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NATURAL KILLER CELL RECOGNITION OF VIRAL INFECTION: PAIRED MHC I RECEPTORS FACILITATE VIRAL DETECTION AND CLEARANCE

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ABSTRACT:

NK cells play a critical role in controlling MCMV infection. We have shown that MHC class I D^k is absolutely required for murine host protection to MCMV infection and MHC class I D^k-dependent MCMV resistance is reliant upon NK cells marked by the MHC class I D^k inhibitory receptor, Ly49G2. Ly49G2 binds MHC class I D^k and educates NK cells on self-MHC class I which causes them to exhibit enhance sensitivity to activation receptor stimulation. Despite this, a role for specific activation receptors in MHC class I D^k-dependent MCMV resistance has not been delineated. We identified the Ly49R activation receptor as a novel mediator of MHC class I D^k-dependent MCMV resistance by selective neutralization via a specific monoclonal antibody. We also precisely define the role of Ly49G2 in MHC class I D^k-dependent MCMV resistance via specific genetic ablation of Ly49G2. Additionally, we discovered that NK cells require simultaneous expression of Ly49G2 and Ly49R to selectively control MCMV infection, proliferate, differentiate to terminal effector cells, and exhibit other features of specific activation. This original work demonstrates that paired MHC I receptors work in tandem to confer viral immunity.

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ABBREVIATIONS:

A	Ly49A
B6	C57BL/6
BrdU	Bromodeoxyuridine
BM	Bone Marrow
class Ib	Nonclassical MHC class I
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
D^k	H-2D^k
Dpi	Days post-infection
G2	Ly49G2
gDNA	Genomic Deoxyribo-Nucleic Acid
GO1	G Out1 – Or – Ly49G2 Knockout
GO2	G Out2 – Or – Ly49G2 Knockout
H	Ly49H
HCMV	Human Cytomegalovirus
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus

HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
hr	hour(s)
HRM	High Resolution Melting
IFN-α/β	Type I Interferon-α/β
IFN-γ	Type II Interferon-γ
IL	Inter-Leukin
ILC	Innate lymphoid cell
IP	Intra-Peritoneal
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
KIR	Killer cell Immunoglobulin-like Receptor
mAb	Monoclonal Antibody
MCMV	Murine Cytomegalovirus
MHC I	Major histocompatibility complex class I
NK	Natural Killer Cell
NKC	Natural Killer Gene Complex

NKD	Natural Killer cell Deficiency
PAM	Protospacer Adjacent Motif
PCR	Polymerase Chain Reaction
PMA/IONO	Phorbol Myristate Acetate and Ionomycin
PFU	Plaque forming units
Qdm	Qa-1 Determinant Modifier
R	Ly49R
RNA	Ribo-Nucleic Acid
scRNA-Seq	Single Cell RNA Sequencing
sgRNA	short guide RNA
Wt	Wildtype

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CHAPTER I: Overview of the NK cell Response to Viral Infection

Defining Features of Natural Killer Cells

Natural killer (NK) cells are prototypical innate lymphoid cells originally identified and described in 1975 for their ability to kill tumor cells without the need for prior activation, hence, *natural* killer cells (Kiessling et al., 1975). They are critical players in the immune system that mediate essential antiviral and antitumor activities as well as support the differentiation and function of other leukocytes.

Over the past 10 years NK cells have been increasingly appreciated for the *nonredundant* role they play in the immune system (Mace and Orange, 2016, 2019). Individuals with an NK cell deficiency (NKD) are generally highly susceptible to Herpesviruses and eventually succumb to infectious pathogens, often with comorbid onco-malignancies (Biron et al., 1989; Cohen et al., 2016; Mace and Orange, 2019; Spinner et al., 2014). There are a number of primary immunodeficiencies caused by genetic abnormalities which result in dysregulation of NK cell function or development (Mace and Orange, 2019). Although many mutations resulting in primary NKD affect other cell lineages, the individuals suffering from NKDs are commonly susceptible to herpes viral infections. Moreover, susceptibility to herpes viral infections is also shared by individuals harboring mutations that specifically impair NK cells, such as GATA2, MCM4, GINS1, IRF8 and FCGR3A (Biron et al., 1989; Cottineau et al., 2017; Gineau et al., 2012; Grier et al., 2012; Mace et al., 2017). Thus, NK cells are a critical arm of the innate immune system that are essential for host survival.

NK cells are cytotoxic effector cells that have to the potential to specifically lyse cells that are virus infected or have undergone a malignant transformation. The majority of the killing potential is mediated via perforin and granzymes which are cytotoxic proteins that are stored as preformed mediators in secretory lytic granules (Prager et al., 2019; Topham and Hewitt, 2009). The contents of these granules can be released after NK cell activation when interfacing with a target cell. Additionally, NK cells express a variety of cell death receptor ligands, such as TRAIL, that promote cell death of cells bearing cell death receptors. Thus, NK cells have multiple mechanisms they may employ to kill abnormal host cells.

In the mouse, conventional NK cells (referred throughout as "NK cells") are defined as group 1 innate lymphoid cells (ILC) due to their ability to promote inflammation by potently secreting type II interferon(IFN)-γ and their developmental and functional reliance on the T-box transcription factor T-bet (Colonna, 2018). These traits are shared with the other group 1 ILC population termed, ILC1s. Although sharing many surface markers to NK cells, other ILCs, including ILC1s, do not exhibit the same degree of cytotoxic effector activity. These ILC populations are recognized as the "helper" cells of the innate immune system. NK cells are further reliant on the T-box transcription factor, eomesodermin, which regulates their functional maturation (Gordon et al., 2012). Mature NK cells are readily identified apart from other ILC populations by their simultaneous expression of both T-bet and eomesodermin (Colonna, 2018; Robinette and Colonna, 2016). Thus, NK cells are unique cytotoxic effector cells that serve a specialize role among ILCs.

Splenic NK cells in C57BL/6 (B6) mice are traditionally identified as $CD3^{-}$ $CD19^{-}$ and either $NK1.1^{+}$ $CD49b^{+}$ or $Nkp46^{+}$. The NK1.1 antigen is not frequently expressed by NK cells in most mouse strains lacking a B6-like Natural Killer Gene Complex haplotype (NKC^{b6}), and thus is not an reliable marker of NK cells is most mouse strains (Carlyle et al., 2006; Higuchi et al., 2010). Although CD49b is an effective marker of NK cells under homeostatic conditions, it can be induced by other cell types during viral infection and thus cannot be considered an exclusive NK cell marker. Nkp46 is a marker of NK cells in all mammalian species tested to date (Pessino et al., 1998; Biassoni et al., 1999; Boysen and Storset, 2009; Storset et al., 2004; Walzer et al., 2007). NKp46 can also be expressed by select ILC populations and NKT cells, but is primarily expressed by NK cells in the spleen (Narni-Mancinelli et al., 2011). Thus, although the vast majority of cells characterized using traditional NK cell markers in the spleen are bona fide NK cells, their identities are not precisely defined. Activation Ly49 receptor surface protein expression, such as Ly49D/R, is strongly correlated with NK cells (Gabrielli et al., 2017; Sojka et al., 2014). Thus, activation Ly49 are suitable markers in addition to the canonical surface proteins that identify NK cells in the spleen.

NK Cell Associated Receptors in Mouse and Human – Bridging the Gap

A salient feature of NK cells is their diverse expression of germline encoded surface MHC I receptors. Allelic variation amongst the MHC I genes has driven the evolution of

MHC I receptor families including the C-type lectin-like Ly49 receptor family found in rodents, and the killer immunoglobulin-like receptor (KIR) family found in humans (Guethlein et al., 2015). Genes for the Ly49 or KIR receptors are found within the NKC or Leukocyte Receptor Complex (LRC), respectively (Yokoyama et al., 1991; Wende et al., 1999). Although Ly49 and KIR receptors are structurally different, they serve analogous functions in regards to their capacity to recognize MHC I molecules as ligands and subsequently drive similar downstream signaling cascades.

MHC I receptor signaling generally results in one of two outcomes: Activation or inhibition of cellular processes. KIR can be divided into groups with short (S) or long (L) cytoplasmic tails. KIR with long cytoplasmic tails contain immunoreceptor tyrosine-based inhibitory motifs (ITIM). Whereas those with short cytoplasmic tails associate with the signaling adaptor molecule, DAP12, which contains immunoreceptor tyrosine-based activation motifs (ITAM). Inhibitory Ly49 receptors likewise contain an ITIM while Ly49 activation receptors directly associate with DAP12. This is facilitated via a transmembrane arginine residue which is common to both activation KIR and Ly49. Ultimately, the effector function of a given Ly49 MHC I receptor is dependent upon the presence of an ITIM within the cytoplasmic tail or a positively charged amino acid residue within the transmembrane region (Vély and Vivier, 1997).

Both Ly49 and KIR are acquired in a semi-stochastic process during NK cell development (Brodin et al., 2012). Despite these being germline encoded receptors, they contribute to a substantial amount of heterogeneity amongst NK cells due to their

variegate expression profiles within the NK cell repertoire. For instance, breaking down an individual's PBMC NK cell population base off KIR expressed and NK cell developmental markers leads to the over 10,000 unique subsets (Horowitz et al., 2013). Thus, the unique regulation of KIR and Ly49 expression provides a layer of functional diversity between individual NK cells.

The composition of the Ly49 and KIR gene loci display incredible genetic diversity. This is reflective of the species-specific expansions and contractions of their component genes which has led to the formation of distinct haplotypes for each separate gene family (Brown et al., 2001a). Although the biological significance for multiple haplotypes is not fully understood, a working hypothesis is that resistance to microbial pathogens and fostering reproduction are dominant selective pressures promoting genetic diversity in these regions (Guethlein et al., 2015). The existence of both receptor families is one of the most fascinating examples of convergent evolution and speaks to the importance for mammalian species to have multiples mechanisms for surveying MHC I expression on host cells.

Structure, Nomenclature and Origin of the Ly49 genes

The Ly49 receptor family encodes for C-type lectin-like type II transmembrane proteins that predominantly recognize MHC class I molecules as their ligands. The *Ly49* gene

cluster is comprised of numerous genes and pseudogenes which together define different haplotypes. For example, the B6 *Ly49* haplotype includes 16 genes and pseudogenes which encode 4 predominant inhibitory (Ly49A, Ly49C, Ly49G, and Ly49I) and 2 activation (Ly49H and Ly49D) receptors (Wilhelm et al., 2002). To date, four NKC haplotypes from inbred mouse strains have been well-characterized and display considerable variability within the Ly49 Locus: C57BL/6(B6), 129, BALB/c and NOD (Brown et al., 2001a; Carlyle et al., 2008; Yokoyama et al., 1990, 1991). The work for this thesis utilizes mice carrying either a NKC^{b6} or the 129-like NKC^{c57l} (NKC^{L}) haplotype. NKC^{L}, like the NKC^{129} haplotype, encodes 6 Ly49 inhibitory (O, V, S, T, G, E) and 3 Ly49 activation (R, U, P) receptors expressed by NK cells (**Figure 1**). $Ly49^{129}$ gene sequences are 85-96% identical to those in B6, with the exception of *Ly49e* which is 100% identical (Makrigiannis et al., 2001). Despite the high gene sequence similarity, the alleles for

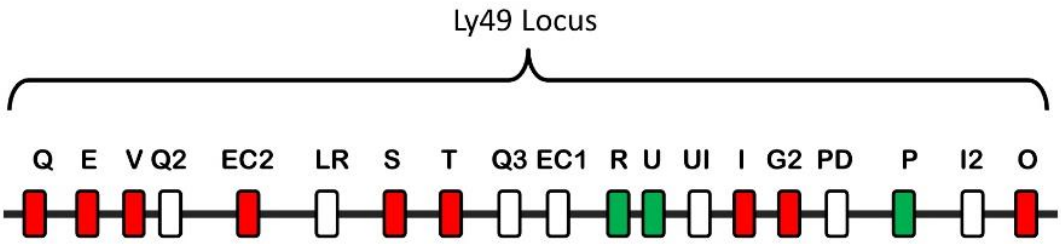


Figure 1. Simple Schematic of the C57L Ly49 Locus

Genes encoding pseudo-genes are shown in white, inhibitory receptors in red, and activation receptors in green.

certain genes, such as *Ly49g*, encode for different allotypes with distinct ligand binding profiles which highlights their functional diversity.

