IFN- γ production by NK cells. NK cells from NKC^{KD} mice exhibited normal production of IFN- γ following stimulation with PMA+ionomycin (Supplementary Fig. S3.3A), as well as various tumor cells: RMA, RMA-Rae-1 β^{low} , RMA-Rae-1 β^{high} , RMA-S, and RMA-S-Rae-1 β (Supplementary Fig.S3B), suggesting that loss of Ly49 does not affect IFN- γ production. A direct comparison of the killing between WT and NKC^{KD} mice was studied through an *in vitro* cytotoxicity assay. The direct killing of the MHC-I-deficient tumor cells, RMA-S (Supplementary Fig. S3.3C) and RMA-S-Rae-1 β (Supplementary Fig. S3.3D) by NKC^{KD}-derived ALAK cells is impaired, while killing of MHC-I-expressing RMA and RMA-Rae-1 β^{high} and RMA-Rae-1 β^{low} is comparable between WT and NKC^{KD}, suggesting a cytotoxicity defect with regards to missing-self recognition. While there is observed killing of RMA-S by NKC^{KD}

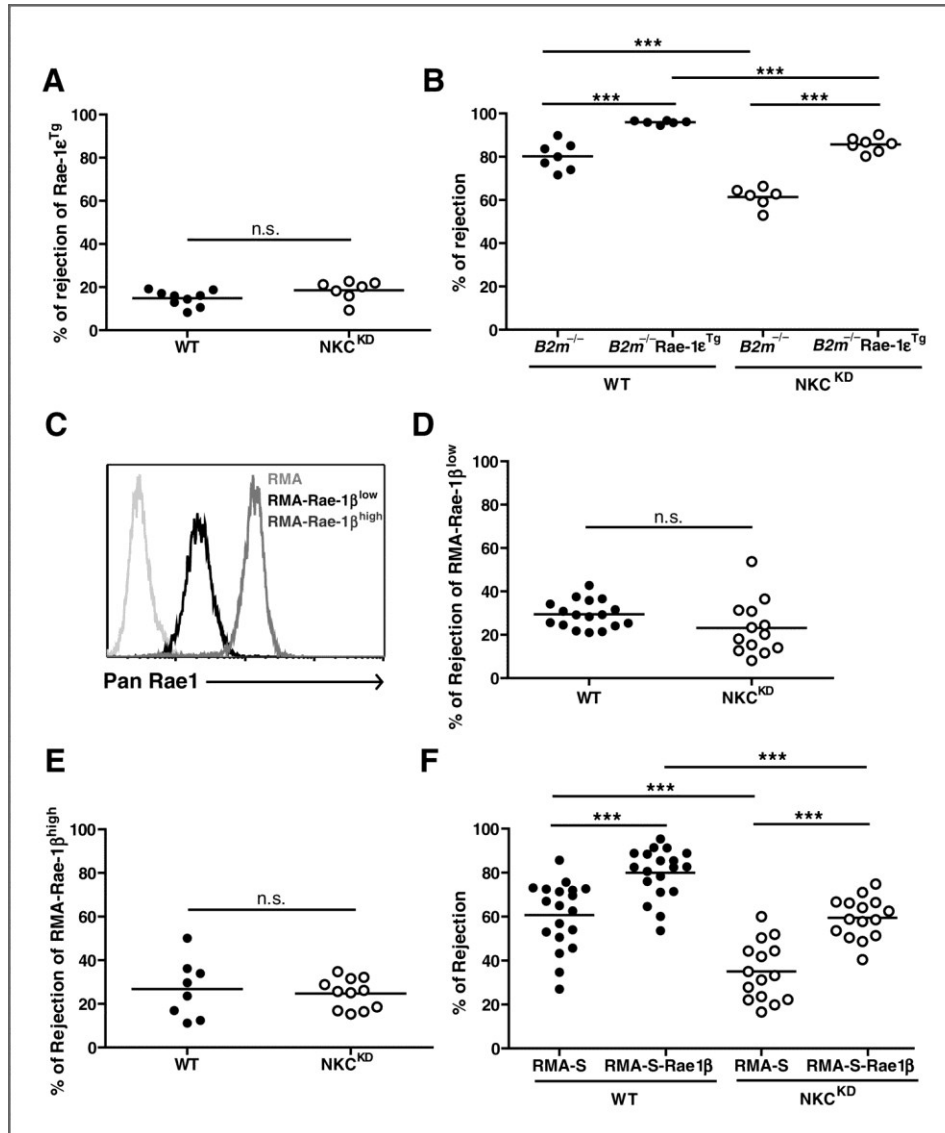


Figure 3.7: Signaling through NKG2D is unaffected in NKC^{KD} mice

In vivo rejection of CFSE-labelled splenocytes from (A) Rae-1 ϵ^{Tg} mice and (B) B2m^{-/-} and B2m^{-/-} Rae-1 ϵ^{Tg} mice relative to B6 mice. (C) Rae-1 expression levels in parental RMA cells, and RMA cells ectopically expressing Rae-1 β^{low} and Rae-1 β^{high} . *In vivo* rejection of MHC-I-expressing RMA (D) Rae-1 β^{low} and (E) Rae-1 β^{high} relative to RMA. (F) *In vivo* rejection of MHC-I-deficient RMA-S and RMA-S-Rae-1 β relative to RMA. Each dot represents a single mouse. Mean $\pm$ SEM. Data are pooled from two to four independent experiments.

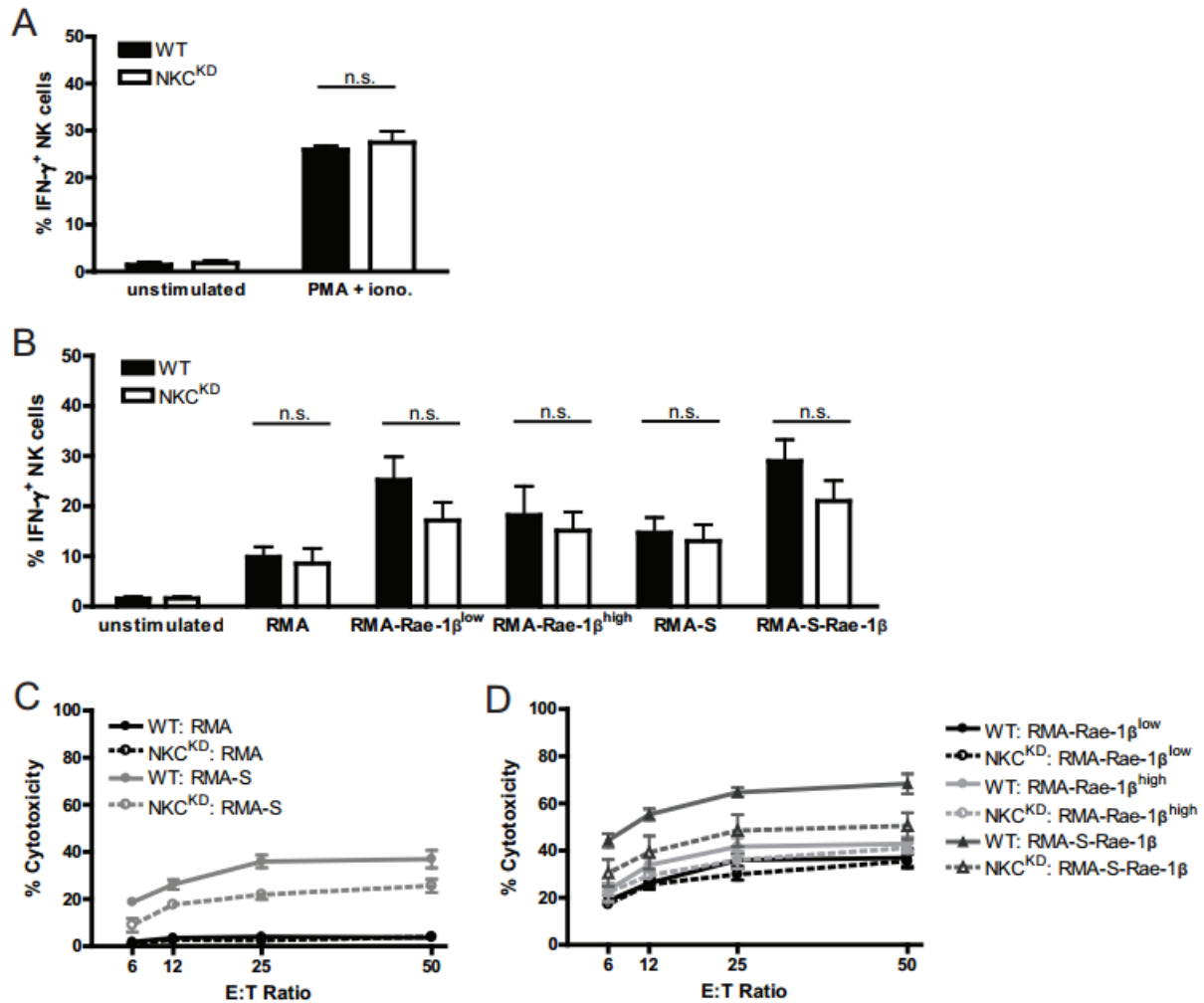


Figure S3.3: Decreased cytotoxicity but not IFN- γ production by NK^{KD} mice

(A) Splenocytes from WT ($n=6$) and NK^{KD} ($n=6$) mice were incubated with phorbol 12-myristate 13-acetate (PMA) and ionomycin. After 4.5 hours, intracellular staining was performed to assess the frequency of IFN- γ^+ NK cells. Data pooled from two independent experiments. (B) Splenocytes from WT ($n=5$) and NK^{KD} ($n=5$) mice stimulated 18 hours previously with 150 μ g poly(I:C) were incubated with RMA, RMA-Rae-1 β^{low} , RMA-Rae-1 β^{high} , RMA-S or RMA-S-Rae-1 β . After 5 hours, intracellular staining was performed to assess the frequency of IFN- γ^+ NK cells. Data are pooled from two independent experiments. (C) *In vitro* ^{51}Cr -release cytotoxicity assay utilizing adherent lymphokine-activated killer (ALAK) cells from WT and NK^{KD} mice to assess killing of RMA, RMA-Rae-1 β^{low} , RMA-Rae-1 β^{high} , RMA-S and RMA-S-Rae-1 tumor cells. Data are represented as the mean $\pm$ SD percentage of chromium release from triplicate wells. Data are representative of three independent experiments.

cells, which is not expected, the levels are lower than that of WT and may be attributable to residual Ly49 expression on approximately 20% of NK cells in NKC^{KD} mice. Overall, these results suggest that loss of Ly49 affects the cytotoxicity of the NK cells in response to missing-self signals.

Discussion

NK cells are known to play an important role in the *in vivo* recognition and control of tumors (Kim et al., 2000). Our genetic NKC^{KD} mouse model provides long-term Ly49 downregulation, allowing a study of NK cell targeting in cancer immunosurveillance. Flank tumor growth induced with MHC-I-deficient tumor cells is accelerated in NKC^{KD} mice; however, those induced with MHC-I-expressing tumor cells were comparable between WT and NKC^{KD} mice. This suggests the uncontrolled tumor growth in NKC^{KD} mice is a result of defective missing-self recognition. In the pulmonary metastasis model, there are a greater number of metastases on the lungs of NKC^{KD} mice than WT mice. However, the number of metastases induced by MHC-I-expressing transfectants in WT mice approached NKC^{KD} levels, since the functional NK cells are now inhibited by MHC-I on the tumor cells. Flank tumors induced with the carcinogen, MCA, exhibited accelerated onset and growth in NKC^{KD} mice, in accordance with previous work demonstrating control of MCA-induced tumors by NK cells (Smyth et al., 2000, 2001). Finally, in the oncogene-driven B cell lymphoma model, we observed that mice on the NKC^{KD} background develop lymphoma earlier than their WT counterparts also possessing the oncogene. The defect in NKC^{KD} mice is due to reduced cytotoxicity of the NK cells since decreased killing is observed in response to missing-self. Signaling through NKG2D is fully intact in NKC^{KD} mice; *in vitro* killing and *in vivo* rejection of RMA Rae-1-expressing target cells is similar between WT and NKC^{KD} mice. The observed differential killing and rejection of RMA-S-Rae-1-expressing target cells by WT and NKC^{KD} mice may be attributable to defective missing-self recognition in NKC^{KD} mice; though there is evidence that signaling through NKG2D may compensate for such defects (Bélanger et al., 2012).