The Ly49 receptors were named according to the order of their discovery. It is possible that uniquely annotated genes from separate NKC haplotypes are truly alleles of one another and arose from a common ancestral gene. Interestingly, there are common framework genes present in all NKC haplotypes, one of these genes being *Ly49g* (Carlyle et al., 2008). Most *Ly49* haplotypes also contain *Ly49d*/*r*-like genes and 99% of wild mice NK cells are reactive to monoclonal antibodies (mAbs) that recognize Ly49G (4D11) or Ly49D/R (12A8) (Abolins et al., 2017). Interestingly, $4D11^{+}$ $12A8^{+}$ were found to be strongly coexpressed, similar to what's seen in common laboratory mouse strains, including those used in the work comprising this thesis. It is possible that Ly49G and Ly49D/R may be a common pairing found within Mus musculus. The KIR family likewise contain framework genes that are common amongst all haplotypes along with variable regions that differ in gene content and the extent of genetic polymorphism (Abi-Rached and Parham, 2005; Guethlein et al., 2015). This suggests that the selective pressures underlying the adaptive mechanism(s) driving genetic diversity amongst Ly49 and KIR may be similar between mice and humans.

Although most of the inhibitory Ly49 receptors are most commonly expressed on NK cells they can be expressed on other lymphocyte populations such as select ILC, NKT, and memory CD8 T cells (Cortez and Colonna, 2016; Coles et al., 2000; Maeda et al., 2001). The role of Ly49 receptors on non-NK cell lymphocytes is poorly understood and

it is unknown whether they serve similar or specialized regulatory functions. There are Ly49 that are uniquely regulated and/or not expressed by NK cells at all. For example, Ly49Q is an inhibitory Ly49 that is not expressed by NK cells but can be found on plasmacytoid dendritic cells and positively regulates type I production (Rahim et al., 2013; Toyama-Sorimachi et al., 2005). Ly49B is a poorly characterized inhibitory Ly49 that is not encoded within the Ly49 locus, exhibits considerable sequence divergence from other Ly49 family members, and is not expressed on NK cells under homeostatic conditions but is found on myeloid cells(Gays et al., 2006; Mickiewicz et al., 2014). Ly49E is an inhibitory Ly49 that is highly expressed on fetal NK cells and is known to support fetal development and can also be found on ILC I in the liver where it limits ILC I IFN-γ production (Van Beneden et al., 2001). Thus, different Ly49 family members play complex roles in regulation of NK cell development and function independent of their role as allospecific MHC class I receptors.

NK Cell Education and Self-tolerance

NK cell education is the process in which NK cells acquire functional maturation and self-tolerance. While the mechanistic basis for education currently remains shrouded in mystery, the phenomenon has been heavily scrutinized over the past 15 years. Education occurs when self-MHC I molecules are recognized by their cognate *inhibitory*

receptors resulting in fully functional and self-tolerant NK cells. The functional capacity of educated NK cells has traditionally been measured in two ways: 1) production of IFN-γ in response to plate-bound antibody mediated stimulation of activation receptors 2) rejection of target cells lacking self-MHC I molecules. It has been shown that the stronger the association, in terms of affinity and avidity, between self-MHC I and their cognate inhibitory receptors, the stronger the NK cell functional response in terms of IFN-γ production and rejection of target cells lacking self-MHC I (Brodin et al., 2009).

Education is a dynamic process. NK cells are constantly surveying for, or lack of, tolerizing signals in their environment and can be "re-educated" when that environment changes (Bern et al., 2019; Wei et al., 2014). For example, mature NK cells that have already undergone education during development in self-MHC I sufficient hosts will *retune* their educational status when adoptively transferred into hosts that are self-MHC I deficient. Likewise, mature NK cells from self-MHC I deficient hosts will retune when adoptively transferred into self-MHC I sufficient hosts. Additionally, in regards to education via MHC I molecules, tolerizing signals from both the hematopoietic and non-hematopoietic compartments are required for optimal education (Wei et al., 2014; Xie et al., 2010). Thus, although NK cells enter an educated state during development, they constantly require tolerizing signals in order to maintain their current state.

Although most of the work on NK cell education has been done in the context of classical MHC I, nonclassical MHC I and nonMHC I-ligands have shown a capacity to promote education as well. NKG2A and Ly49A^{B6} recognize and educate NK cells on

nonclassical MHC class I molecules Qa-1b and H2-M3, respectively (Zhang et al., 2019; Andrews et al., 2012; Vance et al., 1998). NKRP1b is an NK cell associated inhibitory receptor that is part of the NKRP1 receptor family and educates NK cells on its cognate C-type lectin-related ligand, clr-b (Rahim et al., 2015). SLAMF6, 2B4, and TIGIT have been shown to educate NK cells on their respective ligands as well (He et al., 2017; Lee et al., 2004, 2006; Wu et al., 2016). As some of these receptors are commonly expressed on NK cells it is reasonable to presume that the vast majority of NK cells are able to become educated in some capacity, albeit to different extents depending on the affinity and availability of their corresponding ligands.

The educated natural killer cell brings about a natural conundrum to the educated immunologist. If NK cells need to be sufficiently tolerant to self in order to be fully functional, how do they overcome tolerance? Simply put, educated NK cells must sense a shift in the balance of their integrated inhibitory and activation signals towards activation in order to recognize an abnormal target cell. Here are conditions that allow for an educated NK cell to overcome tolerance and recognize a target cell: 1) Gross overexpression or increased affinity of activating ligands for NK cell activation receptors 2) Loss or aberrant expression or decreased affinity of self-inhibitory ligands for NK cell inhibitory receptors.

Education is thought to be a fundamental aspect of natural killer cell biology. Interestingly, NK cells in genetically humanized mice expressing transgenes for both an inhibitory KIR on NK cells, and its cognate human leukocyte antigen (HLA) ligand,

ubiquitously, exhibit an educated NK cell phenotype in regards to enhanced IFN-γ production in response to activation receptor stimulation (van Bergen et al., 2013). This demonstrates that human NK receptors are capable of educating murine NK cells and the underlying mechanism governing education is likely similar between the two species. This adds additional credence to the mouse serving as a suitable model for studying how education regulates NK cell responsiveness in mammals.

Features of Activated Virus Antigen-specific NK Cells

Viral pathogens, such as Murine cytomegalovirus (MCMV), induce the production of type I IFNs when detected by cells expressing pathogen associated molecular pattern receptors, such as TLR9 (Krug et al., 2004). This in turn leads to general activation of the immune system and promotes the release of many inflammatory mediators. NK cells can be directly activated by these inflammatory mediators, the most robust of which being type I IFNs, IL-12 and IL-18 (Andrews et al., 2003). This non subset-specific activation stimulates all NK cells to proliferate, produce IFN-γ, upregulate activation markers such as CD69 and CD25, and traffic to damaged tissues (Lee et al., 2012). During MCMV infection, the bulk of the NK response prior to 48 hours post infection is not induced by direct recognition of viral antigens (Dokun et al., 2001).

NK cells can be activated in an antigen specific manner to directly clear virus infected cells. Although NK cells do not provide sterilizing immunity, they limit viral spread during the acute phase of infection. This allows time for adaptive T cells to accumulate and clear the remaining infection. B6 mice display strong host resistance to MCMV infection due to antigen-specific NK cells that express the activation receptor, Ly49H, which is capable of directly recognizing the MCMV antigen, m157 (Arase et al., 2002; Brown et al., 2001a). By 3-4 days post MCMV infection (dpi) $Ly49H^{+}$ NK cells begin to diverge in terms of their rate of proliferation and surface marker expression based on their ability to specifically recognize viral infection, which manifests as upregulation or maintenance of KLRG1 and CD62L, and down regulation of DNAM-1 (Dokun et al., 2001; Fogel et al., 2013). They continue to expand until 7 dpi, and then permanently maintain a $KLRG1^{hi}$ phenotype throughout the contraction phase and their transition to memory cells (Sun et al., 2009). Thus, we hypothesize that virus-induced cell surface antigens represent useful tools, essentially biomarkers, to identify putative effector NK cells in mouse strains lacking known virus-specific effector NK cell subsets.

MHC I-dependent Viral Resistance

Human genome-wide genetic association (GWAS) studies have shown that individuals with select *KIR* and *HLA* gene combinations can exhibit improved resolution, or delayed progression, of certain viral infections. For instance, individuals that are homozygous

for KIR2DL3 and their ligand, HLA-C1, are associated with spontaneous clearance of HCV infection (Romero et al., 2008). Additionally, HIV infected individuals that harbor select KIR3DL1 and HLA-B alleles show delayed onset to AIDs and lower plasma HIV RNA levels (Martin et al., 2007). An underlying mechanism for how inhibitory receptors and their MHC I ligands may delay disease progression has not been identified. Modeling MHC I-dependent resistance in mice might enhance our understanding of NK receptor-ligand immune contributions in human disease.

Genes within the MHC I locus of the mouse have long been appreciated to play an important role in resistance to MCMV infection (Grundy et al., 1981; Mercer and Spector, 1986). One gene of considerable importance, identified by both the Vidal and Brown labs, encodes for the MHC I molecule, H-2D^{k} (Desrosiers et al., 2005; Xie et al., 2009). Remarkably, mice that harbor the MHC I H-2D^{k} allotype exhibit enhanced resistance to MCMV compared to those expressing MHC I H-2D^{b} or H-2D^{d} molecules. This resistance mechanism requires NK cells as their depletion prior to infection abrogates MCMV resistance. Moreover, this host resistance effect is CD8 T cell-independent during acute infection which stresses the importance of NK cell-mediated immunity.

BALB/c mice, which harbor both BALB/c NKC and H-2^{d} haplotypes, are the prototypical MCMV susceptible mouse strain. However, BALB/c background mouse strains that harbor a congenic H-2^{k} locus have been shown to be MCMV resistant relative to Wt (H-2^{d}) BALB/c. This resistance phenotype is thought to require a specific population of NK

cells bearing the $NKC^{balb/c}$-derived activation receptor, Ly49L (Fodil-Cornu et al., 2011; Pyzik et al., 2011). Interestingly, Ly49L was unable to recognize native H-$2D^k$, yet recognized H-$2D^k$ complexed with an MCMV protein, gp34, on virus infected cells. Gp34 is a viral immunoevasin that escorts MHC I molecule to the surface in order to inhibit NK cells via their self-MHC I receptors. Thus, Ly49L is capable of recognizing virus modified MHC I.

NKC^L encodes for a Ly49L-like activation receptor called Ly49P. It has been posited that Ly49P is responsible for H2-D^k dependent resistance in the MA/MY mouse strain which harbors an NKC with a Ly49 locus that is very similar to NKC^L (Kielczewska et al., 2009). However, Ly49P mRNA expression does not correlate with the putative effector $Ly49G2^+$ NK cell populations in NKC^L mice (Xie et al., 2009). Additionally, Ly49P NK cells have yet to be precisely characterized in vivo due to a lack of molecular tools to do so. Thus, the in vivo role of Ly49P remains tenuous. Although we know that H-$2D^k$ clearly plays a role in NK cell-dependent MCMV resistance in mouse strains such as MA/MY and C57L, the precise mechanism(s) facilitating viral control remain to be elucidated.

Rationale and Specific Aims

NK cells play a nonredundant role in supporting immunity to a variety of viral infections. There are many examples of specific KIR:HLA compound genotypes that are correlated with improved clinical outcome in the context of viral infections (Brown et al., 2019; Nash et al., 2014). How these receptors facilitate NK cell responses to viral infection has not been clearly delineated. In the mouse, we have shown that MHC I D^k is a major host resistance factor that facilitates control of murine cytomegalovirus (MCMV) spread in NKC^L bearing mice. NK cells marked by $Ly49G2^L$ inhibitory receptors mediate this effect since depletion of $Ly49G2^+$ NK cells before infection abolishes virus control. However, whether Ly49G2 plays a direct functional role in coordinating NK cell responses to viral infection is uncertain. Moreover, an *in vivo* role for NK activation receptors in this model has not been precisely defined. We hypothesize that (1) Ly49G2 augments NK cell activation to promote responsiveness against MCMV infection in a D^k-dependent manner and (2) NK cell activation receptors are critical to facilitate specific recognition and clearance of viral infection. These hypotheses are addressed in two specific aims:

Specific Aim 1: Identify the activation receptor(s) and discrete subset(s) of NK cells required for MHC I D^k-dependent MCMV resistance

NK cell responses are governed by a shift in the balance of their integrated signals derived from both inhibitory and activation receptors. For $Ly49G2^+$ NK cells to play a functional role in clearance of MCMV, there must be a loss of inhibition in combination

with concurrent activation signals. In order to assess the role(s) of activation receptors in D^k-dependent MCMV resistance we can screen a panel of neutralizing mAb to various activation receptors. We also generated a novel mouse model system that would allow us to transfer discrete subsets of NK cells into NK cell deficient H-2-matched hosts. Also, we generated activation receptor reporter cell-lines to characterize and identify putative ligands.

Specific Aim 2: Examine how NK cell functional responses are impacted by self-MHC I D^k-specific inhibitory MHC I receptors during MCMV infection

Self-MHC I inhibitory receptors are known to educate NK cells endowing them with a greater capacity to respond to stimulation via their activation receptors. Self MHC I inhibitory receptors have also been shown to limit NK cell responses in other models of MCMV infection. In NKC^L mice, Ly49G2 has been shown educate NK cells and promote control of MCMV infection. We generated a specific Ly49G2 KO mouse which allowed us to assess the direct role of Ly49G2 in regulating NK cell responses to viral infection. We thoroughly characterized proliferative potential, activation status and transcriptomic profiles of NK cells in mice harboring the Ly49G2 inhibitory receptors, its cognate ligand, or both.

CHAPTER II: Ly49R activation receptor drives self-MHC-educated NK immunity against cytomegalovirus infection

ABSTRACT

Natural killer (NK) cells mediate vital control of cancer and viral infection. They rely on MHC class I (MHC I)-specific self-receptors to identify and lyse diseased cells without harming self-MHC I-bearing host cells. NK cells bearing inhibitory self-receptors for host MHC I also undergo education, referred to as licensing, which causes them to become more responsive to stimulation via activation receptor signaling. Previous work has shown that licensed NK cells selectively expand during virus infections and they are associated with improved clinical response in human patients experiencing certain chronic virus infections, including HIV and HCV. However, the importance of inhibitory self-receptors in NK-mediated virus immunity is debated as they also limit signals in NK cells emanating from virus-specific activation receptors. Using a mouse model of MHC I-dependent (H-2D^k) virus immunity, we discovered that NK cells depend on the Ly49G2 inhibitory self-receptor to mediate virus control which coincided with host survival during murine cytomegalovirus (MCMV) infection. This antiviral effect further requires active signaling in NK cells via the Ly49R activation receptor which also binds H-2D^k. In tandem, these functionally discordant Ly49 self-receptors increase NK cell proliferation and effector activity during infection, resulting in selective upregulation of CD25 and KLRG1 in virus-specific Ly49R$^+$ Ly49G2$^+$ NK cells. Our findings establish that paired self-receptors act as major determinants of NK cell-mediated virus sensing and immunity.

INTRODUCTION

Natural Killer (NK) cells are innate lymphocytes that play a nonredundant role in sustaining host immunity to virus infections (Biron et al., 1989; Mace and Orange, 2016). They respond to environmental cues by integrating signals from diverse arrays of activation and inhibitory receptors, including structurally unrelated killer immunoglobulin-like receptor (KIR) or Ly49 receptors expressed in different species. Both human KIR and rodent Ly49 families include germline-encoded inhibitory and activation receptors which bind highly polymorphic host (self) MHC I molecules and control NK effector functions. Adaptive selection for binding self MHC I in the different species presumably underlies convergent diversification of clustered KIR or Ly49 receptor genes which aids in pathogen protection and reproductive functions (Abi-Rached and Parham, 2005; Kelley et al., 2005).

Both KIR and Ly49 inhibitory self-receptors help tune NK cells during interaction with host MHC I (Brodin et al., 2009; Long et al., 2013). "Self-aware" NK cells that have been tuned are said to be educated or licensed, as evidenced by enhanced effector function following activation receptor stimulation, and the ability to kill missing-self (MHC I^-) target cells (Fernandez et al., 2005; Kim et al., 2005; Anfossi et al., 2006). Licensed NK cells may improve clinical outcomes in human patients with chronic virus infections including hepatitis C virus (HCV) or human immunodeficiency virus (HIV) (Khakoo et al., 2004; Martin et al., 2007; Romero et al., 2008). Indeed, licensed NK cells have been found to

respond and accumulate during several different human virus infections including Hantavirus, Chikungunya virus, Hepatitis B virus (HBV), HCV, HIV, and CMV (Béziat et al., 2012, 2013; Björkström et al., 2011; Eller et al., 2011; Petitdemange et al., 2011).

The H-2D^{k} class I molecule promotes NK cell-mediated control of MCMV infection in different mouse strains, including MA/My and C57L.D^{k} (Dighe et al., 2005; Fodil-Cornu et al., 2011; Pyzik et al., 2011; Xie et al., 2009). Host resistance in these strains was found to correspond with the Ly49G2 inhibitory receptor encoded in most Ly49 haplotypes so far studied (Xie et al., 2009; Brown and Scalzo, 2008; Abolins et al., 2017). Importantly, Ly49G2 self-receptor allotypes expressed in MA/My and C57L.D^{k} mice can license NK cells via H-2D^{k}, whereas others (e.g. Ly49G2^{b6}) cannot (Silver et al., 2002; Wei et al., 2014; Xie et al., 2009). This licensing effect correlates with H-2D^{k}-dependent virus control and is abolished by specifically depleting Ly49G2^{+} NK cells prior to MCMV infection (Prince et al., 2013; Teoh et al., 2016; Wei et al., 2014; Xie et al., 2009, 2010).

Although the importance of inhibitory self-receptors for MHC I in virus immunity is still debated, Ly49 activation receptors have been shown to specifically recognize and target NK cell lysis of virus infected host cells. For example, Ly49H, which binds MCMV m157, directs virus-specific NK cell lysis of infected target cells in B6 mice (Arase et al., 2002; Smith et al., 2002). Likewise, Ly49L recognition of MCMV gp34–H-2D^{k} complexes was shown to mediate MHC I-dependent MCMV resistance in BALB.K mice (Fodil-Cornu et al., 2011; Kielczewska et al., 2009; Pyzik et al., 2011). Activation receptors thus might also contribute in Ly49G2^{+} NK cell responses during MCMV infection as predicted (Xie et

al., 2010). The Ly49R activation receptor, encoded in MA/My-related Ly49 haplotypes, is interesting in this regard as it was shown to modestly bind soluble H-2D^{k} tetramers (Brown et al., 2001a; Makrigiannis et al., 2001). Moreover, the Ly49D^{B6} activation receptor, an allele variant of Ly49R, was shown to augment virus-specific NK responses during MCMV infection. Thus, we interrogated Ly49R's role in H-2D^{k}-dependent resistance against MCMV infection. We discovered that the discordant Ly49G2 and Ly49R self-receptors enable NK cells to mediate MHC I-dependent virus control and overall host survival. Our findings highlight a vital role for such paired self-receptor systems which rely on licensing to increase activation receptor-driven antiviral NK cell effector activities.

RESULTS

Generation of *Ly49g2*-deficient mice.

Ly49G2^{+} NK cells were previously shown to mediate H-2D^{k}-dependent MCMV resistance in MA/My, C57L.D^{k} and B6.NKC^{L}-D^{k} mice (Xie et al., 2009; Teoh et al., 2016). A specific role of Ly49G2 in virus control, however, remained poorly defined. Thus we used CRISPR/Cas9 genome editing to initially generate B6.NKC^{L} (NKC^{L}) mice deficient in Ly49G2 expression. The C57L allele of *Ly49g2^{L} exon 4* was selectively targeted in NKC$^{B6/L}$ heterozygous embryos which aided in genotypic and allotypic screening for mutant

founders (**Figures 2A, Figure 3A-C**). Two NKC$^{B6/L}$ founders carrying *exon 4* indels were identified using *Ly49g2*-specific high-resolution melting (HRM) PCR and the resultant mutant alleles were termed G Out1 and G Out2 (GO1 and GO2) (**Figure 3B**).

Ly49G2 allotype-specific staining showed that NK cells from GO founder offspring had reduced cell surface Ly49G2^{L} expression (**Figure 3C**). Direct sequencing revealed identical cytosine insertions in GO1 and GO2 *Ly49g2* alleles at the anticipated CRISPR/Cas9 target site, resulting in Ly49G2 truncation within the stalk region prior to a critical dimerization domain (**Figures 2B, 2C**). Both GO founders transmitted their mutations through the germline to establish homozygous *Ly49g2*^{GO1} and *Ly49g2*^{GO2} null mice which can be identified from littermates carrying *Ly49g2*^{L} alleles using HRM PCR (**Figure 2D**).

GO mice were further crossed with NKC^{L}-D^{k} to establish NKC^{GO1}-D^{k} and NKC^{GO2}-D^{k} strains for virus resistance studies. We found NK cells from both strains lack Ly49G2^{L} NK cell surface expression (**Figure 2E**). Whole genome exome sequencing confirmed *Ly49g2* cytosine insertions in both GO strains. Moreover, only wild-type (WT) exome sequences (i.e. no mutations) were detected in highly related *Ly49* genes for the regions spanning the CRISPR target site in *Ly49g2* (**Tables 1-3**). Highly specific *Ly49* gene-editing thus selectively abolished Ly49G2 surface expression on GO NK cells.

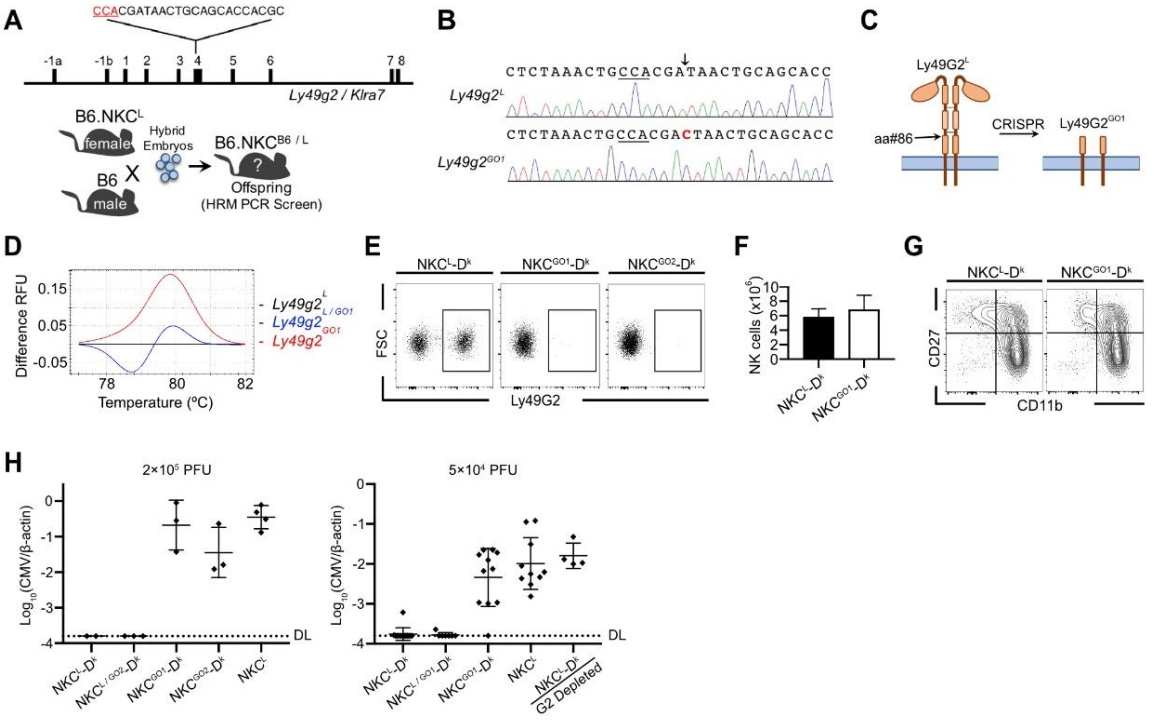


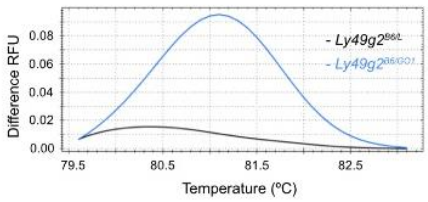
Figure 2. NK cells develop normally in *Ly49g2*-deficient GO mice but fail to control MCMV infection. (A) Diagram of CRISPR/Cas9-mediated editing of *Ly49g2*^{L} genomic DNA (gDNA) and the breeding scheme used to generate *Ly49g2*-mutant founders. The protospacer adjacent motif (PAM) sequence is indicated in red. (B) Sequence flanking the CRISPR sgRNA/Cas9 target site of WT *Ly49g2*^{L} and mutant *Ly49g2*^{GO1} alleles. The PAM sequence is underlined and a single cytosine insertion is shown in red. (C) Schematic of putative truncation site. (D) *Ly49g2 exon 4*-specific HRM PCR was performed with tail gDNA from WT (*Ly49g2*^{L}), heterozygous (*Ly49g2*$^{L/GO1}$), and GO1 (*Ly49g2*^{GO1}) mice. (E) Representative flow plots show Ly49G2 staining of NK cells from the spleens of uninfected NKC^{L}-D^{k}, NKC^{GO1}-D^{k}, and NKC^{GO2}-D^{k} mice. (F) Spleen NK cell numbers in uninfected NKC^{L}-D^{k} and NKC^{GO1}-D^{k} mice. (G) CD27 and CD11b profiles of spleen NK cells from uninfected NKC^{L}-D^{k} and NKC^{GO1}-D^{k} mice. (H) Mice were infected i.p. with

2×10^5 PFU (left panel) or 5×10^4 PFU (right panel) MCMV and evaluated for spleen virus levels 90hr post-infection (p.i.). Each symbol represents an individual mouse and error bars indicate mean ± SD. DL, detection limit. Data in D are representative of >20 independent experiments. Data in E – G are representative of three independent experiments with three to four mice per group. (H) Left panel, two to four mice per group. Right panel, combined data from three separate experiments with three to five mice per group. Error bars indicate mean ± SD.