Studies have reported that NK cells must undergo an education process in which interaction between inhibitory self-specific Ly49 and MHC-I molecules during development results in the acquisition of NK cell function (Fernandez et al., 2005; Kim et al., 2005). In our studies, following Ly49I^{Tg} restoration in NKC^{KD} mice, overall increased tumor control was observed, confirming that *in vivo* education is mediated by inhibitory self-specific Ly49. As well, the ability of Ly49I to rescue hyporesponsive NK cells supports that the phenomenon observed is the result of a loss of Ly49 expression and not due to the partial silencing of two other adjacent gene families, encoding the CD94/NKG2 and KLRI/E molecules (Bélanger et al., 2012). While flank tumor size in the NKC^{KD}-Ly49I^{Tg} mice is comparable to WT, suggesting near complete restoration of NK cell control, NKC^{KD}-Ly49I^{Tg} mice display slightly faster tumor growth and greater number of metastases, possibly due to the absence of other self-specific Ly49 receptors. In a 129-strain Ly49 repertoire, Ly49I binds to H-2K^b, and Ly49V, G2, and O bind to H-2D^b (Makrigrannis et al., 2001), and so these three other Ly49 receptors may also be important for NK cell education. As well, the contribution of other non-self Ly49s remains unclear; there is evidence that Ly49A recognizes the non-classical MHC-I molecule H2-M3 and mediates NK cell education (Andrews et al., 2012; Xu et al., 2006). H2-M3-deficient mice exhibit defective cytotoxicity and tumor control (Andrews et al., 2012; Xu et al., 2006).

MHC-I downregulation in various mouse and human cancers has been shown to be a common mechanism for tumor escape (Festenstein and Garrido, 1986; Restifo et al., 1993). B lymphoma cells isolated from NKC^{KD}.E μ -myc^{Tg} exhibit marked down-regulation of both H-2K^b and H-2D^b, in comparison to WT.E μ -myc^{Tg} mice as well as normal B cells from non-E μ -myc^{Tg} mice. The hyporesponsive state of NK cells in our NKC^{KD} model removes any benefit of a tumor

retaining MHC-I expression to evade NK cells, permitting these tumors to more readily downregulate MHC-I to escape effector T cell recognition.

NK-mediated immunoediting has been reported in the context of the strong activating receptor NKG2D, with respect to expression of its ligands H60 and Rae-1 (Guerra et al., 2008; O'Sullivan et al., 2011). Expression of H60a was reduced following passage in *Rag2*^{-/-} mice, which lack mature T and B lymphocytes, suggesting pressures from innate immune cells towards reduced ligand expression (O'Sullivan et al., 2011). Increased expression of Rae-1 was observed in NKG2D-deficient mice with prostate adenocarcinoma but not in mice with B cell lymphoma (Guerra et al., 2008), suggesting different immune evasion mechanisms are undertaken by different cancers. In our analysis of B cell lymphomas from NKC^{KD} and WT mice, no difference in NKG2D ligand expression was observed, which further highlights the differential MHC-I expression seen between NKC^{KD}.E μ -*myc*^{Tg} and WT.E μ -*myc*^{Tg}. Some cancers secrete NKG2D ligands as a way to nullify the function of NKG2D⁺ effector cells, and in such circumstances MHC-I expression may be the determining factor in tumor clearance by NK cells (Groh et al., 2002). Understanding the effects of MHC-I expression on clinical outcomes could help to improve current treatments employing Ly49 and KIR receptor signaling (Benson et al., 2012; Vey et al., 2012). Low MHC-I expression is highly correlated with increased tumor-associated necrosis and poor prognosis (Morabito et al., 2009; Speetjens et al., 2008); however, complete loss of MHC-I expression is an indicator of good prognosis (Madjd et al., 2005; Ramnath et al., 2006). Such observations suggest that total loss of MHC-I renders the tumor sensitive to NK cells. In contrast, partially reduced MHC-I allows them to potentially evade both NK and T cells: the allele for antigen presentation is lost thus eluding T cell recognition, and the remaining allele can inhibit NK cells. While cancer therapy clinical trials at present do not include tumor MHC-I expression analysis as part of

treatment monitoring, such practice could potentially improve the success of current therapies with targeted regimens towards specific MHC-I level and allele alterations.

This work contributes to a growing understanding of the role and importance of the Ly49 family in NK cells, not only as inhibitory receptors but also as necessary mediators of NK cell function. This study provides *in vivo* support for the importance of Ly49 in NK cell-mediated tumor immunosurveillance and MHC-I-directed tumor immunoediting as a result of loss of Ly49 expression.

Acknowledgments

The authors thank Dr. Lee-Hwa Tai and Christiano de Souza for guidance with the pulmonary metastases model and helpful discussion.

Chapter 4 : Ly49-dependent licensing alters NK cell immunosurveillance in breast cancer

Preface

The following chapter consists of original research looking at the role of the Ly49 receptors on NK cells in the control of breast cancer. The manuscript which comprises this chapter has been submitted for publication. The specific contributions of each author to this manuscript are listed below:

Tu MM: Designed, performed and analyzed data from all experiments. Wrote manuscript, and constructed all the figures.

Rahim MM: Assisted with the harvest and cell preparation of the mammary tumors from MMTV-PyVT mice. Performed intracellular staining for IFN γ on tumor-infiltrating cells. Provided critical reading of the manuscript.

Sayed C: Summer student who assisted with tumor measurements and performed qPCR analysis of the mammary tumors from MMTV-PyVT

Mahmoud AB: Provided the NK1.1 antibody and performed intracellular staining for IFN γ on tumor-infiltrating cells.

Makrigiannis AP: Supervised experimental design, execution and analysis. Provided critical reading of the manuscript.

Abstract

Natural killer (NK) cells are an integral part of the innate immune response, and are characterized by their ability to detect and kill transformed and virally-infected cells. Based on the missing-self hypothesis, NK cells survey for cells lacking class I major histocompatibility complex (MHC-I) molecules. Down-regulation of MHC-I in abnormal cells functions as a kill signal for NK cells. The inhibitory Ly49 receptor family recognizes MHC-I and plays a crucial role in developing fully functional NK cells. This work addresses the importance of Ly49 receptors in immune surveillance to cancer. Mice with knocked-down expression of Ly49 receptors (NKC^{KD}) were used to assess how immune variation, specifically of the Ly49 receptor in NK cells, can affect tumor development and surface receptor expression. NK cells were found to play a key role in the control of mammary tumors. Antibody-mediated depletion of NK cells led to accelerated mammary tumor development compared to wildtype mice. In the oncogenic mouse mammary tumor virus (MMTV) transgenic model, NKC^{KD} mice develop palpable mammary tumors earlier than wildtype background mice also possessing the transgene. Upon *ex vivo* analysis, tumors from NK-depleted and NKC^{KD} mice exhibited a decrease in expression levels of MHC-I. This work provides evidence supporting an MHC-I-directed tumor immunoediting phenomenon, where in the absence of a functional NK response, tumor cells preferentially downregulate expression of MHC-I, possibly to avoid recognition by other immune subsets.

Introduction

Breast cancer is one of the most commonly diagnosed cancers worldwide and is the leading cause of cancer-related deaths among women globally (Bray et al., 2004). While detection and treatment of the primary tumors has yielded many successes, many tumors are not so readily eliminated with directed adjuvant treatment. Relapse of the disease can lead to metastatic disease, and thus greater complications. Tumors are heterogeneous in nature and composed of a diverse and varied population of malignant cells (Al-Hajj et al., 2003). As a result of their different characteristics and properties, each cell may respond differently to the conventional treatments of surgery, chemotherapy and radiation. The prospect of harvesting the host's own immune cells to treat cancer provides new avenues for treatment, particularly in light of the discovery of tumor cell populations which are highly propagative and resistant to radiation therapy (Bao et al., 2006; Hambardzumyan et al., 2006; Woodward et al., 2007).

Cancer immunosurveillance describes the process in which the hosts' immune cells control tumor cell outgrowth and cancer progression. These immune cells act as an extrinsic tumor suppressor; however, due to the pressures exerted on the tumor cells by these immune cells, they are also helping to shape and mold the tumors. This cancer-immune interaction proceeds in three phases, known as the "three Es" of cancer immunoediting. First, the immune system is able to eliminate many of the tumor cells. Next, the immune system enters an equilibrium with those cancerous cells that are immune-resistant. Finally, the cancer cells develop enough resistance that they can escape the immune system, leading to a failure of immune-mediated cancer control. The three Es of elimination, equilibrium, and escape describe how the immune system is initially able

to control a tumor, but that this same tumor control can ultimately lead to an immune-resistant tumor.

Adaptive immunity is known to be crucial in the control of tumor cells and overall cancer progression - the presence of tumor-infiltrating CD8⁺ T cells is prognostic of disease-free survival and overall survival (Fridman et al., 2012). For this reason, adaptive immunity is being successfully targeted for clinical interventions in an attempt to break the pattern described by the three Es, leading to robust anti-cancer immune responses and improved clinical outcomes. The success of immune checkpoint inhibitors such as anti-CTLA-4 and anti-PD-1/PD-L1 in the clinic emphasizes the importance and the potency of T cells and adaptive immunity in treating cancer, and has brought cancer immunotherapy to the forefront of the future of cancer treatments, alongside the well-established practice of surgery, radiation and chemotherapy.

However, adaptive immunity is only one arm of the immune system, and there is mounting evidence for innate immune cells' involvement in cancer immunosurveillance. In particular, natural killer (NK) cells are known to be involved in antitumor immunity, and the therapeutic potential of this population against cancers warrants a great deal of study. In humans, NK cells comprise 10–15% of the lymphocytes in the peripheral blood. Murine studies have shown that NK cells can control various tumor cell lines, spontaneously-arising tumors, and tumors induced by the chemical carcinogen methylcholanthrene (Kaplan et al., 1998; Shankaran et al., 2001). In humans, the importance of NK cells is evidenced by correlative studies of disease and NK absence. Low levels of NK cell-mediated cytotoxicity are associated with an increased risk of cancer development (Imai et al., 2000). Similar to the evidence for T cells, NK infiltration into tumors is also prognostic of improved survival (Coca et al., 1997; Ishigami et al., 2000; Villegas et al., 2002),

though their numbers and percentages remain low compared to that of other lymphocytes (Albertsson et al., 2003; Esendagli et al., 2008).

The mouse lectin-like Ly49 family receptors control the reactivity of NK cells. Like their human homologues, the killer-cell immunoglobulin-like receptors (KIRs), Ly49 receptors function by recognizing class I major histocompatibility complex (MHC-I) molecules on potential target cells. NK cell activation is dependent on a balance of activating and inhibitory signals, with the dominant signal determining the response. Because MHC-I expression transmits an inhibitory signal to the NK cell through its Ly49 receptors, these NK cells are able to detect aberrant loss of MHC-I expression through a lack of an inhibitory signal; this phenomenon is known as “missing-self” recognition (Kärre et al., 1986). NK cells are then able to elicit direct cytotoxicity through the production of perforin and granzymes, as well as induction of other immune cells through the release of pro-inflammatory cytokines. In addition to missing-self recognition, NK cells are also able to recognize stress markers which are exhibited on the surface of stressed cells, such as tumors or virally-infected cells. These stress ligands are recognized by activating receptors on NK cells, which upon interaction with their ligands, induce NK activation, a response known as “induced-self” recognition (Raulet and Vance, 2006). Using mice deficient in the Ly49 family receptors, we have previously shown the importance of NK cells and the Ly49 family receptors in the control of leukemia and lymphoma (Tu et al., 2014). Building upon that previous work, we have now expanded our investigation to show a critical role for NK cells in controlling breast cancer. Breast cancer is a leading cause of death for women over the age of 35 (Tirona et al., 2010). Gene expression profiling of breast carcinomas have revealed their heterogeneity, as well as a varied molecular subtype. The five well-known molecular subtypes, luminal A, luminal B, ERBB2, basal, and normal-like, each exhibit distinct gene expression patterns and, more clinically relevant,

result in different survival outcomes (Sørle et al., 2006). Furthermore, solid tumors are more immunologically challenging than blood cancers like leukemia and lymphoma, and so it was important to test the clinical relevance of NK cell activity in controlling a solid tumor. We provide evidence for the role of NK cells and their Ly49 family receptors both in control of pre-established tumor cells, as well as in the early suppression before the tumor cells have become established through a transgenic oncogene mouse model. We provide data supporting the role of not only NK cells, but also their Ly49 receptors in the control of this solid tumor. In particular, there is compelling evidence for the role of both NK cells and their receptors in selective tumor immunoediting, wherein a different tumor surface expression signature is observed between mice possessing or lacking NK cells and the Ly49 receptors. We show that NK cells are able to infiltrate solid tumors and play a critical role in breast tumor development and intrinsic characteristics.

Materials and Methods

Mice

C57BL/6 (B6), MMTV-PyVT transgenic [strain: B6.FVB-Tg(MMTV-PyVT)634Mul/Lel1J], RAG1-deficient [B6.129S7-*Rag1*^{tm1Mom}/J] and IFN γ -deficient [strain: B6.129S7-*Ifng*^{tm1Ts}/J] mice were purchased from the Jackson Laboratory. B6.Ly49¹²⁹ and B6.NKC^{KD} (Klra15^{tm1.1Apm}) mice have been previously described (Bélanger et al., 2012). Because of linkage disequilibrium, B6.NKC^{KD} mice possess a 129-derived NKC. Therefore, the B6.Ly49¹²⁹ congenic mouse strain, which harbors a 129-derived NKC on the B6 background, serves as the genetically correct wild-type (WT) control. For brevity, B6.NKC^{KD} and B6.Ly49¹²⁹ are referred to as NKC^{KD} and WT.