A

Gene	Target Sequence (incl. PAM)	Mismatches
N/A (sgRNA)	GGCGTGGTGCTGCAGTTATCG	---
Ly49g2^{L}	T----------TGG	1
Ly49g2^{B6}	T------G----------TGG	2
Ly49t	T----------TTG	2
Ly49a	T--A--T----------T-TGG	4
Ly49i	T--A--T----------G-TGG	4
Ly49o	T--A--A----------T-TGG	4
Ly49p	T--A--A----------T-TGG	4
Ly49q	T--A-------A-------G-TGG	4
Ly49r	T--A--A----------T-TGG	4
Ly49s	T--A--T----------G-TGG	4
Ly49u	T--A--T----------G-TGG	4
Ly49v	T--A--T----------T-TGG	4
Ly49e	T--A--T----------G-TTG	5

B



C

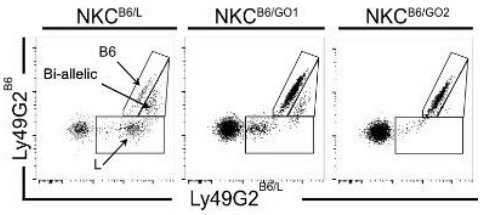


Figure 3. CRISPR/Cas9-mediated editing of *Ly49g2*^{l}. (A) Alignment of Ly49 sequences at the *Ly49g* sgRNA target site with variant nucleotides shown. (B) HRM PCR genotyping for the indicated *Ly49g2* alleles. Data are representative of >5 independent experiments with five mice per group. (C) Flow phenotyping of splenic NK cells for B6 (mAb Cwy-3) and pan-Ly49 (mAb 4D11) Ly49G2 allotypes in WT and GO founders. Data are representative of 3 independent experiments.

Table 1. Details for GO1 *Ly49* exome consensus sequences overlapped at the CRISPR target site.

Predicted Gene / Locus [1]	No. Exome Seq reads	Consensus length (bp)	% identity - Ref Seq	Ref Seq (Strain)	Ref Seq Accession No.
Ly49q1/Klra17	132	171	97.7	129x1/SV	AB193832.1
Ly49v/Klra22	84	178	100	129	AF288381.1
Ly49s/Klra19	48	181	100	129	BC116824.1
Ly49t/Klr20	116	190	99.5	129	AF288379.1
Ly49r/Klra18	109	188	100	129	NM_053153.2
Ly49r-related_a	37	193	97.4	129	NM_053153.2
Ly49r-related_b [2]	33	126	89.7	129	NM_053153.2
Ly49r-related_b [2]	33	126	99.2	B6	AC087336.5 (BAC RP23-44607) & AC134336.2 (BAC RP23-134A10)
Ly49g2^{GO1} [3]	232	202	99.5	C57L	GU434662.1
Ly49p/d	91	142	100	129	AF425096.1
Ly49p/Klra16	35	168	100	129	BC119242.1
Ly49o/Klra15	112	187	99.5	129	NM_013793.2

[1] GO1 exome sequence details for *Ly49g* and related transcripts overlapping the CRISPR target site are listed in chromosomal order.

[2] Exome consensus sequence aligned best to B6 genomic sequences, and *Ly49r*^{129}.

[3] Only *Ly49g2*^{GO1} was found mutated (single cytosine insertion) in comparison to its reference sequence.

Table 2. Details for GO2 *Ly49* exome consensus sequences overlapped at the CRISPR target site.

Predicted Gene / Locus [1]	No. Exome Seq reads	Consensus length (bp)	% identity - Ref Seq	Ref Seq (Strain)	Ref Seq Accession No.
Ly49q1/Klra17	57	167	100	129	AB193832.1
Ly49q1/q2-related	107	100	97.7	129x1/SV	AB193832.1
Ly49e/Klra5	48	194	99.5	B6	NM_008463.2
Ly49v/Klra22	36	188	100	129	AF288381.1
Ly49s/Klra19	30	178	99.4	129	BC116824.1
Ly49t/Klr20	50	189	99.5	129	AF288379.1
Ly49r/Klra18	61	204	100	129	NM_053153.2
Ly49r-related_a	20	200	97.5	129	NM_053153.2
Ly49r-related_b [2]	12	107	89.7	129	NM_053153.2
Ly49r-related_b [2]	12	107	99	B6	AC087336.5 (BAC RP23-44607) & AC134336.2 (BAC RP23-134A10)
Ly49g2^{GO2} [3]	90	205	99.5	C57L	GU434662.1
Ly49p/d	34	142	100	129	AF425096.1

[1] GO2 exome sequence details for *Ly49g* and related transcripts overlapping the CRISPR target site are listed in chromosomal order.

[2] Exome consensus sequence aligned best to B6 genomic sequences, and *Ly49r*^{129}.

[3] Only *Ly49g2*^{GO2} was found mutated (single cytosine insertion) in comparison to its reference sequence.

Table 3. GO *Ly49* exome consensus sequences detailed in Tables 1 and 2.

>Ly49q1/Klra17

AAAAACATGAACTGCAGGAAACTCTAAACTGCCACCATAACTGTAGCACCATGCAAAATGAC
ATCAACGCAAAGGAAGAAATGCTGAGAAATATGCCTCTAGAGTGTAGTACAGGAGATGATCT
TCTGAAATCCCTCAACAGAGAACAGAAGAGATGGTACAGTGAA

>Ly49q1/q2-related *(97.7%)*

CAAGAAAAACATGAACTGAGGGAAACTCTAAACTGCCACCATAACTGTAGCACCATGCAAAG
TGACATCAACGCAAAGGAAGAAATGCCGAGAAATATGCCTCTAGAGTGTAGTACAGGTGATG
ATCTTCTAAAATCCCTCAACAGAGAACAGAAGAGATGGTACAGTGAA

>Ly49e/Klra5

TTTTTCAGTATAGTCAACACAAACAAGAAATACACGAAACTCTAAACCACAACCATAACTGCA
GCAACATGCAAAGTGACATCAAATTAAAGGAAGAAATGTTGAGAAATAAGTCTATAGATTGCA
GTCCAGGTGAGGAACTTCTGGAATCCCTCAACAGAGAACAGAACAGATGGTACAGTGAAAC
CAAGACA

>Ly49v/Klra22

AAACTGCAGGAAATTCTAAACCACCACAATAACTGCAGCAACATGCAAAGTGACATCAACTT
GAAGGATGAACAGCTGAAAAATAAGTCTATAGAGTGTAATCTTCTGGAATCCCTCAACAGGG
ATAAGAACAGATTGTATAATAAAACCAAGACTGTTTTAGATTCCTTGCAGCACA

>Ly49s/Klra19

TCAACACAAACAAGAAATCCATGAACCTCTAAACTACCACCATAACTTCAGCAACATGCAAAG
TGATTTCAACTTAAAGGAAGAAATGTTGACAAATAAGTCTATAGACTCTAGGTCAGGCAATGA
ACTTCTGGAATCCCTCAACAGAGAACAGAACAGAGGGTACAGTGAAACTAAGACA

>Ly49t/Klr20

CAACAAAAACATGAACTGCAAGAAACTCTAAACTGCAACGATAACTGCAGCACCACGCAAAG
TGACATCAACTTAATGGATGAACTGCTGAGAAATAAGTCTATAGAATGTAGGCCAGGCAATG
ATCTTCTGGAATCCCTCAACAAGGAACAGAGCAGATGGTACAGTGAAACCAAGACTCTTTTA
GATT

>Ly49r/Klra18 (100%)

TCAACAAAAACATGAACTGCAGGAATTTCTAAAACACCACAATAACTGCAGCATCATGCAAAG
TGACATCAACTTGAAGGATGAACTGCTGAAAAATAAGTCTATAGAGTGTAATCTTCTGGAATC
CATCAACAGGGATCAGAACAGATTGTATAATAAAACCAAGACTGTTTTAGATTCCTTACAGC

>*Ly49r*-related_a *(97.4%)*

GTCAACAAAAACATGAACTGCAGGAATTTCTAAAACACCACAATAACTGCAGCATCATGCAAA
GTGACATCAACTTGAAGGATGAACAGCTGAAAAATAAGTCTCTAGAGTGTAATCTTCTGGAAT
CCCTCAACAGGGATCAGAACAGATTGTATAATAAAACCAGGACTGTTTCAGATTCCTTACAG
CACAC

>*Ly49r*-related_b *(89.7%)*

TTTTTCAGTGTAGTCAACAAAAGAATGAACTGCAGGAAATTCTAAACCGCCACCATAACTGCA
GCATCATGCAAAGTGACATCAGCTTAAAGGAAGAACTGCTGAGAAATAAGTCTATAGTGTGT
A

>*Ly49g2*$^{GO1\_GO2}$

TTTTTCAGCATATTCAACAAAAACATGAACTACAGGAAACTCTAAACTGCCACGACTAACTGC
AGCACCACGCAAAGTGACGTCAACTTGAAGGATGAACTGCTGAGAAATAAGTCTATAGAGTG
TAGGCCAGGCAATGATCTTCTGGAATCCCTCAACAGGGATCAGAAAAGATGGTACAGTGAAA
CTAAGACTTTTTCAG

>*Ly49p/d*

TCACCAAAAACATGAACTGCAGGAATTTCTAAAACACCACAATAACTGCAGCATCATGCAAAG
AGACATCAACTTGAAGGATGAACTGCTGAAAAATAAGTCTATAGAGTGTAATCTTCTGGAAAC
CCTCAACAGGGATCAG

>*Ly49p/Klra16*

TTTTTCAGTATGGTCAACAAAAACATGAACTGCAGGAATTTCTAAACCACCACAATAACTGCA
GCATCATGCAAAGTGACATCAAATTGAAGAATGAACTGCTGAAAAAGAAGTCTATAGAGTGT
AATCTTCTGGAATCCCTCAACAGGGATCAGAACAGATTGTATA

>*Ly49o/Klra15*

TCAACAAAAAGAACTGCAGGAAATTCTAAACCACCACAATAACTGCAGCAACATGCAAAGTG
ACATCAACTTGAAGGATGAACTGCTGAAAAATAAGTCCATAGAGTGTGATCTTCTGGAATCC
CTCAACAGGGATCAGAACAGATTGTATAATAAAACCAAGACTGTTTTAGATTCCTTAAAGCAC

NK cells develop normally in *Ly49g2*-deficient GO mice but fail to control MCMV infection.

Homozygous GO mice breed well and develop normally, without obvious health defects. Additionally, NK cell numbers and CD27 and CD11b expression profiles are similar in NKC^{GO1}-D^k and NKC^L-D^k mice (**Figures 2F, 2G)**. Thus, Ly49G2 deficiency did not appreciably alter NK cell development at baseline.

We next assessed Ly49G2's effect on host resistance by comparing spleen virus levels several days after MCMV infection. In comparison to WT Ly49G2 (NKC^L-D^k), both GO strains displayed higher MCMV levels as in NKC^L or NKC^L-D^k mice depleted of $Ly49G2^+$ NK cells (**Figure 2H**). Thus, the Ly49G2 inhibitory self-receptor is required for NK cells to mediate MHC I-dependent MCMV control.