MMTV-PyVT mice were crossed with WT and NKC^{KD} mice to produce WT.MMTV-PyVT and NKC^{KD}.MMTV-PyVT mice, respectively. Genotyping for the MMTV-PyVT transgene was performed by PCR using primers: forward (5' to 3') GGA AGC AAG TAC TTC ACA AGG G and reverse (5' to 3') GGA AAG TCA CTA GGA GCA GGG to attain a PCR amplicon size of 556 bp. All *in vivo* experiments were initiated in mice between 6 and 9 weeks of age. All mice were maintained in a specific-pathogen-free environment. Breeding and manipulations performed on animals were in accordance with and approved by the University of Ottawa Animal Ethics Committee (Ottawa, Ontario, Canada).

Purification of monoclonal antibody (mAb)

Anti-NK1.1 mAb was produced in the lab from the hybridoma clone PK136. PK136 was cultured in DMEM supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids,

0.1 mM β -mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Culture supernatants were then centrifuged (10,000x g for 20 min) and filtered through a 0.45 μ m filter. Monoclonal Ab was purified using Protein G sepharose chromatography (ExalphaBiologicals). Purified mAb was dialyzed in 1x PBS buffer (pH 7.4) and concentrated using an Amicon ultra-15 centrifugal filter unit with an ultracel-100 kDa membrane (EMD Millipore). The concentration was determined by SDS-PAGE and spectrophotometric measurement at 280 nm. For NK cell depletion, 100 μ L of 100 μ g/mL anti-NK1.1 mAb was injected intraperitoneally, at days -2, 0, 4, and every 3 days thereafter to maintain NK cell depletion in these mice.

Cells, antibodies and flow cytometry

The C57BL/6-derived breast cancer cell line E0771 was kindly provided by Dr. Ratna B. Ray (Saint Louis University), who originally obtained them from Dr. Rong Xiang (Scripps Institute).

MHC-I-deficient E0771 cells were generated by disrupting the H-2K^b and H-2D^b alleles of MHC-I using the CRISPR-Cas9 system as previously described (Ran et al., 2013). A 20-bp guide sequence (5'GCCCCGACTCAGACCCGCGC 3') targeting DNA within the first exon of both H-2K and H-2D was selected using the CRISPR Design Tool (<http://tools.genome-engineering.org>) and checked using the UCSC Genome Browser for specificity and off-target hits. The single guide RNA (sgRNA) was ligated into the pSpCas9(BB) (also known as pX330; Addgene plasmid ID: 42230) vector, and transformed into competent Stbl3 cells for storage. The sgRNA-containing vector was transfected into E0771 cells using Lipofectamine (Invitrogen), as per the manufacturer's instructions. E0771 cells lacking expression of both H-2K and H-2D were

identified by antibody labelling and cell sorting. Cells were single-cell cloned, expanded, and checked once again for absence of both H-2K and H-2B expression.

Antibody staining was performed using anti-CD45 (30-F11; BioLegend), anti-CD49b (DX5; eBioscience), anti-NKp46 (29A1.4; eBioscience), anti-TCR β (H57-597; BioLegend), anti-CD8 (53-6.7; BioLegend), anti-CD4 (GK1.5; eBioscience), Live/Dead (eBioscience), anti-Clr-b (4A6; kindly provided by Dr. Carlyle (University of Toronto)), anti-H-2K^b (AF6-88.5.5.3; eBioscience), anti-H-2D^b (KH95; BD Biosciences), anti-MULT1 (5D10; eBioscience), and anti-pan Rae-1 (186107; R&D Systems). Cells were acquired on a CyAN-ADP flow cytometer with Summit 4.3 software (Beckman Coulter) or LSR Fortessa (BD Biosciences) with FACSDiva software (BD Biosciences). Flow data was analyzed using Kaluza 1.2 software (Beckman Coulter)

Mammary fat pad injections

Groups of female B6, RAG1-deficient, IFN γ -deficient, WT (congenic) and NKC^{KD} mice were injected with 5×10^4 WT or CRISPR-mediated H-2K and H-2D knockout E0771 cells in the third left thoracic mammary fat pad. Some groups were depleted of NK cells using anti-NK1.1 mAb as described above. Mice were monitored for palpable tumors every three days post-injection. Tumor size was determined using an electronic caliper (Marathon) to measure length and width.

Spontaneous mammary tumor model

MMTV-PyVT mice were monitored weekly for tumor development and scored as tumor positive upon tumor width exceeding 5 mm and exhibiting progressive growth. Tumor size was determined using an electronic caliper (Marathon) to measure length and width. Tumor growth rate (mm²/day) was calculated based on the tumor area with respect to number of days since initial

tumor development. Tumor-bearing mice were euthanized upon exhibiting progressively enlarged tumors exceeding 15 mm in width or length. The spleen was dissociated to attain a single cell suspension. The tumors were removed and minced in 5 ml RPMI with 200 µg/mL collagenase D (Roche) and 20 µg/mL DNaseI (Roche), followed by incubation for 30 min at 37°C. The minced tumors were passed through a 100 µm cell strainer in order to obtain single cell suspensions.

Splenocytes and tumor cells were incubated with Fc block (anti-CD16/CD32), then stained with antibodies as described above. Mean fluorescence intensity (MFI) for H-2K^b, H-2D^b, Clr-b, Rae-1, and Mult1 expression in the spleen and tumors of MMTV-PyVT mice was standardized to spleen and mammary fat pad expression levels in WT control mice.

***In vitro* NK cell cytotoxicity assay**

Adherent lymphokine (IL2)-activated killer (ALAK) cells were grown in culture and used as effector cells in [⁵¹Cr]-release cytotoxicity assays, as previously described (Belanger et al., 2008). Intracellular staining for IFNγ was performed as previously described (Belanger et al., 2008). NKp46⁺TCRβ⁻ cells were analyzed for IFNγ by flow cytometry.

***In vitro* stimulation of tumor infiltrating lymphocytes**

Lympholyte-M (Cedarlane) was used to purify lymphocytes from the single cell tumor suspension. The isolated lymphocytes were incubated with YAC-1 cells at 1:1 ratio or with phorbol 12-myristate 13-acetate (PMA, 10 µg/ml) and ionomycin (1 µg/ml) in the presence of anti-CD107a mAb and brefeldin A (eBioscience) for 4 h. Cells were stained for surface markers as described above, followed by intracellular staining for IFNγ using IC fixation and permeabilization reagents (eBioscience) following the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from WT mammary fat pads (control) and mammary tumor cells from MMTV-PyVT mice. Extraction of mRNA from cells was completed using RiboZol RNA Extraction Reagents (Amresco), as per the manufacturer's protocol. mRNA concentrations following extraction were calculated using a Nanodrop Spectrophotometer (ND-1000) (Thermo Fisher Scientific). Quantitect Reverse Transcription Kit (Qiagen) was used to reverse transcribe 1 µg of mRNA into cDNA as per the manufacturer's protocol using the T100 Thermal Cycler (Bio-Rad). For qPCR analysis, 1/400 of the cDNA generated was used as a template and amplified with the KAPA SYBR® FAST Universal qPCR Master Mix (Kapa Biosystems) as per the manufacturer's protocol. PrimerQuest Tool was used to generate primers that were validated before use. Reactions were performed and data collected using the Mastercycler Realplex² qPCR machine (Eppendorf). Samples were analyzed in duplicates and the average of the two values was used for further analysis. Data was analyzed using the delta-delta CT method to quantify gene expression and graphs were made using Microsoft Excel (Scheffe et al., 2006). For this method, values obtained were first normalized to the values obtained for the amplification of 18S rRNA, and then normalized to WT mammary fat pads. These values were expressed for each respective gene. qPCR primer sequences: H-2K^b Forward (5' to 3'): CAG ATA CCT GAA GAA CGG GAA C, H-2K^b Reverse (5' to 3'): GCA CCT CAG GGT GAC TTT AT, H-2D^b Forward (5' to 3'): CTC TTG GGA AGG AGC AGA ATT A, H-2D^b Reverse (5' to 3'): AGC AAC GAT CAC CAT GTA AGA, 18S rRNA Forward (5' to 3'): CGCCGCTAGAGGTGAAATC, and 18S rRNA reverse (5' to 3'): CCAGTCGGCATCGTTTATGG.

Statistical analysis

Statistical comparisons were performed on Kaplan–Meier plots depicting tumor onset using the log-rank test with Prism (GraphPad Software). For the remainder, statistical significance was determined by a two-tailed t test with cutoff P value of 0.05. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

Results

Accelerated tumor growth and altered MHC-I expression on E0771 tumor cells in the absence of NK cells

Known NK cell targets include a variety of tumor cells of hematopoietic origins (Bélanger et al., 2012; Kärre et al., 1986; Kiessling et al., 1975b). The E0771 cell line is a medullary breast adenocarcinoma cell line which was originally isolated from a spontaneously arising tumor from a C57BL/6 mouse, and subsequently cultured and utilized for *in vitro* and *in vivo* studies of anti-cancer treatments (Ewens et al., 2005; Sirotnak et al., 1984).

Flow analysis revealed that E0771 cells express low to moderate levels of both MHC-I molecules, H-2K^b and H-2D^b (Figure 1A). As well, they expressed low levels of Clr-b—the ligand for the inhibitory NKR-P1B receptor—and Rae1, the ligand for the activating NKG2D receptor on NK cells (Figure 1A). The ability of NK cells to recognize and kill E0771 cells was assessed through an *in vitro* cytotoxicity assay in which the E0771 tumor cells were labeled with chromium-51 and incubated with adherent lymphokine-activated killer cells (ALAKs). It was observed that ALAKs were capable of recognizing and killing E0771 cells, with increased cytotoxicity at increased effector to target ratios (Figure 1B).

We asked whether NK cells can also effectively control these mammary tumor cells *in vivo*. 5×10^4 E0771 cells were injected into groups of B6 mice which were either treated with anti-NK1.1 mAb to deplete NK cells, or untreated. We observed that NK cell depletion resulted in increased tumor growth compared with the untreated mice, thus supporting a role for NK cells in the control of mammary tumor development *in vivo*. The tumor progression in each mouse (Figure 1C), as well as the mean tumor size over time (Figure 1D) shows the NK-depleted group

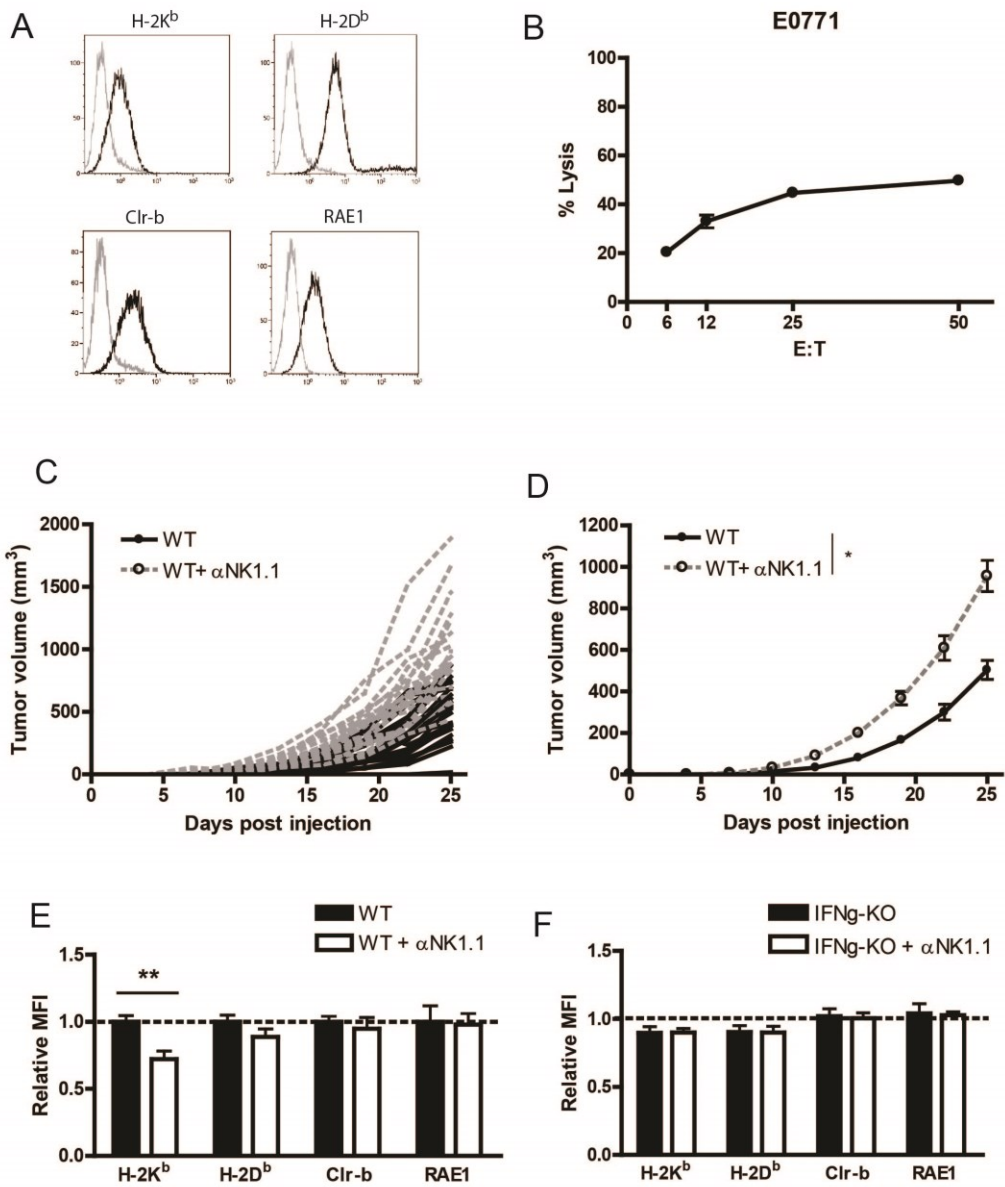


Figure 4.1: Control of E0771 tumor cells is NK-dependent

(A) Surface expression of H-2K^b, H-2D^b, Clr-b, and Rae1 on E0771. (B) Cytotoxicity assay showing killing of E0771 cells by B6-derived ALAKs. Data representative of four independent experiments. (C) Formation of mammary tumors following injection of 5×10^4 E0771 cells into the mammary fat pad of groups of B6 (n=22) and NK-depleted B6 mice (n=23). Left panel, each line represents a single mouse. Right panel, mean $\pm$ SEM. Data pooled from six independent experiments. *Ex vivo* tumor analysis for surface expression of H-2K^b, H-2D^b, Clr-b, and Rae1 from (E) B6 (n=18) and NK-depleted B6 (n=19) mice. Data pooled from five independent experiments. (F) IFN γ -KO (n=14) and NK-depleted IFN γ -KO (n=13) mice. Data pooled from three independent experiments.

bearing larger tumors than the untreated group at comparable times. These tumors were analyzed following endpoint at day 25, and the presence of tumor infiltrating lymphocytes (TILs) was assessed. On average, 3% of the tumor infiltrating immune cells were identified as NK cells, whereas less than 0.3% were found in the NK-depleted mice (Supp 1A). No difference was seen in the percentage of CD4 or CD8 T cells in the NK-depleted or undepleted group (Supp 1B). The quantitative difference in NK cell but not T cell proportion between the two groups supports that the accelerated tumor progression observed is a result of loss of NK cells.

MHC-I is expressed on the surface of all nucleated cells. In the case of a tumor cell, the MHC-I molecule can play a role in CD8 T cell recognition and killing of the tumor cell. Non-self and altered-self peptides can be presented by the MHC-I molecule, thus identifying this tumor cell as aberrant and alerting the CD8 T cell to kill the tumor cell. Conversely, MHC-I also plays a role in NK cell recognition of tumor cells. While CD8 T cells recognize the non-self peptide presented by the MHC-I molecule, NK cells recognize the loss of MHC-I on the cell surface as a marker of an aberrant cell (Kärre et al., 1986). NK cells surveil the body for cells which lack MHC-I, which is considered to be a marker of health as it is normally expressed on all nucleated cells. The absence of MHC-I on the cell surface leads to the recognition of this tumor cell as aberrant due to its ‘missing-self’ and induces NK cell killing of this cell (Kärre et al., 1986). NK cell recognition of MHC-I is peptide dependent for stable MHC-I presentation on the cell surface, but not peptide-specific, as is the case with CD8 T cells. It is hypothesized that in the absence of NK cells, tumor cells which lack MHC-I expression will thus be the most successful at immune evasion, as they will be able to evade CD8 T cell recognition, since there is no MHC-I molecule to present non-self peptides which would alert the CD8 T cells. Conversely, in the case of a wildtype environment with both functional CD8 T cells and NK cells, the tumor cells will need to maintain a moderate

level of MHC-I expression since they still have the requirement to evade NK cells. Analysis of the surface expression of the tumors in NK-depleted and undepleted mice revealed this differential H-2K^b expression which is in line with our hypothesis (Figure 1E). We believe that in the absence of NK cells, the tumor cells which express higher levels of H-2K^b were recognized and killed by CD8 T cells, whereas the tumor cells which have lower levels of H-2K^b were able to evade detection and killing, and thus they comprise the bulk of the tumor at endpoint analysis. Analysis of the lungs for pulmonary metastases revealed no quantifiable difference in metastases due to their low numbers (Supp 1C).

Interferon γ (IFN γ) is well known to play a role in MHC-I expression on cells. In some tumor cell lines, treatment with IFN γ can increase MHC-I levels (Zhou, 2009). We questioned whether the reduction in H-2K^b levels in the tumors from the NK-depleted could be the result of changes in IFN γ levels in the tumor microenvironment. In order to assess this, we analyzed tumor infiltrating CD4 and CD8 T cells for IFN γ production. The percentage of IFN γ expressing CD4 (Supp 1D) and CD8 (Supp 1E) were similar between the undepleted and NK-depleted groups. We then went on to look at tumors isolated from IFN γ -KO mice. We hypothesized that if the differences in H-2K^b expression levels observed in NK-depleted mice were the result of a difference in IFN γ levels in the tumor microenvironment, this difference between the NK-depleted and undepleted group would thus be abrogated in an environment lacking IFN γ . We tested this in IFN γ -KO mice, which were injected with 5×10^4 E0771 cells into the mammary fat pad to induce mammary tumors, and the tumors analyzed. As expected, we no longer saw a difference in H-2K^b levels between the NK-depleted or undepleted group in the IFN γ -KO mice (Figure 1F).

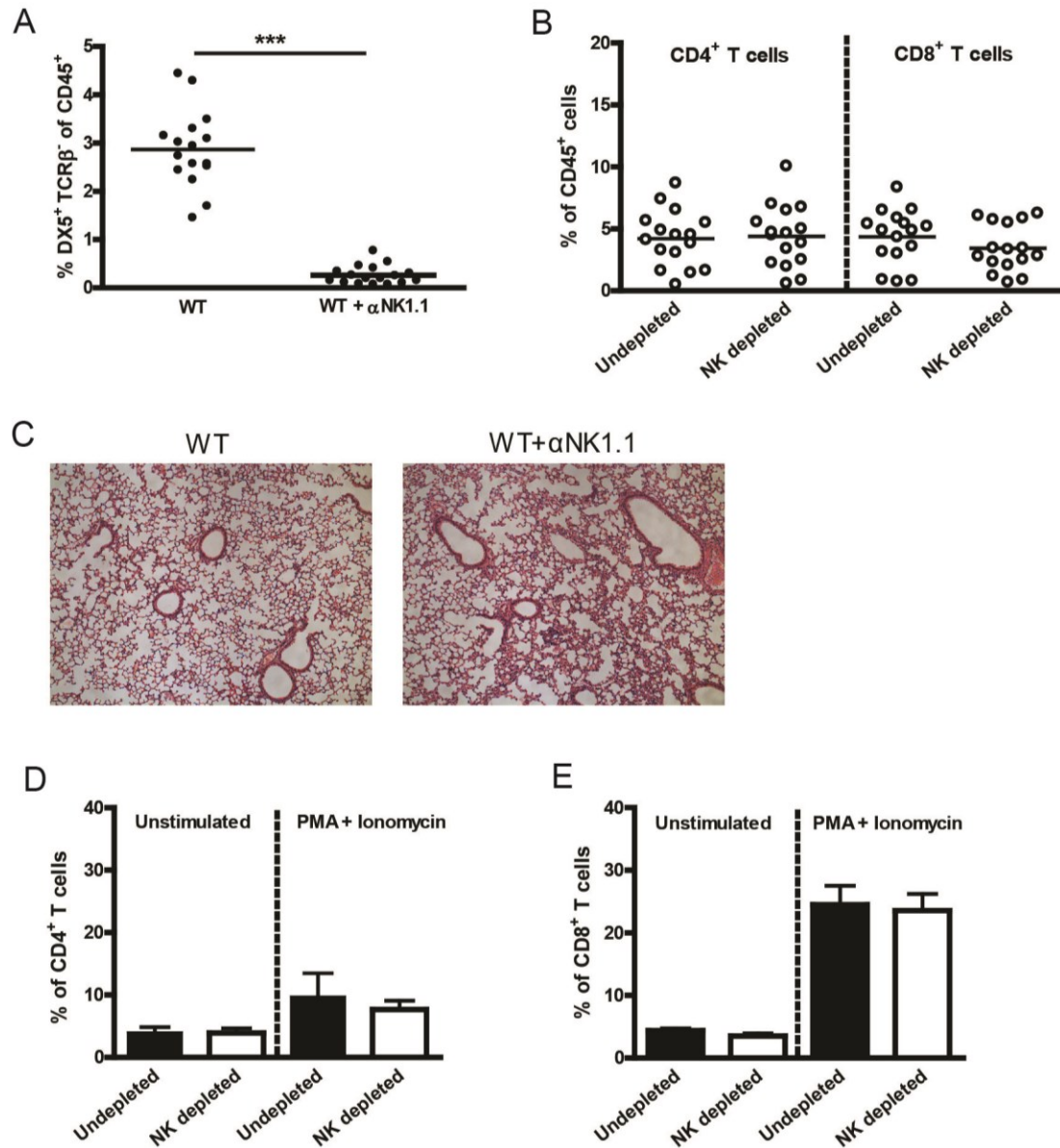


Figure Supp 4.1: *Ex vivo* analysis of tumors from B6 and NK-depleted B6 mice for tumor-infiltrating

(A) NK cells, and (B) CD4⁺ and CD8⁺ T cells. Each dot represents a single tumor. Horizontal line represents mean. (C) H&E stained lung cross sections from tumor-bearing B6 and NK-depleted B6 mice. *Ex vivo* analysis of 5×10^4 E0771-induced tumors from B6 and NK-depleted B6 mice for interferon γ (IFN γ) production by the tumor-infiltrating (D) CD4 (n=14) and (E) CD8 (n=14) T cells. Mean $\pm$ SEM. Data pooled from four independent experiments.

Defective recognition and control of MHC-I-deficient E0771 tumors in mice lacking Ly49 receptors

While E0771 cells were identified to be good targets for NK cells, for our purposes of studying the role of the Ly49 receptor family, the expression of MHC-I molecules on the cell surface would render these cells not useful since our previous work has established that mice which lack the Ly49 receptor family are defective in missing-self recognition of MHC-I-deficient tumors, but are otherwise normal in response to MHC-I expressing tumors (Tu et al., 2014). Using CRISPR-Cas9 mediated technology, we knocked-out expression of the two MHC-I molecules H-2K and H-2D in E0771 cells. Analysis by flow cytometry showed decreased H-2K^b and H-2D^b levels in the CRISPR-Cas9 knockout, compared to WT empty vector (Figure 2A). Cytotoxicity assays of the killing of WT E0771 cells by WT and NKC^{KD}-derived ALAKs suggested no difference between NK cells from WT or NKC^{KD} mice, as expected (Figure 2B). However, killing of the MHC-I-deficient E0771 cells by NKC^{KD} was much less than that of WT ALAKs at comparable effector to target ratios (Figure 2C). Loss of MHC-I expression renders the E0771 target cells more susceptible to killing by WT NK cells. *In vivo* analysis of the growth of E0771 in the mammary fat pads of WT and NKC^{KD} mice revealed similar tumor growth, as expected (Figure 2D). However, when MHC-I-deficient E0771 cells were injected into the mammary fat pads of WT and NKC^{KD} mice, we observed that the tumor developed and grew more rapidly in NKC^{KD} than WT mice (Figure 2E).