MHC I D^k-dependent MCMV control is comparable to that mediated via Ly49H and is abolished by Ly49R neutralization.

MHC I-independent MCMV resistance has been well characterized in B6 mice, however, less is known about the extent of viral control conferred by MHC I-dependent mechanisms in comparison. As expected, NKC^L NK cells which lack m157-specific Ly49H receptors failed to control MCMV in the absence of H-$2D^k$ (**Figure 4A**). In contrast, NKC^L-

D^k Ly49G2$^+$ NK cells controlled MCMV as effectively as virus-specific NK cells in B6 mice (**Figure 4A**). These data demonstrate H-2D^k-dependent MCMV resistance is as robust as that provided by Ly49H.

We then interrogated a role for activation receptors in NKC^L-D^k mice. Strikingly, the Ly49R-specific mAb 12A8 selectively abolished MCMV resistance in comparison to NKp46- or NKG2D-blocking mAbs (**Figure 4B**). The extent of mAb 12A8's effect was comparable to selective depletion of Ly49G2$^+$ NK cells which suggested both receptors may be important in MCMV control. In contrast to Ly49G2$^+$ NK cells depletion, however, total NK cell numbers were unaffected by the α-Ly49R mAb. Rather, a comparable subset of Ly49R$^+$ NK cells remained after treatment which was readily detected using another Ly49R-reactive mAb, clone 4E5 (**Figure 5A**)**.** Moreover, prolonged 12A8 treatment in NKC^L-D^k mice did not interfere with IFN-γ production during NK cell stimulation via the Nkp46 activation receptor, nor did it alter the ability of Ly49G2$^+$ NK cells to reject non-D^k bone marrow cell targets in vivo (**Figures 5B-5D**). These data indicate mAb 12A8 treatment functionally neutralizes Ly49R signaling without depleting or broadly impairing NK effector cells. We infer that Ly49R signaling in Ly49G2$^+$ NK cells is required to mediate MHC I D^k-dependent MCMV resistance.

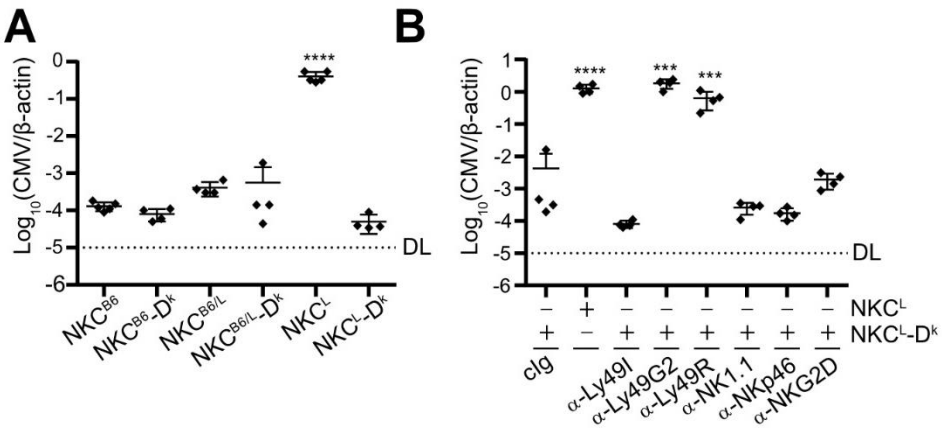


Figure 4. MHC I D^k-dependent MCMV control is abolished by Ly49R neutralization. (A) Quantification of viral genomes in the spleens of the indicated NKC congenic ± D^k-transgene expression. (B) Quantification of viral genomes in the spleens of mice that were treated with depleting or neutralizing antibodies against NK cell surface receptors prior to infection. Mice in A and B were infected i.p. with 2×10^5 PFU MCMV and evaluated for spleen virus levels 90hr p.i. All data are representative of 2 to 5 independent experiments with four to five mice per group. Error bars indicate mean ± SD.

Ly49G2 and Ly49R receptors engage MHC I D^k.

Prior work showed that both Ly49G2 and Ly49R receptors can bind soluble H-2D^k tetramers (Makrigiannis et al., 2001). We hypothesized that these discordant self-receptors for a shared MHC I ligand may be an important element for specific virus control. To pursue this question, we established a reporter cell system (Iizuka et al., 2010) to examine whether MA/My- or C57L-derived self-receptor allotypes bind D^k. In comparison to control J7 cells, J7.ZG^M, J7.ZG^L and J7.ZR^L reporter cells were selectively stained and stimulated with Ly49-specific mAbs 4D11 or 12A8 (**Figures 6A, 6B**). Moreover, each of these reporter cell lines specifically responded when co-cultured with YB2/0-D^k cells, but not YB2/0 cells (**Figure 6B**). Thus, Ly49G2 and Ly49R self-receptors both bind H-2D^k.

Considering that Ly49 activation receptor expression on NK cells is sensitive to the presence of its cognate ligand in the host (George et al., 1999a; Sun and Lanier, 2008a; Tripathy et al., 2008), we further examined Ly49R expression on NK cells in H-2D^k-disparate mice. Consistent with the results obtained using reporter cells, we found that Ly49R expression varied in direct relation to host H-2D^k (**Figures 6C, 6D**), confirming that D^k is a cognate ligand for both self-receptors. Since licensed NK cells display greater responsiveness to activation receptor stimulation than their unlicensed counterparts (Fernandez et al., 2005; Kim et al., 2005; Anfossi et al., 2006), we next assessed whether Ly49G2 is a primary licensing receptor in NKC^L-D^k mice. Following stimulation with plate-bound mAbs specific for Nkp46 or Ly49R, we observed a significantly higher

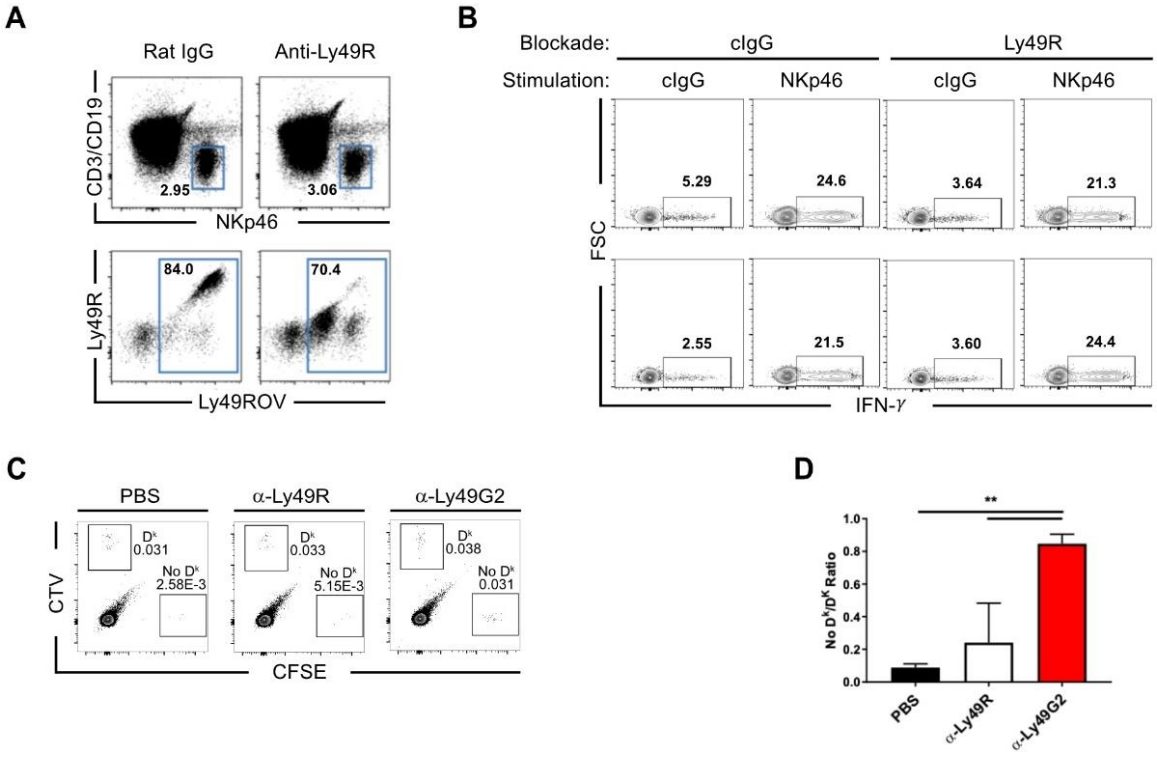


Figure 5. Functional normalcy of NK cells during Ly49R blockade. (A) NK cells were stained with anti-Ly49ROV (mAb 4E5) after 3 days of anti-Ly49R (mAb 12A8) or control IgG pretreatment in vivo. Data are representative of 2 independent experiments. (B) NKC^{L}-D^{k} splenocytes were stimulated with plate bound antibodies after 3 days of mAb 12A8 or control IgG pretreatment in vivo. NK cells were assessed for IFN-γ production. Each row is representative of one mouse per group. Data are from a single experiment with two mice per group. (C and D) Flow plots and quantification of rejected target cells 20 hr after transfer of BM targets. Data are from a single experiment with three mice per group.

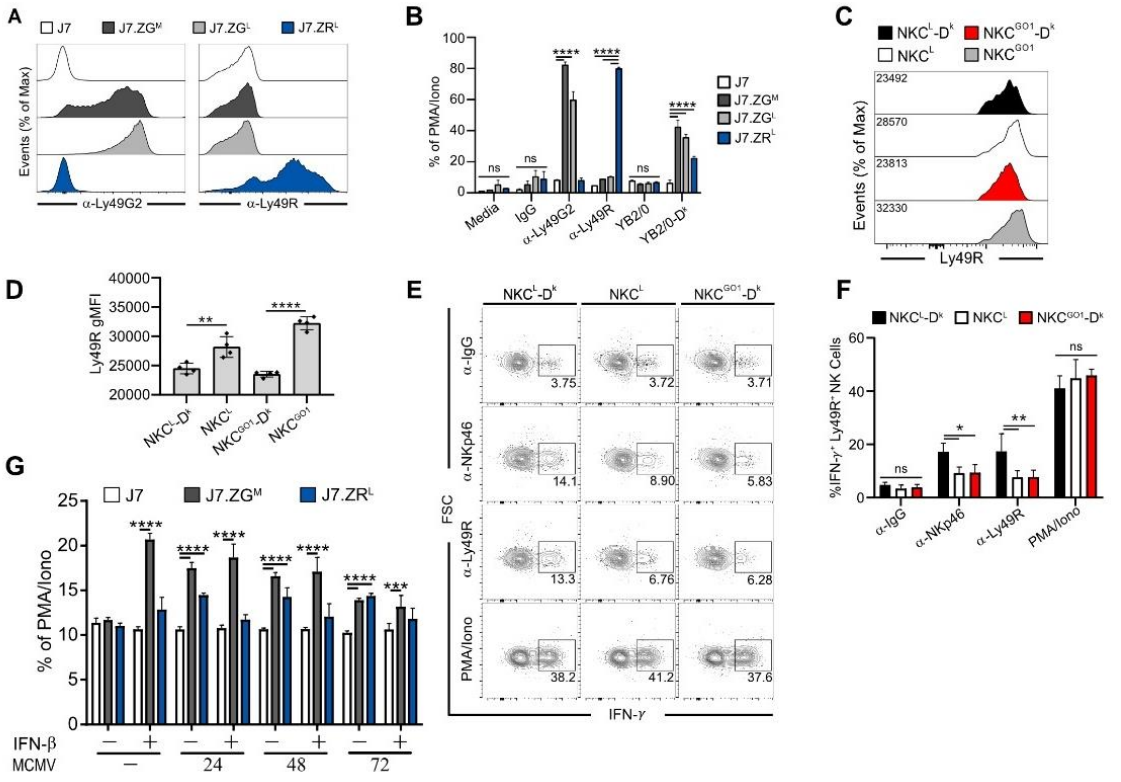


Figure 6. Ly49G2 and Ly49R receptors specifically bind MHC I D^k. (A) Surface expression of chimeric Ly49 receptors on J7 reporter cells, as determined by anti-Ly49G2 (4D11) and anti-Ly49R (12A8) mAb staining of J7.ZG^M, J7.ZG^L, or J7.ZR^L reporter cells. (B) Reporter cells were stimulated with plate-bound mAbs or target cells. (C) Representative histograms of Ly49R expression by splenic NK cells. (D) Quantification of Ly49R gMFI from (C). (E) Representative intracellular IFN-γ staining of spleen NK cells from uninfected NKC^L-D^k, NKC^L, and NKC^{GO1}-D^k mice following stimulation with the indicated plate-bound mAbs or PMA/Ionomycin. (F) Percentages of splenic $Ly49R^+$ NK cells that express IFN-γ from (E). (G) Reporter cells were co-cultured for 12hr with infected M2-10B4 cells pretreated with IFN-β 16hr before co-culture as indicated. Target cells were infected for the indicated times prior to co-culture with reporter cells. Data in

A and B are representative of 3-5 independent experiments with 2-5 samples per group. Data in C and D are representative of three experiments with 3-4 mice per group. Data in E and F are representative of two independent experiments with 4 mice per group. Data in G is representative of two experiments with 3-6 samples per group. Error bars indicate mean ± SD.

percentage of responsive Ly49R$^+$ NK cells from mice which express the licensing receptor and its cognate ligand (**Figures 6E, 6F**). A small fraction of responsive NK cells (2-4%) from each strain were also observed to respond during control stimulation, which suggests immobilized IgG may elicit low-level CD16 signaling. However, background stimulation via control Ig was inadequate to elicit disparate responses in NK cells from the different strains. The Ly49G2 licensing self-receptor thus enhances Ly49R$^+$ NK cell responsiveness in NKC^L-D^k mice.