Ly49-dependent control and immunoediting of mammary tumors

Using the MMTV-PyVT transgenic mice, we were able to monitor the development of mammary tumors in mice on the WT or NKC^{KD} background to better understand the effect

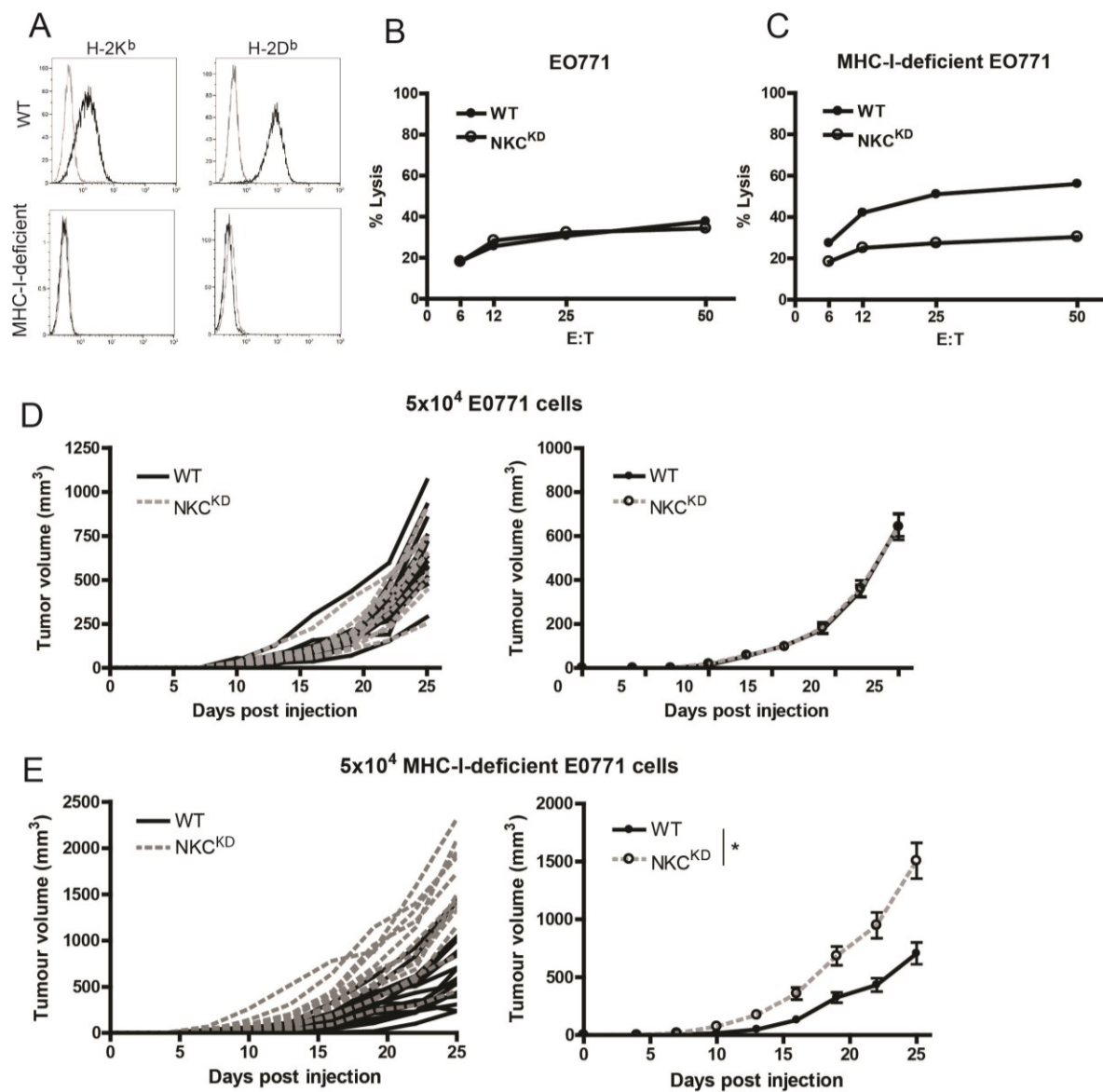


Figure 4.2: Control of MHC-I-deficient E0771 is dependent on Ly49-mediated licensing

(A) Surface expression of H-2K^b and H-2D^b on WT and CRISPR-Cas9-mediated H-2K and H-2D knockout E0771. (B) Cytotoxicity assay showing killing of E0771 tumor cells by WT- and NKC^{KD}-derived ALAKs. Data representative of four independent experiments. (C) Cytotoxicity assay showing killing of MHC-I-deficient E0771 tumor cells by WT- and NKC^{KD}-derived ALAKs. Data representative of three independent experiments. (D) Formation of mammary tumors following injection of 5×10^4 E0771 cells into the mammary fat pad of groups of WT (n=15) and NKC^{KD} (n=11) mice. Left panel, each line represents a single mouse. Right panel, mean $\pm$ SEM. Data pooled from three independent experiments. (E) Formation of mammary tumors following injection of 5×10^4 MHC-I-deficient E0771 cells into the mammary fat pad of groups of WT (n=16) and NKC^{KD} (n=13) mice. Left panel, each line represents a single mouse. right panel, mean $\pm$ SEM. Data pooled from three independent experiments.

which silenced Ly49 expression had on breast cancer development. The MMTV-PyVT mice express the Polyoma virus middle T antigen under the control of the mouse mammary tumor virus promoter (Guy et al., 1992). Female mice were monitored weekly for palpable tumors in any one of their ten mammary glands. The detectable presence of a tumor exceeding 3mm in diameter with progressive growth was scored as tumor positive. Mice lacking the Ly49 receptor families developed palpable tumors earlier on than their WT counterparts (Figure 3A). The earliest tumor onset was detected in NKC^{KD} mice at day 62, compared to day 88 in WT mice. The first-arising tumor in each mouse was measured weekly and the tumor size was plotted relative to the age of the mice (Figure 3B). The tumor growth rate of tumors arising in NKC^{KD} mice was greater than that of WT mice (Figure 3C).

NKC^{KD} mice lack MHC-I-educated NK cells due to their loss of the Ly49 receptors, and as such, we hypothesized that the tumors which develop in WT and NKC^{KD} mice will exhibit different properties as a result of their different microenvironment and immunoediting. The early stage tumors and end stage tumors from WT and NKC^{KD} mice were analyzed for expression of the MHC-I molecules H-2K^b and H-2D^b, as well as other ligands for activating and inhibitory receptors on NK cells—Clr-b, MULT1, and RAE1. The early stage tumors allowed for insight into the initial tumors which developed and act as a baseline for comparison of changes which occurred as the tumors underwent the cancer immunoediting stages of elimination, equilibrium, and escape (Dunn et al., 2004). Ligand expression in early stage tumors from both WT and NKC^{KD} were comparable with both exhibiting overall high levels of H-2K^b expression and low levels of the other ligands analyzed (Figure 4A, B). In comparison, the end stage tumors were quite distinct between WT and NKC^{KD} . The tumors isolated from WT mice retained their higher levels of H-2K^b on the tumor cell surface (Figure 4C), whereas the end stage tumors from

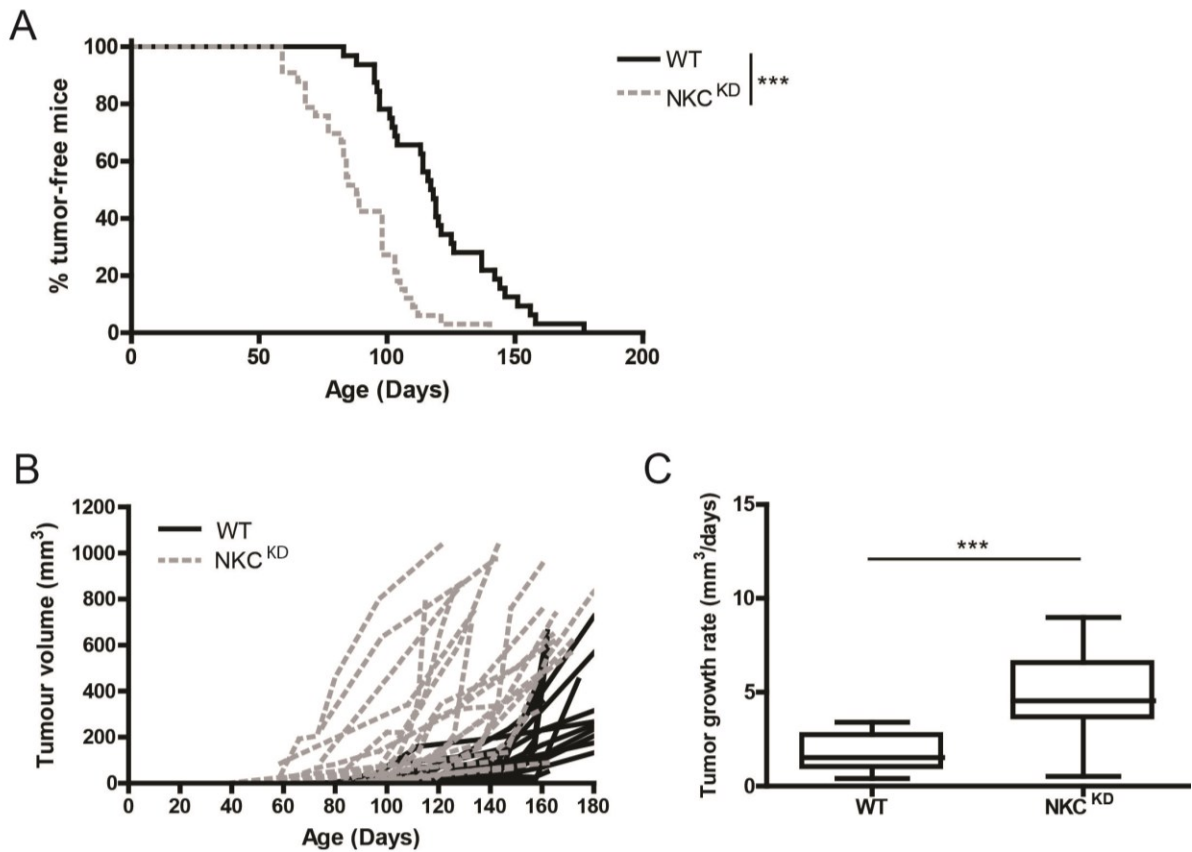


Figure 4.3: NK cell-mediated control of mammary tumor development is Ly49-dependent

(A) Mammary tumor development in NKC^{KD}.MMTV-PyVT^{Tg} (n=31) and WT.MMTV-PyVT^{Tg} (n=31) mice. (B) Individual tumor growth was measured weekly in WT (n=20) and NKC^{KD} (n=21) mice, with each line representing a single tumor. (C) Tumor growth rate calculated from tumor size in (B), with respect to time. Top and bottom limits of the box represent 25th and 75th percentiles, with whiskers indicating minimum and maximum values.

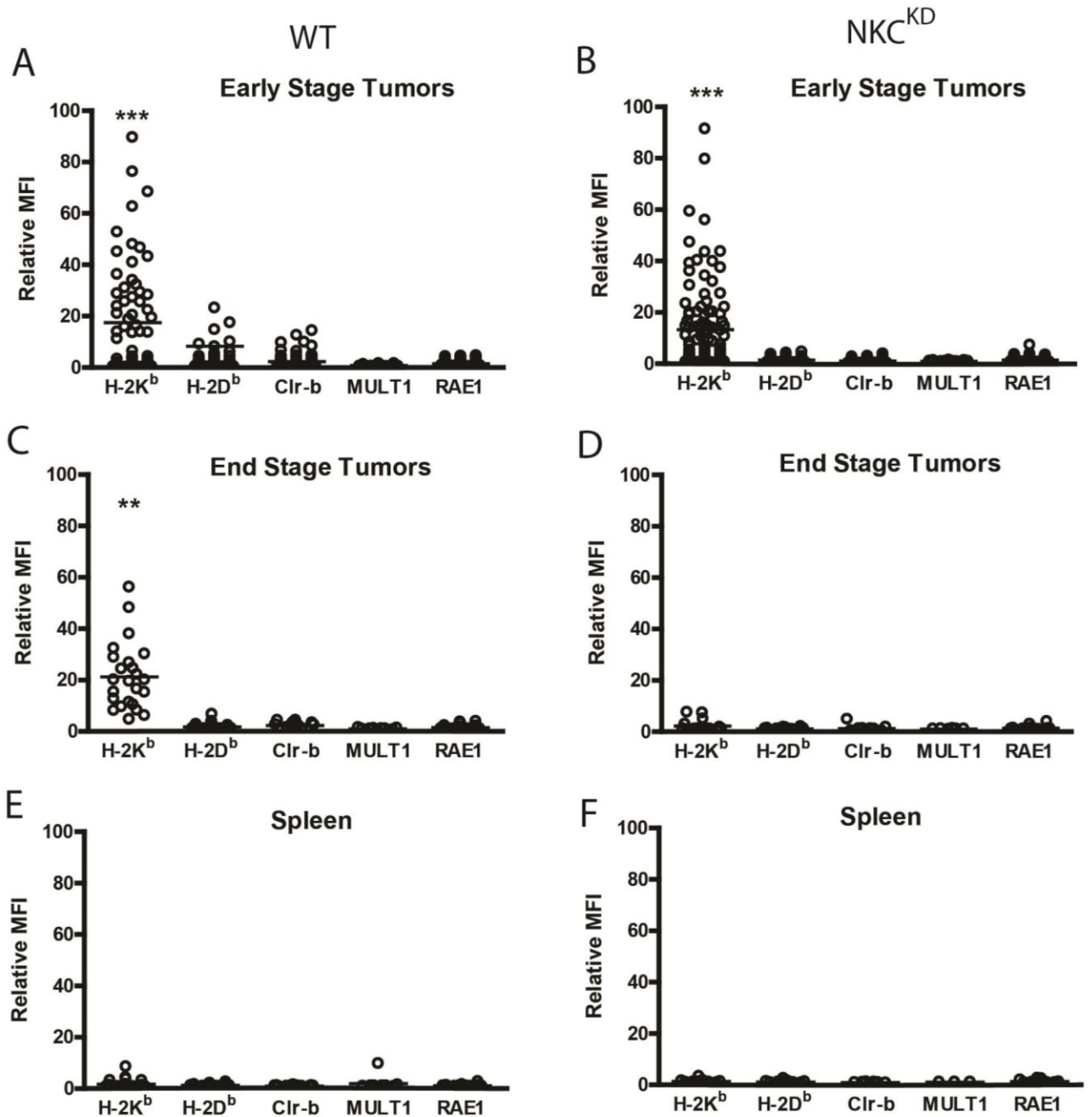


Figure 4.4: Alterations in MHC-I expression on mammary tumors over time due to altered NK cell receptor expression

(A, B) *Ex vivo* analysis of early stage mammary tumors from WT (n=92) and NKC^{KD} (n=101) mice, (C, D) endpoint tumors from WT (n=24) and NKC^{KD} (n=26) mice, and (E, F) spleens from WT (n=24) and NKC^{KD} (n=26) mice. Surface expression was analyzed by antibody labelling and flow cytometry. For tumors, MFI was standardized to expression on mammary fat pads from WT, non-MMTV transgenic mice. For spleens, MFI was standardized to expression on spleens from WT, non-MMTV transgenic mice. Each dot represents a single tumor. Horizontal line represents mean.

NKC^{KD} exhibited low level expression of H-2K^b (Figure 4D). The spleen was also analyzed to ensure that any differences seen were not the result of varied antibody staining, flow acquisition or analysis (Figure 4E, F). These observations support the gradual process of cancer immunoediting following initial tumor development. The similarities in the tumors from WT and NKC^{KD} mice in early stage tumors, and the observed digression at the end stage tumors suggests that the initial tumors which develop, will over time, be molded into different paths by the selective pressures exerted on them by the immune cells within the tumor microenvironment. In this case, due to the loss of the Ly49 receptors rendering the NK cells hyporesponsive, tumors which developed in the NKC^{KD} mice may be preferentially downregulating H-2K^b expression in order to avoid recognition and killing by CD8 T cells, since there was a reduced threat from NK cells.

It was then of interest to determine what is the cause of this H-2K^b downregulation. Either the H-2K^b molecule was no longer being produced at the RNA level, or else was simply not being presented to the cell surface and thus not detectable through antibody cell surface staining. The MFI attained flow values were compared to the qPCR attained expression levels from the same tumor. It was expected that if the RNA is being made, but just at lower levels, and then being presented to the cell surface, we would observe a plot where the overall slope would be close to 1. In contrast, if RNA was being made, but possibly due to some defect in antigen processing, was not being presented to the cell surface, we would expect that the slope of the line would be much greater than 1, as the qPCR values would be vastly exceeding that for MFI. Overall, a linear correlation was observed for both H-2K^b and H-2D^b in WT and NKC^{KD}-derived tumors (Figure 5). This suggested that the changes in MFI surface expression levels are

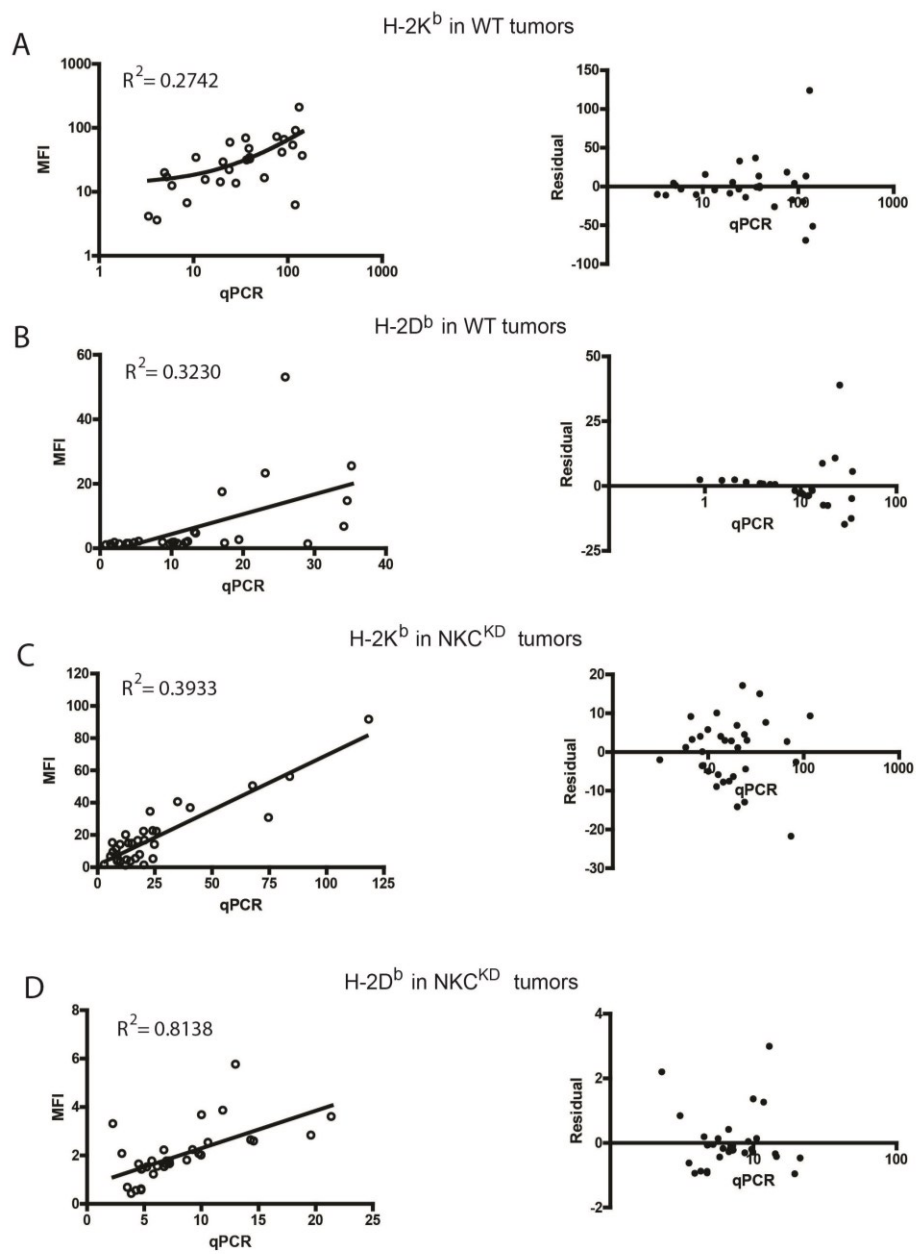


Figure 4.5: Comparison of mammary tumor H-2K^b and H-2D^b expression using qPCR and flow cytometry

Analysis of tumors isolated from WT mice for (A) H-2K^b and (B) H-2D^b expression. NKC^{KD} mice for (C) H-2K^b and (D) H-2D^b expression. Left panels represent comparison of data acquired from qPCR compared to flow cytometry. Right panels represent residual plots of the corresponding comparison.

reflective of the changes in the RNA levels within the cell. The residual plot displays a random patterning indicative of a linear regression pattern.

MHC-I expression positively correlates with tumorigenesis of primary mammary tumors

We then wondered whether the observed differences in H-2K^b in end stage tumors from WT and NKC^{KD} affected their overall tumorigenicity. Tumors were resected from WT and NKC^{KD} mice, and single cell tumor suspensions injected into the mammary fat pads of groups of naïve WT mice. The MFI for MHC-I expression of the primary tumor which was isolated from the initial-tumor bearing mouse was compared to the MFI following growth for 40 days in the secondary host (Figure 6A, B). Primary tumors from WT mice, which originally exhibited higher levels of H-2K^b expression, retained their high level (Figure 6A). In comparison, primary tumors from NKC^{KD} mice, which originally exhibited low levels of H-2K^b expression, also retained their low expression levels (Figure 6B). The levels of H-2D^b, Clr-b and RAE1 remained low and unchanged following both WT and NKC^{KD}-derived tumor transplantation into naïve WT (Figure 6A, B). The WT-derived tumors grew much better following transplantation into WT naïve recipients, compared to NKC^{KD}-derived tumors (Figure 6C). We hypothesize that the tumors which developed in the NKC^{KD} mice had undergone tumor immunoediting in response to a tumor microenvironment with hyporesponsive NK cells with their low MHC-I expression levels, and thus when transplanted into a WT environment with fully functional NK cells, these MHC-I-low tumors were quickly recognized and killed by NK cells in the WT host (Figure 6D). Analysis of the tumor-infiltrating NK cell and T cell comparing WT and NKC^{KD}, and early versus late revealed no difference in cell percentages (Supp 2A-D). Analysis of IFN γ production by CD4 and CD8 T cells revealed no distinct differences (Supp 2E-H). These observations suggest that the overall differences in tumor control are the result of hyporesponsive NK cells in NKC^{KD} due to the

silencing of the Ly49 receptors, which play a critical role in NK cell recognition of aberrant cells through 'missing-self'.

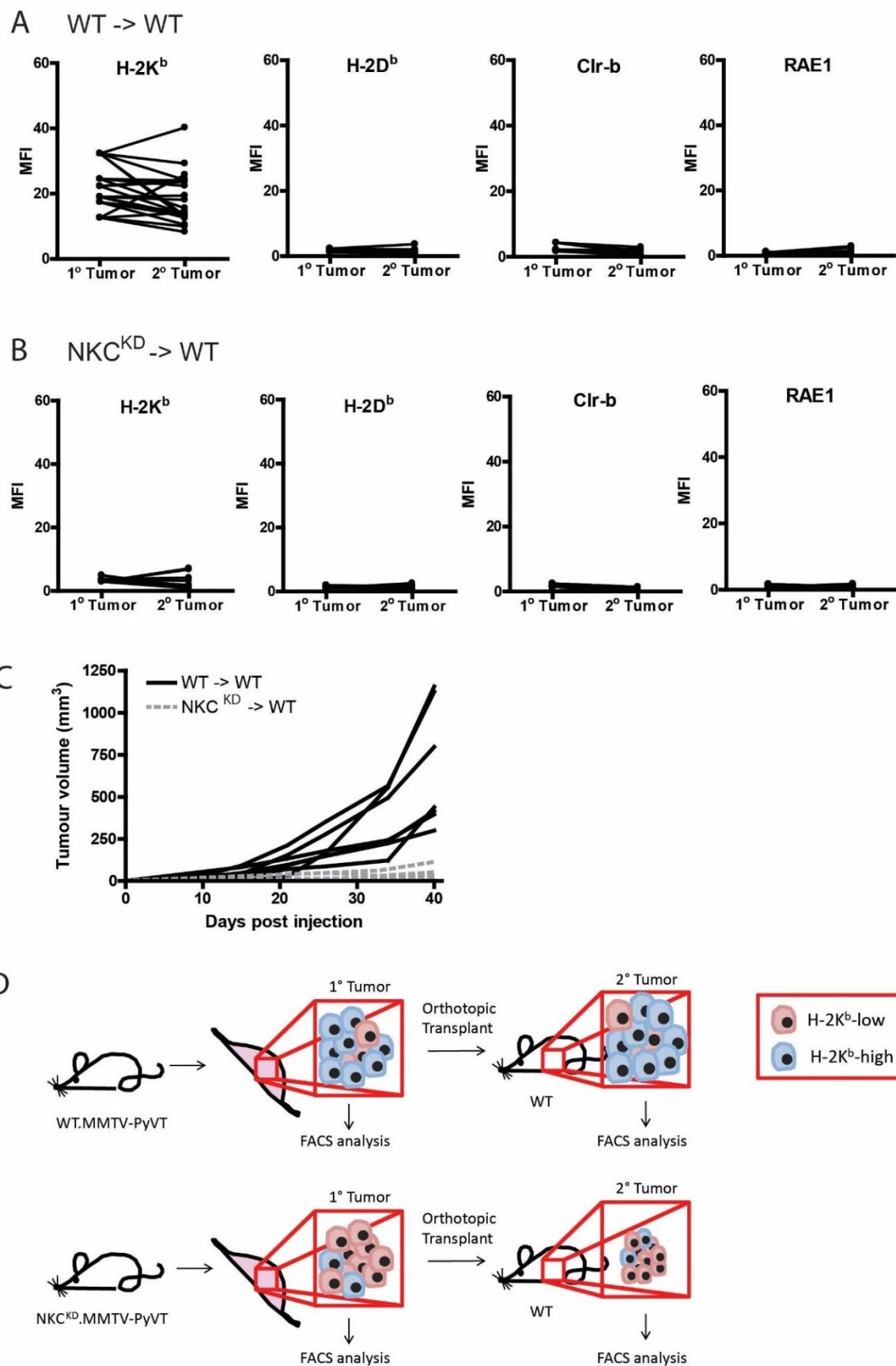


Figure 4.6: Altered MHC-I expression of primary tumors affects tumorigenicity in naïve recipients

(A) Initial expression levels of H-2K^b, H-2D^b, Clr-b, and Rae1 in WT-derived primary tumor and MHC-I expression levels of transplanted tumors following growth in recipient WT mice (n=23). (B) Initial expression levels of H-2K^b, H-2D^b, Clr-b, and Rae1 in NKC^{KD}-derived primary tumor and MHC-I expression levels of transplanted tumors following growth in recipient WT mice (n=15). (C) Tumor growth curves of secondary tumor following transplantation into WT recipient. Each line represents a single mouse. (D) Schematic model of WT- or NKC^{KD}-derived primary tumor transplantation into wildtype naïve recipient mice.