We next tested whether Ly49G2 and Ly49R self-receptors can recognize MCMV-infected M2-10B4 targets bearing H-2D^k ligands. Despite IFN-β-induced H-2D^k surface expression on M2-10B4 cells (**Fig 7**), only the Ly49G2 reporters significantly responded. Additionally, despite both Ly49G2 and Ly49R reporters responding to targets infected with MCMV for 24-72 h (**Fig 6G**) Ly49G2 signals diminished as MCMV infection progressed and H-2D^k cell surface expression declined (**Fig 7**), whereas Ly49R signaling was maintained throughout. Intriguingly, IFN-γ treatment of MCMV-infected targets prevented Ly49R-specific recognition, whereas Ly49G2 reporters were undeterred. Together, these data demonstrate that while both self-receptors recognize H-2D^k, their binding affinities and MCMV response patterns differ.

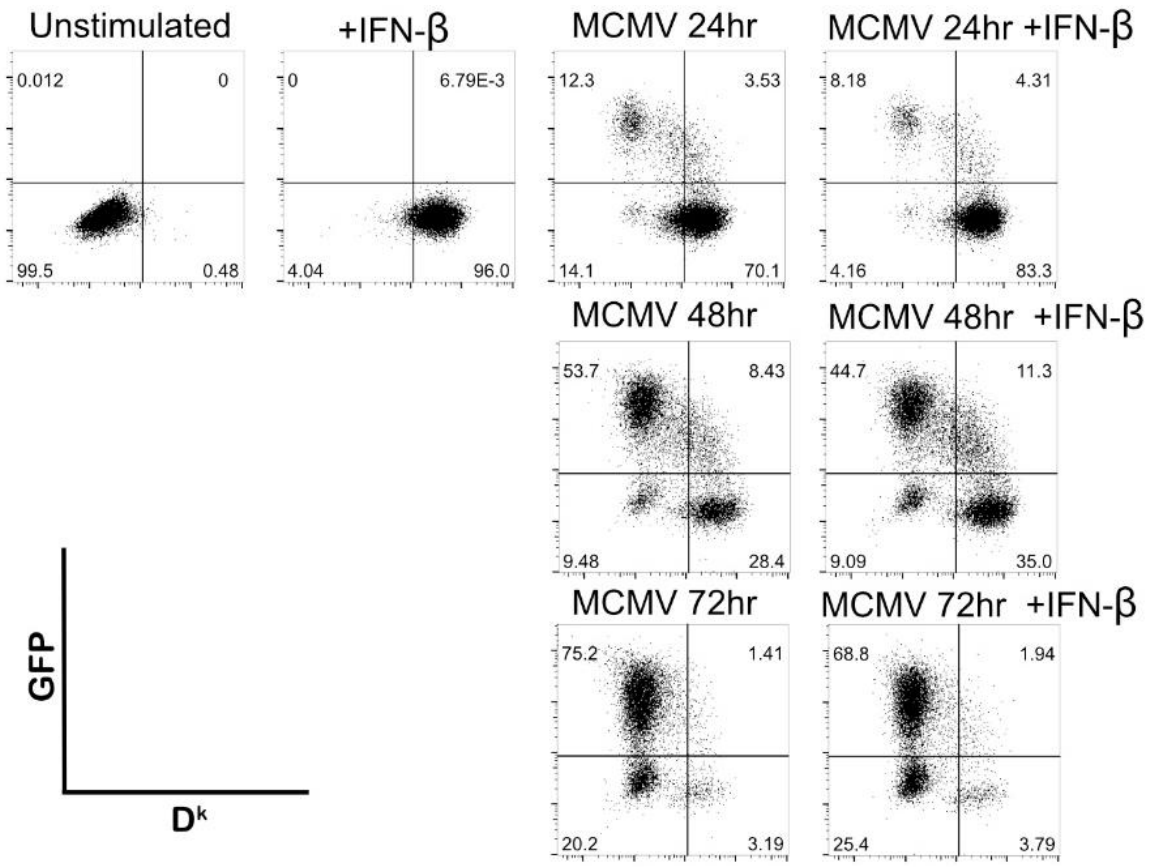


Figure 7. Modulation of Dk surface expression on M2-10B4 cells during MCMV infection. Representative flow plots are shown for M2-10B4 cells infected with MCMV-GFP ((Henry et al., 2000), kindly provided by Oscar Aguilar and Lewis Lanier) for the indicated durations. Some samples were treated with IFN-β 16 hr prior to co-culture (i.e. 8, 32, or 56 hr post-infection). Data are representative of 2-3 experiments with 2-6 samples per group.

Ly49R^{+}Ly49G2^{+} NK cells are specifically activated during MCMV infection.

Because NK cells differentiate in response to virus-induced inflammation (Dokun et al., 2001; Fogel et al., 2013), we examined the impact of Ly49G2-coexpression on Ly49R^{+} NK cell responses during MCMV infection. First, we assessed differentiation profiles for Ly49R^{+}Ly49G2^{+} (R^{+}G2^{+}) and Ly49R^{+}Ly49G2^{-} (R^{+}G2^{-}) NK cells. Since the proportions of NK cells in immature (CD27^{+} CD11b^{-}), transitional (CD27^{+} CD11b^{+}), and more mature (CD27^{-} CD11b^{+}) differentiation stages (Chiossone et al., 2009; Hayakawa and Smyth, 2006) were similar amongst R^{+}G2^{+} and R^{+}G2^{-} cells at 4 days post-infection (p.i.) (**Figures 8A, 8B**), these data suggest that the different NK subsets undergo similar maturation during infection. We also measured KLRG1 since virus-specific Ly49H^{+} NK cells were shown to acquire and maintain KLRG1^{hi} expression during MCMV infection (Fogel et al., 2013; Nabekura and Lanier, 2016). We observed a significantly higher fraction of NKC^{L}-D^{k} R^{+}G2^{+} NK cells exhibited KLRG1 terminal differentiation than their counterpart R^{+}G2^{-} NK cells, and this difference was D^{k}-dependent as both subsets displayed similar KLRG1^{hi} frequencies in NKC^{L} mice (**Figures 9A, 9B**).

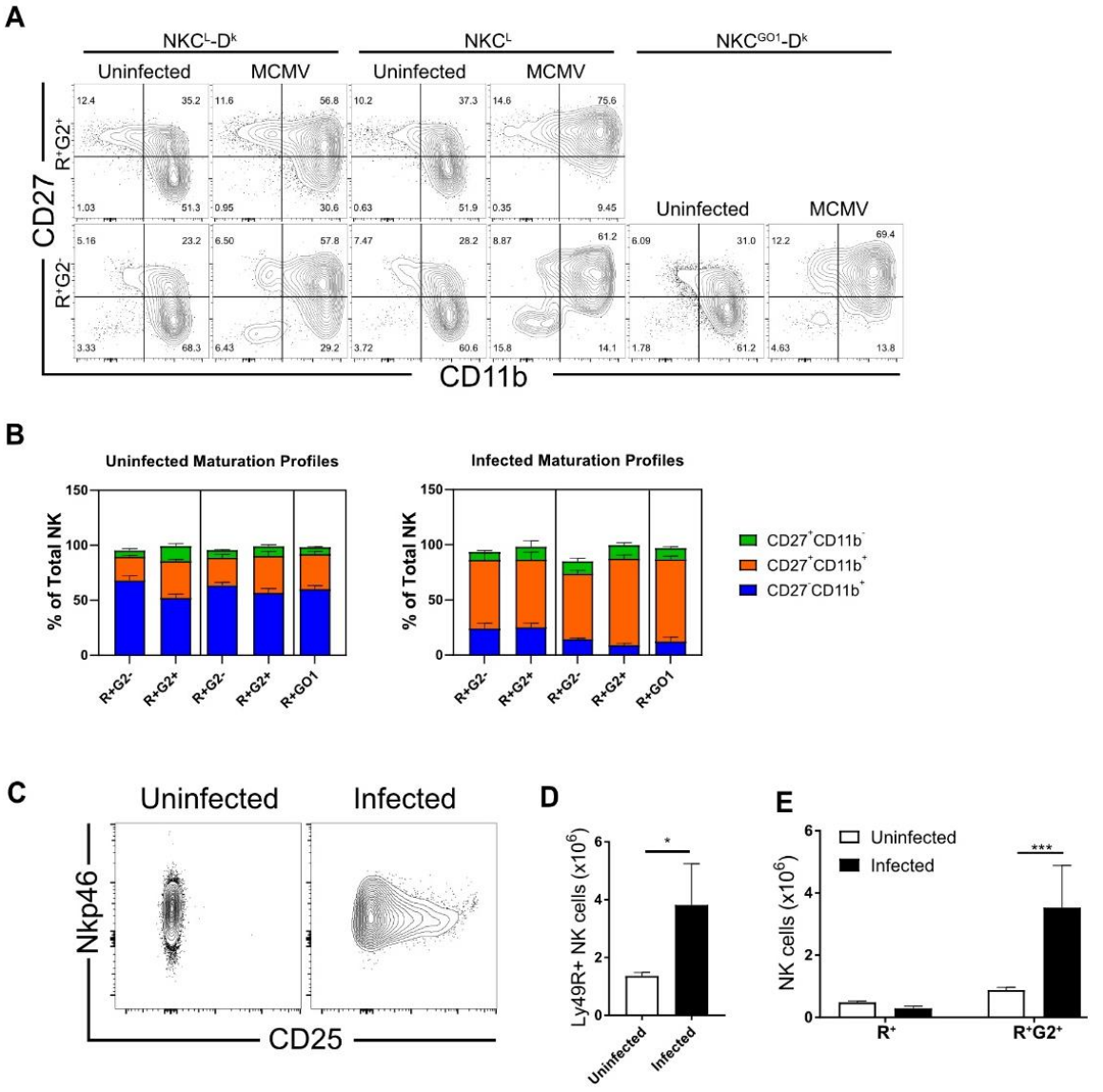


Figure 8. NK cell response profiles. (A and B) Representative flow plots and quantification of CD27 by CD11b splenic NK subsets from uninfected or day 4 p.i. mice. Data are representative of 3 independent experiments with 3-4 mice per group. (C) CD25 expression on splenic NK cells in uninfected or MCMV infected NKCL-Dk mice. Data are representative of 2 experiments. (D) Accumulation of total Ly49R+ splenic NK cells 6 days p.i., and (E) accumulation of R+G2+ splenic NK cells 6 days p.i. in NKCL-Dk mice. Data are representative of 3 independent experiments with 3-4 mice per group.