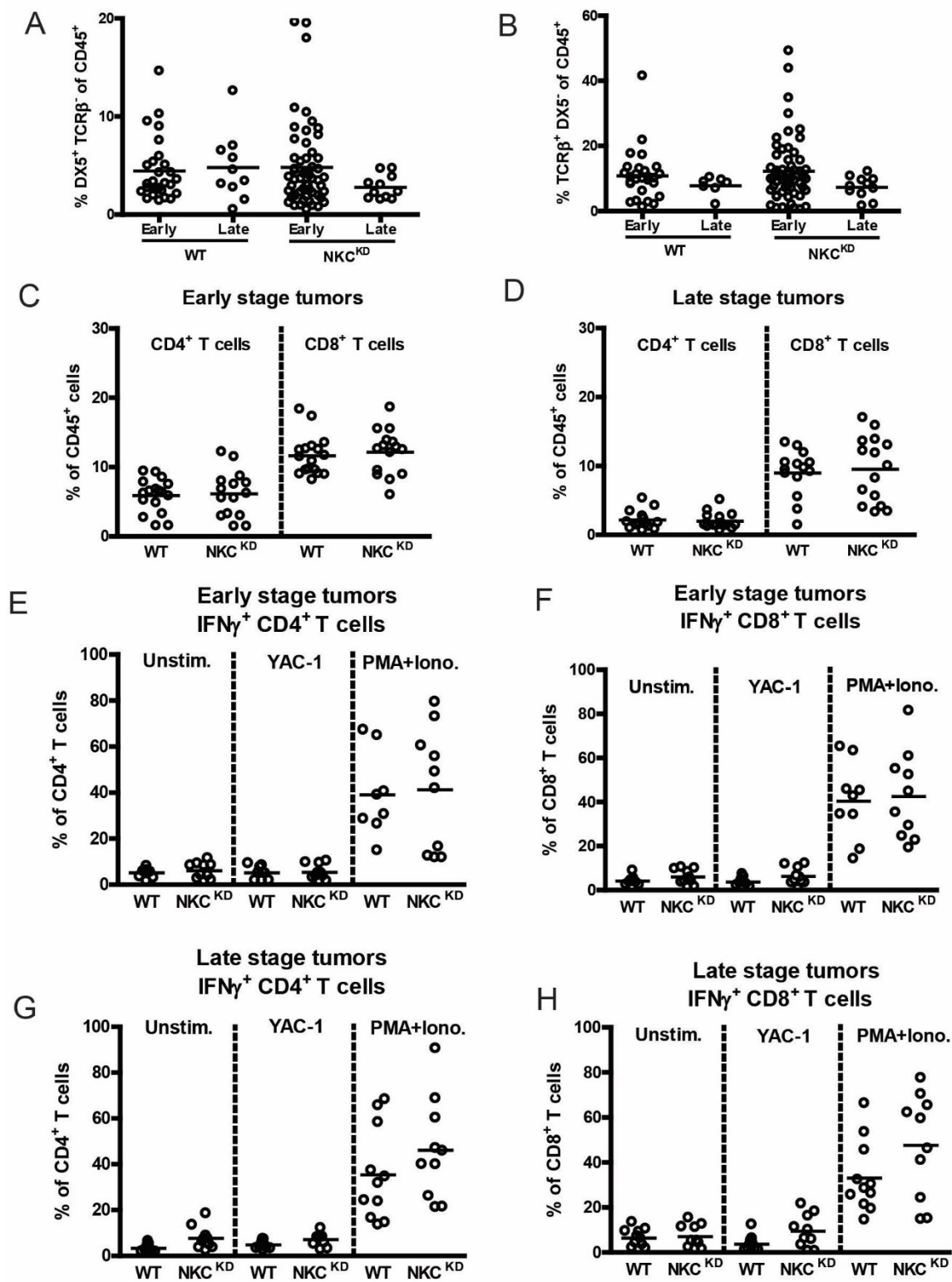


Figure Supp 4.2: Analysis of tumor-infiltrating lymphocytes

(A) NK cell infiltration. (B) T cell infiltration. CD4 and CD8 infiltration in (C) early stage and (D) end stage tumors. IFN γ production by tumor-infiltrating (E) CD4 T cells and (F) CD8 T cells in early stage tumors, and (G) CD4 T cells and (H) CD8 T cells in end stage tumors.

Lymphocytes were unstimulated, or stimulated with YAC-1 cells or PMA + ionomycin. Each dot represents a single tumor.

Discussion

Due to the known role of NK cells in anti-tumor immunity, NK cells have been well studied in response to various forms of malignancies. In addition to general immune defects such as reduced peripheral lymphocyte numbers and impaired IFN γ production, NK-specific alterations have also been identified in breast cancer patients (Caras et al., 2004). In this study we provide *in vivo* data showing the importance of NK cells in breast cancer, which supports published clinical observations and correlative assessments from human patient tumors. We show that breast tumor cells express markers which are recognized by both activating and inhibitory receptors on NK cells. In the absence of NK cells, E0771-induced tumors grow much more rapidly. NKC^{KD} mice exhibit a defect in the control of MHC-I-deficient E0771 tumors. In an oncogene-driven model of spontaneous breast cancer, NKC^{KD} mice develop tumors earlier than their wildtype counterparts, and these tumors appear to grow at a more accelerated rate. Upon analysis of the tumors which developed in a wildtype and NKC-deficient NK cell tumor microenvironment, clear differences can be seen in the expression level of the MHC-I molecule, H-2K^b. While early stage tumors from both mice exhibit an upregulation of H-2K^b expression, in late stage tumors, H-2K^b expression appears to be lost in NKC^{KD} mice, while maintained in WT mice. Similarly, H-2K^b is downregulated on E0771-induced tumors in the absence of NK cells. This provides direct evidence for the phenomenon of tumor immunoediting. In the absence of a selective pressure via MHC-I receptors (Ly49) in the NK cell-depleted and NKC^{KD} mice, the tumors have evolved to a more collectively low MHC-I phenotype, since MHC-I levels do not need to be maintained to evade NK cell recognition. Conversely, they may preferentially downregulate MHC-I to avoid recognition by the cytotoxic T cells, which remain fully intact and functional in NKC^{KD} mice. MHC-I expression on MHC-I-low tumors, which arise in NKC^{KD} mice, remain low, even following

transplantation into a WT environment; however, due to their low MHC-I profile, they are greatly susceptible to recognition and killing by NK cells in WT mice which have fully functional missing-self recognition.

In a retrospective study comparing the molecular tumor signatures of breast cancer patients with relapse or relapse-free survival, it was observed that expression of molecules involved in the activating signaling of NK cells and in NK:target interaction are increased in patients with a favorable prognosis. Increased expression of the NK activating receptors, NKp30 and NKp44, and NK adhesion molecule, LFA-1, is observed in patients who do not experience a recurrence (Ascierto et al., 2013). In addition to differential NK cell properties and function between breast cancer patients and healthy control individuals, differences in NK cells can be seen based on the stage of the disease. When comparing patients with breast malignancies and those with benign tumors, those with the malignant disease have significantly decreased NK cell-mediated lysis capabilities (Garner et al., 1983). As well, it appears to be correlative with disease stage. NK cells from patients with early cancers (stage I) have more lysis potential than those from advanced breast cancer (stage II, III, and IV) (Garner et al., 1983). NK cell functions are profoundly altered in advanced breast cancer patients. Advanced breast cancer patients have an increased proportion of immature and non-cytotoxic NK cell subsets in their peripheral blood (Mamessier et al., 2013). In a comparison study of the NK cells found in the peripheral blood and tumor infiltrate of breast cancer patients, NK cells found within the tumor exhibited a pronounced defect in cytotoxicity when compared to NK cells from healthy donors, and most interestingly, from the peripheral blood of breast cancer patients. Tumor-infiltrating NK cells exhibited lower levels of the activation marker CD69, the degranulation marker CD107, IFN γ and TNF α (Mamessier et al., 2011a). While mammary tissue from breast cancer patients express various activating ligands, such as MICA/B

and ULBP, for NK cell receptors which should activate the NK cell to eliminate these cells; however, expression of these activating NK cell receptors, NKp30, NKG2D, DNAM-1 and CD16, were decreased (Mamessier et al., 2011a). This decrease in activating receptors is interestingly correlative with disease progression, suggesting that NK cells play an important role in the control and delayed progression of breast cancer in humans (Mamessier et al., 2011a).

We and other groups have shown that breast tumor cells express protein ligands for both activating and inhibitory NK cell receptors (Mamessier et al., 2011b). Mamessier et al have identified HLA receptors (KIRs and/or NKG2A), NKG2D, and DNAM-1 as the most common and important receptors regulating the recognition of breast tumor cells by NK cells (Mamessier et al., 2011b). The activating NKG2D and NKp30, and DNAM receptors have been reported to be downregulated following co-culture with various breast cancer cell lines (Mamessier et al., 2011b).

Another study has also observed the alteration of the NK cell phenotype and function in a murine model of spontaneously developing invasive mammary tumors using the MMTV/Neu, which utilizes the neu protooncogene, similar to the Py-VT in our study (Mamessier et al., 2011a). Studies by Hoover et al have shown that mice deficient in the NK lytic-associated molecule (LAM) are more susceptible to primary and metastatic tumor growth (Hoover et al., 2009, 2012). While we observed similar E0771 primary tumor control defects in our knockdown mice to NK-LAM-deficient mice, we saw no difference in the metastasis levels. Our analysis of gross pathology of the lung may not have been sensitive enough to detect significant difference between our wildtype and NKC^{KD} group, as GFP expression was used in the aforementioned study (Hoover et al., 2012).

Further work into the NK cells which localize to the tumor environment could provide more insight into the properties of these NK cells, their preferential localization to the tumor in

comparison to the periphery and a better understanding of their functional capabilities in cancer patients. One group has shown that the tumor-infiltrating NK cells in human breast cancer possess an uneducated phenotype (Mamessier et al., 2013). Due to the plastic nature of NK cells and their ability to become re- or un- educated, dependent upon their environment, it may be that uneducated NK cells preferentially localize to the tumor site (Elliott et al., 2010; Joncker et al., 2010). Alternatively, the educated NK cells, upon tumor infiltration and exposure to the tumor microenvironment, become uneducated. The latter may be a result of the known low levels of MHC-I exhibited by late stage tumors (Goepel et al., 1991).

While approaches to the treatment of breast cancer in the clinic are widely dependent on the cancer subtype - luminal A, luminal B, ERBB2, basal, and normal-like - immunology-based analysis of these various subtypes has revealed a heterogeneity in the levels of expression of NK cell-associated markers, such as the inhibitory human leukocyte antigen (HLA), and ligands for the activating NKG2D and DNAM receptor on NK cells (Mamessier et al., 2011b).

Similar to what we have shown of the capability of breast tumor cells to modulate their cell surface proteins to avoid recognition by NK cells, work by Mamessier et al suggests that breast tumor cells are capable of secreting soluble inhibitory factors which alter NK cell function (Mamessier et al., 2011b). This is not surprising since tumor cells are known to secrete soluble ligands for the activating receptor NKG2D in order to dampen NK cell response to aberrant cells (Groh et al., 2002).

Heterogeneity and the plasticity of NK cell subsets, which appear to be dependent on the microenvironment, should be considered as an advantage in patient monitoring because these cells would thus be amenable to modulation. This strongly suggests that a better understanding of the

NK cell educational processes is of major importance to design future therapies based on anti-tumor immunity. In NK cell biology, it is well known that the NK cell's response to an encounter with another cell is dependent on a balance of activating and inhibitory receptors. In this study, we provide the direct *in vivo* evidence for the unique phenomenon of MHC-I-directed immunoediting in a solid cancer due to hyporesponsive NK cells. Our study, in conjunction with studies discussed previously, highlight the necessity for NK cells in the cooperation between the innate and adaptive immune components within the tumor microenvironment for cancer treatment.

Acknowledgments

We would like to thank Ms. Rashida Rajgara for assistance with qPCR, and Dr. Vera Tang from the University of Ottawa Flow Core Facility for assistance with cell sorting.

Chapter 5 : General Discussion

This body of work contributes to a better understanding of the interactions between MHC-I molecules and their Ly49 receptors, as well as their impact on NK cell education and NK-mediated control of cancer cells. In Chapter 2, it is shown that the five Ly49 receptors studied, Ly49G, Ly49I, Ly49O, Ly49V, and Ly49Q, are capable of binding with greater frequency to MHC-I molecules than previously believed. Furthermore, Ly49 receptors and MHC-I molecules, which can be expressed on the same NK cells as all nucleated cells express MHC-I, are engaging in *cis* interactions, thus limiting the detection of and dampening observed results of tested interactions between Ly49 receptors and MHC-I in *trans*-binding based experiments. In Chapter 3 and 4, I build upon this previous work and explore the role of NK cells in cancer immunosurveillance, particularly focusing on the importance of the Ly49 receptors on NK cells for their functionality. While NK cells are part of the innate immune system, and are capable of recognizing and killing aberrant cells without having previously encountering them, new research has shown that NK cells do undergo an education-like process which involves the Ly49 receptor and self-specific MHC-I (Kim et al., 2005). This education process is believed to be required for NK cell functionality. Using the genetic Ly49-deficient mouse model (NKC^{KD}) developed in the lab, I show that loss of the Ly49 receptors results in defective tumor cell recognition and killing, earlier cancer onset, and accelerated development of the disease. The NK cells are normal in all other aspects, such as stimulation through other pathways. This cancer controlling defect was observed not only for hematopoietic-based cancers, but also for solid cancers.

Most notable in these studies is the observation of tumor immunoediting. In Chapter 3, the B cell lymphomas which developed in NKC^{KD} mice exhibited marked decreases in levels of MHC-I, compared to WT mice. In a continuation of this study in breast cancer in Chapter 4, it was of particular interest to compare MHC-I levels in early versus end stage tumors. While in our previous

study, only the end stage tumors were analyzed, in accordance with the premise of tumor immunoediting, it is expected that the early tumors would possess similar phenotypes between the WT and NKC^{KD} groups. It is only after a period of time allowing for the three stages of elimination, equilibrium and escape, that the immune-mediated selective pressures and differences in the tumor microenvironment would eventually lead to the diversion of the tumor types which propagate in WT and NKC^{KD} mice. Analysis of the early stage tumors showed similar moderate levels of MHC-I expression in both WT and NKC^{KD} mice, as expected. It is only upon analysis of the late stage tumors, that a phenotype similar to the earlier lymphoma study in which the MHC-I level is decreased, is observed, but only in the NKC^{KD}-derived tumors. MHC-I levels are maintained in WT-derived tumors, comparable to that of early stage tumors. Thus, it was hypothesized that in the absence of effective Ly49-mediated missing-self recognition by NK cells, the tumor cells in NKC^{KD} do not require expression of MHC-I to avoid NK cell recognition. Conversely, tumor cells which express low levels of MHC-I are better capable of eluding cytotoxic T cell recognition of antigen presentation by MHC-I. The MHC-I expression levels observed in the early stage tumors are believed to be that of a more heterogeneous population which hasn't undergone selective immune pressures due to the characteristics of the immune cells present in the tumor microenvironment.

While cancer immunotherapy has grown widely in popularity over the recent years, focus has mostly been placed on the use of cytotoxic T cells in such therapy. Treatments with checkpoint inhibitors, such as PD-1/PD-L1 and CTLA4, specifically target T cells (Hodi et al., 2010; Topalian et al., 2012).

Adoptive transfers of T cells expressing chimeric antigen receptors are being widely undertaken in clinical trials. Treatments with NK cells are much more sparse in comparison.

Notable studies include the use of IL-2 or IL-15 to stimulate non-expanded autologous or allogeneic NK cells, as well as *ex vivo* expansion of autologous or allogeneic NK cells for infusion into the patient (Childs and Carlsten, 2015).

The expansion of NK cells is considered to be a major obstacle for their use in cancer immunotherapy. In humans, only 10-15% of the lymphocyte population are NK cells, thus already a small number to begin with. Studies into the possible use of the NK cell line NK-92, derived from a patient with a rare case of NK cell lymphoma, hope to bypass the expansion difficulties associated by NK cells using a cell line. NK-92 cells are able to quickly expand and grow, while still retaining the ability to kill traditional NK cell targets (Gong et al., 1994). Furthermore, antibody-mediated techniques have been used for NK cells. HER2-specific trastuzumab and pertuzumab antibodies, which are used in the treatment of HER2⁺ breast cancer, can be recognized by NK cells leading to tumor cell killing through antibody-dependent cell-mediated cytotoxicity (ADCC) (Diessner et al., 2013). The ADCC properties of NK cells has also been exploited with the antibody rituximab, a CD20-specific antibody, used in the treatment of B cell lymphomas. Furthermore, human monoclonal antibodies, 1-7F9 and lirilumab, which interact with KIR2DL1, -L2, and -L3 receptors and impair their inhibitory signaling by preventing their binding to HLA-C, have demonstrated the potential for use in cancer immunotherapy (Benson et al., 2012; Romagné et al., 2009).

While T cell-based therapies have many documented successes, the use of NK cells in the treatment of late stage tumors, which commonly express low levels of MHC-I, could prove to be beneficial. Treatment for cancers may not be benefitted from classifying them based on their cancer types and subtypes alone, but also on the MHC-I immune profile of these tumors. Tumors with high levels of mutations and thus neoepitope targets would better benefit from a T cell-based

therapy, whereas, conversely, a tumor which exhibit significantly low levels of MHC-I would benefit from an NK cell-based therapy.

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For Tu MM, Mahmoud AB, Makrigiannis AP. Licensed and unlicensed NK cells: Differential roles in cancer and viral control. *Frontiers in Immunology*. 2016 May 2;7:166.

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Curriculum Vitae

Publications

First author

- Tu MM, Rahim MM, Sayed C, Mahmoud AB, Makrigiannis AP. Natural killer cells mediate direct immunoediting of breast cancer via class I MHC receptors (Submitted).
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Co-author

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(Recommended by the *Faculty of 1000* as a ‘must read’)

Honours & Awards

- Canadian Institutes of Health Research Forum National Poster Competition – Nomination from the University of Ottawa (2016)
- Syed Sattar PhD Student Award (2016)

- Canadian Student Health Research Forum National Poster Competition – Honourable Mention (2015)
- Canadian Student Health Research Forum Travel Award (Canadian Institutes of Health Research) (2015)
- Canadian Student Health Research Forum National Poster Competition– Nomination from the University of Ottawa (2015)
- 15th Meeting of the Society for Natural Immunity Travel Award (2015)
- Canadian Cancer Society Travel Award (2015)
- Faculty of Medicine Award of Excellence in Graduate Studies for the PhD Microbiology and Immunology Program (University of Ottawa) (2014)
- Faculty of Medicine Graduate Studies Leadership Award (University of Ottawa) (2014)
- Microbiology & Immunology Graduate Studies Poster Day – 3rd place (2014)
- Canadian Society for Immunology 27th Annual Conference Travel Award – 1st place (2014)
- Cedarlane Poster Award - Canadian Society for Immunology (2013)
- Canadian Society for Immunology 26th Annual Conference Travel Award – 2nd place (2013)
- Microbiology & Immunology Graduate Studies Seminar Day – 2nd place (2013)
- Ontario Graduate Scholarship – PhD (Government of Ontario) (2012-2016)
- Admission Scholarship - Graduate Studies at Doctoral Level (University of Ottawa) (2012-2016)
- Faculty of Medicine Graduate Studies Leadership Award (University of Ottawa) (2012)
- Dean's Scholarship (Faculty of Graduate and Postdoctoral Studies, University of Ottawa) (2012)
- Microbiology & Immunology Graduate Studies Poster Day – 1st place (2012)
- International Graduate Student Immunology Conference Travel Award (Harvard University) (2012)
- Excellence Scholarship (University of Ottawa) (2011-2016)
- Ontario Graduate Scholarship –MSc (Government of Ontario) (2011-2012)

- Admission Scholarship - Graduate Studies at Masters Level (University of Ottawa) (2011-2012)

Oral Presentations

- Ly49-dependent cancer immunosurveillance and tumour immunoediting by NK cells (2014). Invited talk at the 27th Canadian Society for Immunology Meeting. March 6-9 2014. Quebec City, Quebec. Canada.
- Ly49 family receptor expression is required for cancer immunosurveillance mediated by NK cells (2013). Invited talk at the 26th Canadian Society for Immunology Meeting. April 5-8 2013. Whistler, British Columbia. Canada.

Conference Abstracts (**denotes presenter*)

- Tu MM*, Sayed C, Rahim MM, Mahmoud A, Makrigiannis AP. Ly49 receptor-dependent cancer immunosurveillance and tumor immunoediting in lymphoid and non-lymphoid cancers (2016) Canadian Student Health Research Forum. June 7-9 2016. Winnipeg, Manitoba. Canada.
- Tu MM*, Sayed C, Rahim MM, Mahmoud A, Makrigiannis AP. Impaired cancer immunosurveillance and MHC-I-targeted tumour immunoediting in the absence of NK cells and its Ly49 receptors (2016) Canadian Society for Immunology. April 1-4 2016. Ottawa, Ontario, Canada.
- Tu MM*, Mahmoud A, Wight A, Mottashed A, Makrigiannis AP. Ly49-dependent cancer immunosurveillance and tumour immunoediting by NK cells (2015) Canadian Student Health Research Forum. June 1-4 2015. Winnipeg, Manitoba. Canada.

- Tu MM*, Rahim MM, Mahmoud AB, Makrigiannis AP. Ly49 receptor-dependent cancer immunosurveillance and tumor immunoediting in lymphoid and non-lymphoid cancers (2015) 15th Meeting of the Society for Natural Immunity. May2-6 2015. Montebello, Quebec. Canada.
- Mahmoud AB*, Tu MM, Zein HS, Wight A, Rahim MM, Lee SH, Sekhon HS, Brown EG, Makrigiannis AP. Ly49:MHC-I interactions are deleterious during influenza virus infection: Superior protection by unlicensed NK cells (2015) 15th Meeting of the Society for Natural Immunity. May2-6 2015. Montebello, Quebec. Canada.
- Wight A*, Mahmoud AB, Tu MM, Rahim MM, Makrigiannis AP. Investigating the role of inhibitory Ly49 receptors in T and B cell-independent immune memory (2015) 15th Meeting of the Society for Natural Immunity. May2-6 2015. Montebello, Quebec. Canada.
- Wight A*, Mahmoud AB, Tu MM, Rahim MM, and Makrigiannis AP. The role of Ly49 receptors in natural killer-mediated immune memory (2014). 27th Annual Canadian Student Health Research Forum. June 16-18 2014. Winnipeg, Manitoba. Canada.
- Mahmoud AB*, Tu MM, Zein HS, Rahim MM, Abou-Samra E, Sekhon HS, Gomes M, Sentman C, Lee S-H, Brown EG, and Makrigiannis AP. Disrupting the interaction between class I MHC and NK cell inhibitory receptors protects mice from lethal influenza virus infection (2014). 30th Annual Clinical Virology Symposium April 27-30 2014. Daytona Beach, Florida. U.S.A.
- Tu MM*, Mahmoud AB, Wight A, Mottashed A, Bélanger S, Rahim MM, Abou-Samra E, and Makrigiannis AP Ly49-dependent cancer immunosurveillance and tumour immunoediting by NK cells (2014). 27th Canadian Society for Immunology Meeting. March 6-9 2014. Quebec City, Quebec. Canada.
- Tu MM, Mahmoud AB, Wight A, Mottashed A, Abou-Samra E, Stintzi A, and Makrigiannis AP*. Ly49 family receptor expression is required for cancer immunosurveillance mediated by natural killer cells (2013). 14th Meeting of the Society for Natural Immunity. September 17-21 2013. Heidelberg, Germany.

- Mahmoud AB, Tu MM, Zein HS, Rahim MM, Abou-Samra E, Sekhon HS, Gomes M, Sentman C, Lee S-H, Brown EG, and Makrigiannis AP*. Disrupting the interaction between class I MHC and NK cell inhibitory receptors protects mice from lethal influenza virus infection (2013). 14th Meeting of the Society for Natural Immunity. September 17-21 2013. Heidelberg, Germany.
- Lima PDA, Rahim MMA, Tu MM, Peng A, Croy BA* and Makrigiannis AP. KIR-like Ly49 Receptors: Insights into pregnancy complications linked to inhibition of uterine natural killer cell functions (2013). International Federation of Placenta Associations Meeting. September 11-14 2013. Whistler, British Columbia. Canada.
- Lima PDA*, Rahim MMA, Tu MM, Peng A, Croy BA and Makrigiannis AP. Reduced expression of Ly49 receptors on natural killer cells leads to impaired vascular remodeling in the pregnant mouse uterus (2013). Society for the Study of Reproduction's 46th Annual Meeting. July 22-26 2013. Montreal, Quebec. Canada.
- Mahmoud AB*, Tu MM, Zein HS, Rahim MM, Abou-Samra E, Sekhon HS, Gomes M, Sentman C, Lee S-H, Brown EG, and Makrigiannis AP. Disrupting the interaction between class I MHC and NK cell inhibitory receptors protects mice from lethal influenza virus infection (2013). Canadian Society for Microbiology Annual Conference. June 17-20 2013. Ottawa, Ontario. Canada.
- Lima PDA*, Rahim MMA, Tu MM, Peng A, Croy BA and Makrigiannis AP. Gestational outcomes from Ly49 receptor knockdown in mouse uterine natural killer cells (2013). International Society for Immunology of Reproduction Meeting. May 28 - June 1 2013. Boston, Massachusetts. U.S.A.
- Tu MM*, Mahmoud AB, Mottashed A and Makrigiannis AP. Ly49 family receptor expression is required for cancer immunosurveillance mediated by NK cells (2013). 26th Canadian Society for Immunology Meeting. April 5-8 2013. Whistler, British Columbia. Canada.

- Tu MM* and Makrigiannis AP. Role of class I MHC receptor on NK cells in cancer immunosurveillance (2012). International Graduate Student Immunology Conference. Harvard University. Boston, Massachusetts. USA.
- Tu MM* and Makrigiannis AP. Role of class I MHC receptor on NK cells in cancer immunosurveillance (2012). Biochemistry, Microbiology and Immunology Graduate Studies Poster Day. University of Ottawa. Ottawa, Ontario. Canada.

Education

- Ph.D. Microbiology and Immunology – University of Ottawa
- B.Sc. with Honours in Biopharmaceutical Sciences, Genomics – University of Ottawa