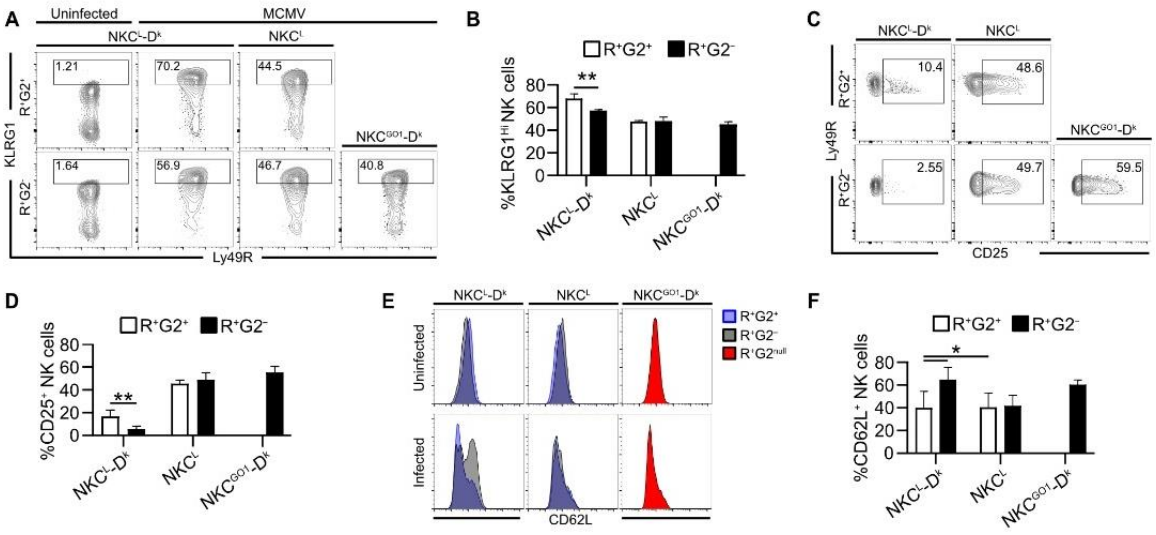


Figure 9. Ly49R$^+$ Ly49G2$^+$ NK cells are specifically activated during MCMV infection. (A and B) Flow plots and quantification of KLRG1^{hi} splenic NK cell subsets from uninfected or day 4 p.i. mice. (C and D) Flow plots and quantification of CD25$^+$ splenic NK cell subsets from day 4 p.i. mice. (E) Histograms of CD62L expression by splenic NK cell subsets from uninfected or day 4 p.i. mice. (F) Frequency of NK cells that remain CD62L$^+$ at day 4 p.i. Mice were infected with 5 x 10^4 PFU of MCMV. All Data are representative of two to three independent experiments with three to four mice per group. Error bars indicate mean $\pm$ SD.

in NKC^{L}-D^{k} mice (**Figures 9C, 9D**). This is likely due to the sustained inflammatory environment in mice lacking Ly49G2 or D^{k} caused by unfettered viral spread (**Figure 2G**)(Nash et al., 2017). In NKC^{L}-D^{k} mice, however, CD25 selectively increased on R^{+}G2^{+} cells. We also observed lower CD62L on R^{+}G2^{+} NK cells in comparison to R^{+}G2^{-} NK cells only in NKC^{L}-D^{k} mice (**Figures 9E, 9F**), similar to virus-specific NK cells in MCMV-infected B6 mice (Francois et al., 2013). Together, these data suggest D^{k}-licensed R^{+}G2^{+} NK cells experienced virus-specific activation, as opposed to more general, cytokine-mediated stimulation resulting from virus-induced inflammation.

Ly49G2 promotes Ly49R^{+} NK cell accumulation and proliferation during MCMV infection.

We showed in prior work that D^{k}-licensed Ly49G2^{+} NK cells selectively accumulate in response to MCMV (Gillespie et al., 2016; Prince et al., 2013). We thus examined whether Ly49G2 governs this expansion. Whereas R^{+}G2^{+} NK cells significantly increased in NKC^{L}-D^{k} spleens by 4 days p.i., there was a notable decrease in mice lacking either the self-receptor or its cognate ligand (**Figures 10A, 10B**). This finding is consistent with previous work showing that high viral burden induces splenic lymphopenia and lymphoid architecture collapse (Gillespie et al., 2016). Additionally, there was a greater representation R^{+}G2^{+} NK cells within the NK cell compartment in NKC^{L}-D^{k} mice (**Figures 10C, 10D**). This skewing is also seen near the peak of NK cell expansion 6 days p.i.

(**Figures 8D, 8E**). These results suggest D^k-licensed R^+G2^+ NK cells are the dominant responding subset during MCMV infection.

We assessed whether cell survival differences might explain subset variation during infection. R^+G2^+ and R^+G2^- NK cells from infected NKC^L-D^k mice exhibited similar caspase activation which indicated that apoptosis does not explain differential subset accumulation (**Figures 11A, 11B**). We next measured NK cell incorporation of BrdU to gauge whether increased proliferation accounts for selectively expanded R^+G2^+ NK cells. We observed a greater proportion of NKC^L-D^k R^+G2^+ NK cells incorporated BrdU during infection than their R^+G2^- counterparts (**Figures 10E, 10F**). Variation in subset proliferation was not seen in NKC^L or GO1-D^k mice, suggesting D^k-licensed R^+G2^+ NK cells selectively increased proliferation during infection.

To test whether subset skewing was due to an intrinsic defect in R^+G2^- proliferation, we injected mice with PolyI:C (pIC) to mimic virus-induced inflammation and again measured NK cell uptake of BrdU. In contrast to the results obtained during MCMV infection, both subsets responded equivalently following pIC treatment which indicated R^+G2^- NK cells are competent to undergo rapid proliferation (**Figure 10G**). Taken together, these data demonstrate that the selective accumulation of R^+G2^+ NK cells resulted from enhanced proliferation in response to MCMV infection.

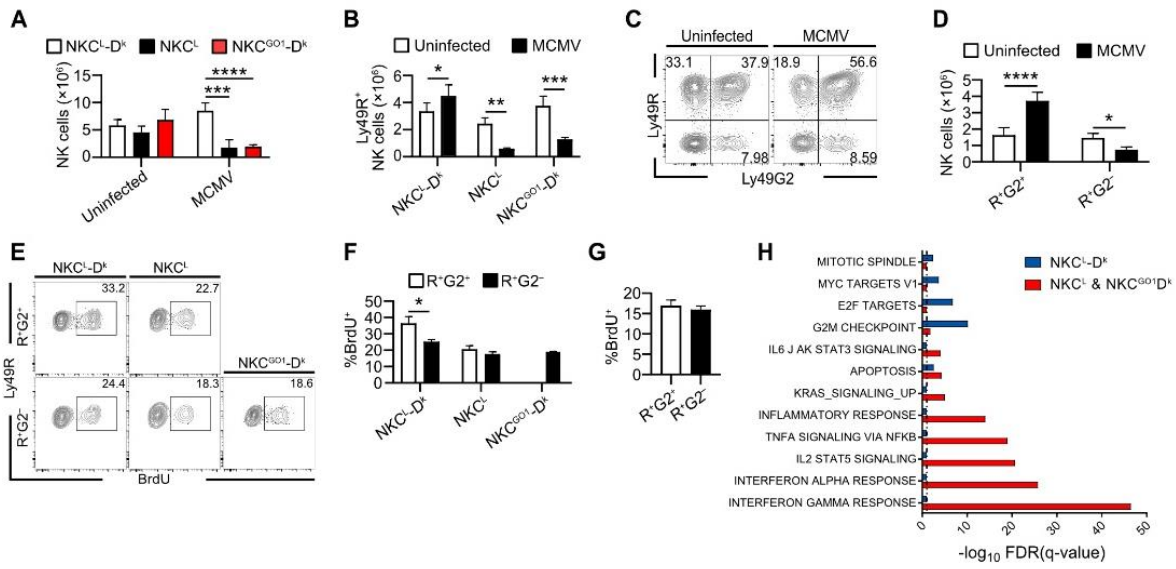


Figure 10. Ly49G2 promotes Ly49R$^+$ NK cell accumulation, proliferation and differential gene expression during MCMV infection. (A and B) Total NK cells and Ly49R$^+$ NK cells from the spleens of uninfected and day 4 p.i. mice. (C and D) Distribution profiles of Ly49R and Ly49G2 on NK cells in naïve or day 4 p.i. NKC^L-D^k mice. Representative flow plots are shown in (C). Quantified numbers of R$^+$G2$^-$ and R$^+$G2$^+$ subsets are shown in (D). (E and F) BrdU incorporation after 3hr pulse BrdU treatment 4 days p.i. (G) BrdU incorporation after 3hr pulse BrdU treatment three days post pIC injection. (A-F) Mice were infected with 5 x 10^4 PFU of MCMV. (H) Selected hallmark genes and corresponding gene enrichment analysis of NKC^L-D^k splenic NK cells. Data are representative of three to six independent experiments with three to four mice per group. (G) Data are representative of two independent experiments with three to four mice per group. Error bars indicate mean ± SD.

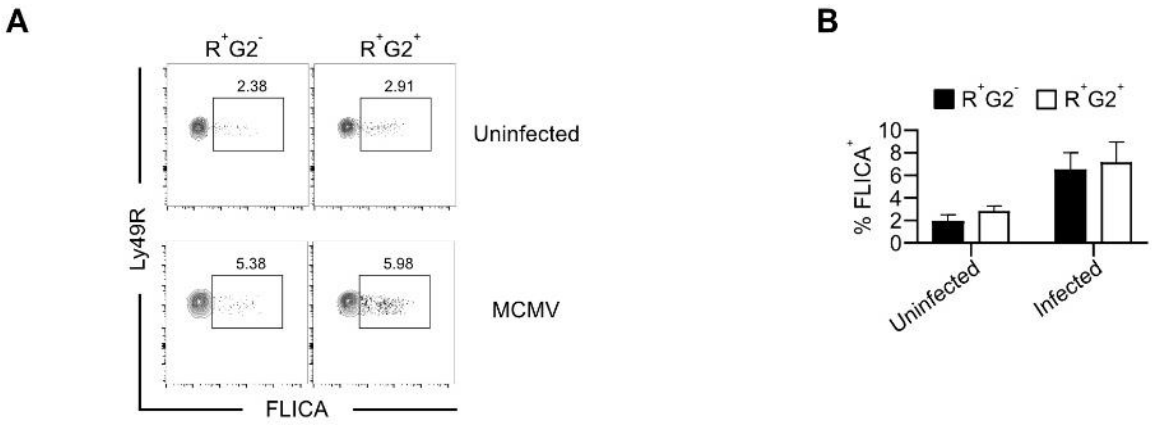


Figure 11. Ly49R+ Ly49G2– and Ly49R+ Ly49G2+ exhibit similar caspase activation during MCMV infection. (A and B) Flow plots and quantification of NK subsets with activated caspases in day 4 p.i. NKCL-Dk mice. Data are representative of 2-3 independent experiments with 3-4 mice per group.

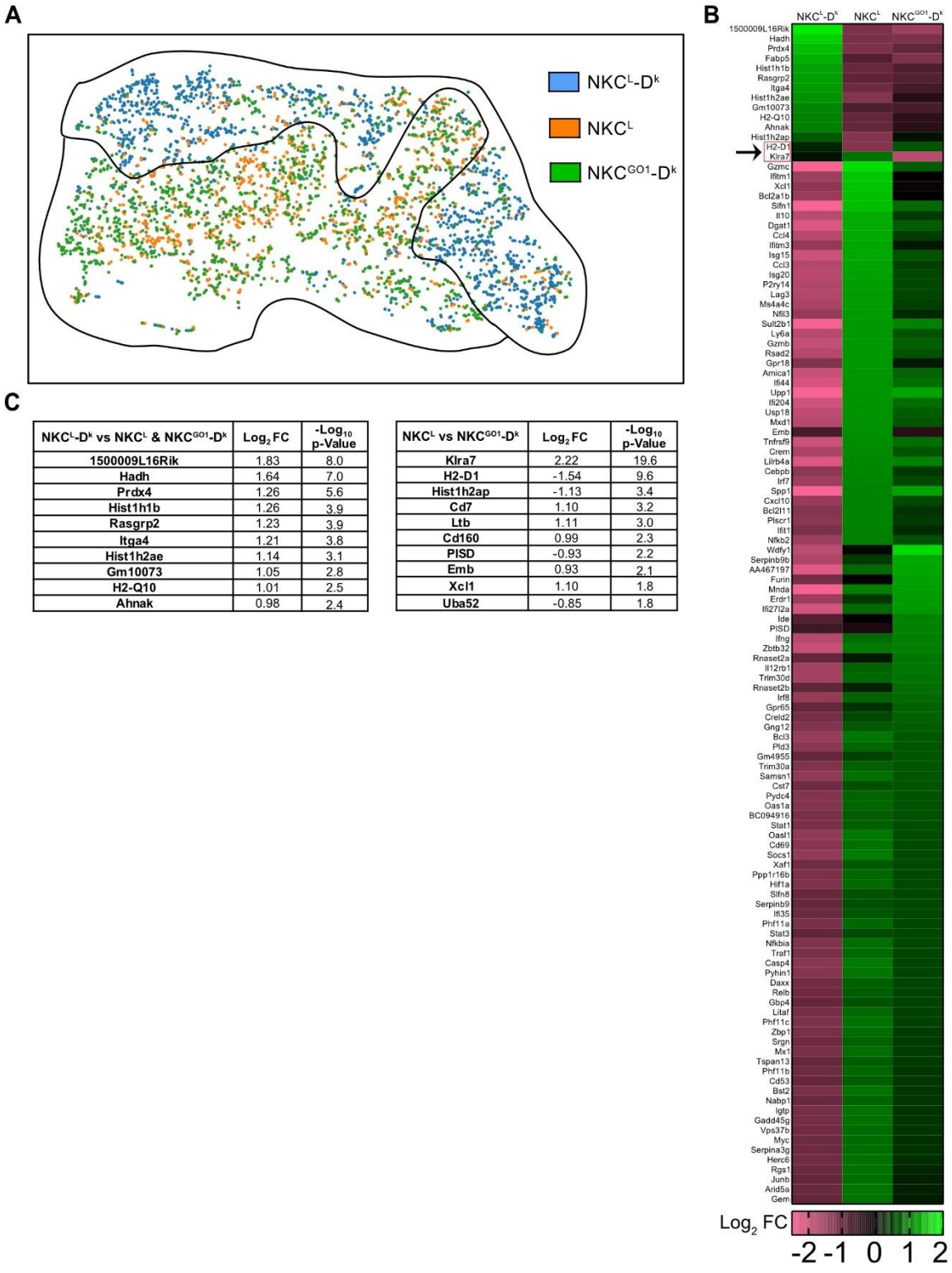
High-dimensional transcriptomic profiling of mouse splenic NK cells during MCMV infection.

Single cell RNA sequencing (scRNA-Seq) was used to evaluate transcriptomic differences in NK cells responding to MCMV. An unbiased t-SNE approach was applied to analyze scRNA-seq data. We observed NK cells from MHC I- or Ly49G2-disparate mouse strains clustered on the basis of sample, such that NKC^{L}-D^{k} clusters differed from those in NKC^{L} and NKC^{GO1} (**Figure 12A**). This suggests that NKC^{L} and NKC^{GO1} NK cells are transcriptionally similar during infection, in contrast to NK cells from infected NKC^{L}-D^{k} mice (**Figure 12B**). Indeed, examination of gene differences in NKC^{L} and NKC^{GO1}, aside from *H-2D* or *Klra7*, revealed little substantive variation (**Figure 12C**).

Gene set enrichment analysis (GSEA) using published hallmark gene sets revealed NKC^{L} and NKC^{GO1} NK gene expression is significantly skewed towards upregulation of an inflammatory response, including signaling via IL-2-STAT5, and strong TNF-α and interferon signatures (**Figure 10H**). NKC^{L}-D^{k} mice with licensed $R^{+}G2^{+}$ NK cells, on the other hand, upregulated genes associated with cell cycle control and proliferation. A net effect of self-receptor-dependent virus control thus resulted in profoundly altered gene expression to enable NK cell expansion.

To ascertain whether strain-specific differences in NK cell gene expression were simply due to different extrinsic signals based on host environment, we performed a similar anlaysis using NK cells from infected NKC^{L}-D^{k} mice only. Three distinctive t-SNE clusters were identified for comparison (**Figure 13A**). GSEA of hallmark genes showed that

Cluster 1 (C1) was highest in genes associated with upregulation of cell cycle control, DNA repair and metabolic activity (**Figures 13B-13D**), similar to data obtained for all NKC^{L}-D^{k} NK cells in **Figure 10H**. As a dominant responding subset with enhanced proliferative and metabolic function, C1 NK cells likely contributed significant MCMV control in NKC^{L}-D^{k} mice. Analysis of Clusters 2 and 3 (C2 and C3) NK cells, in contrast, revealed significant upregulation of complement and inflammatory response pathways as was observed for NKC^{L} and NKC^{GO1} NK cells (**Figures 10H, 13D**). Together these data indicate C2- and C3-type NK cell responses were not limited only to a highly inflammatory environment as in infected NKC^{L} or NKC^{GO1} mice. Rather, they represented a significant NK cell gene expression signature, possibly underpinning key antiviral activities. We infer C2- and C3-type NK responses may be overactivated in the absence of highly specific antiviral NK cells. More importantly, these data demonstrated that the Ly49G2 self-receptor itself drives an intrinsic difference in NK cells specifically responding to infection, whereas extrinsic factors were also involved.



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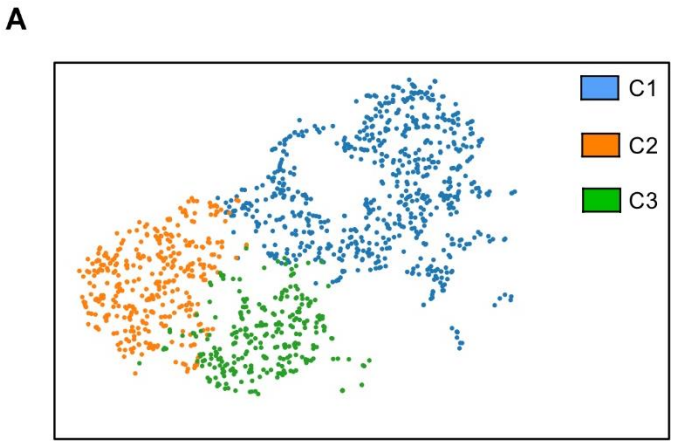
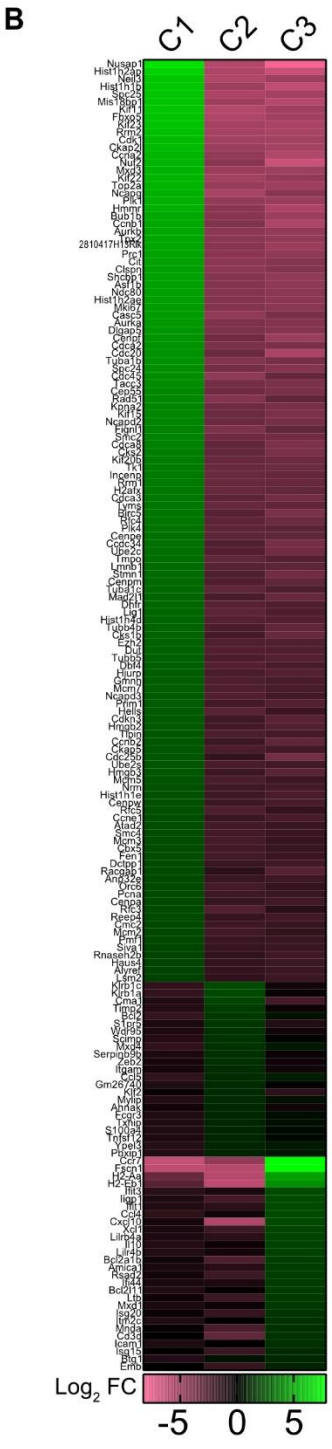
NKC^{L}-D^{k} vs NKC^{L} & NKC^{GO1}-D^{k}	Log$_2$FC	-Log$_{10}$ p-Value
1500009L16Rik	1.83	8.0
Hadh	1.64	7.0
Prdx4	1.26	5.6
Hist1h1b	1.26	3.9
Rasgrp2	1.23	3.9
Itga4	1.21	3.8
Hist1h2ae	1.14	3.1
Gm10073	1.05	2.8
H2-Q10	1.01	2.5
Ahnak	0.98	2.4

NKC^{L} vs NKC^{GO1}-D^{k}	Log$_2$FC	-Log$_{10}$ p-Value
Klra7	2.22	19.6
H2-D1	-1.54	9.6
Hist1h2ap	-1.13	3.4
Cd7	1.10	3.2
Ltb	1.11	3.0
Cd160	0.99	2.3
PISD	-0.93	2.2
Emb	0.93	2.1
Xcl1	1.10	1.8
Uba52	-0.85	1.8

Figure 12. High-throughput scRNA-Seq analysis of gene expression patterns for virus-responsive NK cells from host mice distinguished by licensed NK-mediated virus

control. (A) t-SNE plot of 4195 mouse splenic NK cells negatively enriched from the indicated strains day 4 p.i. (B) Heatmap showing Log_2 fold-change of 120 differentially expressed genes. Samples are separated by mouse strain (S1- NKC^{L}-D^{k}, S2- NKC^{L}, S3- NKC^{GO1}-D^{k}). Benjamin-Hochberg correction for multiple tests was performed to establish statistical significance. (C) Top 10 upregulated genes of each cluster according to p-value.





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C1	Log_2 FC	$-Log_{10}$ p-Value	C2	Log_2 FC	$-Log_{10}$ p-Value	C3	Log_2 FC	$-Log_{10}$ p-Value
Nusap1	7.0	30.0	**Klrb1c**	2.3	6.2	**Fscn1**	7.8	8.7
Hist1h2ap	6.4	30.0	**Klrb1a**	2.3	5.8	**Ccr7**	7.8	8.7
Hist1h1b	6.1	26.3	**Cma1**	2.1	4.7	**Lilrb4a**	2.1	4.5
Rrm2	5.9	26.3	**Bcl2**	1.7	3.6	**Ifit1**	2.3	4.5
Cdk1	5.8	26.3	**Timp2**	1.8	3.4	**Iigp1**	2.3	4.3
Fbxo5	6.0	25.5	**S1pr5**	1.7	3.2	**Ifit3**	2.3	4.2
Top2a	5.5	25.4	**Scimp**	1.5	2.8	**Ccl4**	2.2	4.0
Kif11	6.0	25.4	**Mxd4**	1.5	2.7	**H2-Aa**	4.9	3.9
Ccna2	5.6	25.1	**Wdr95**	1.6	2.6	**Lilr4b**	2.0	3.7
Spc25	6.0	24.0	**Zeb2**	1.4	2.2	**Amica1**	1.9	3.6

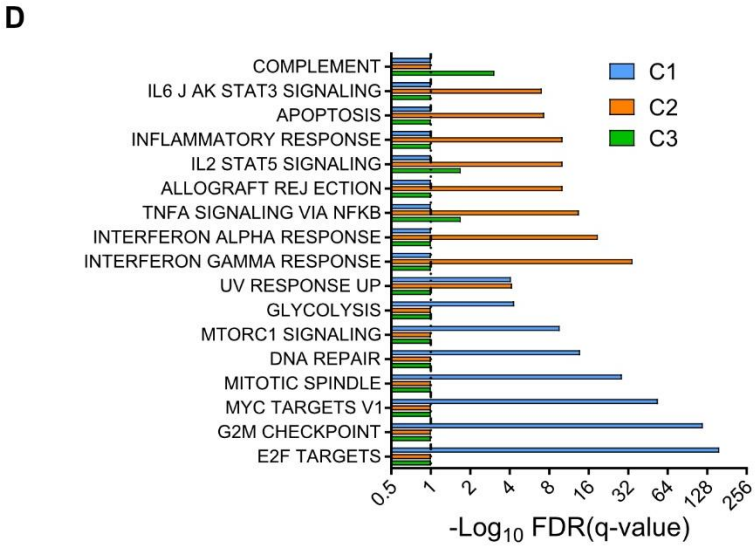

Figure 13 - High-throughput scRNA-Seq analysis of gene expression patterns for differently t-SNE-clustered virus-responsive NK cells from NKC^{L}-D^{k} host mice. (A) t-SNE plot of 1312 mouse splenic NK cells negatively enriched from NKC^{L}-D^{k} day 4 p.i. (B)

Heatmap showing the Log_2 fold-change of 172 differentially expressed genes. Individual clusters are being compared. Benjamin-Hochberg correction for multiple tests was performed to establish statistical significance. (C) Top 10 upregulated genes of each cluster according to p-value. (D) Selected hallmark gene sets and corresponding gene enrichment analysis.

Ly49G2 receptor licensing enables Ly49R^{+} NK cell activation, virus control and host survival during MCMV infection.

To verify Ly49G2's role in the activation and expansion of Ly49R^{+} NK cells, we co-transferred differentially labeled NKC^{L}-D^{k} and NKC^{GO1}-D^{k} NK cells into NKC^{L}-D^{k} or NKC^{GO1}-D^{k} recipients prior to MCMV infection (**Figure 14A**). Remarkably, R^{+}G2^{+} NK cells rapidly responded and displayed enhanced proliferation and expansion in NKC^{GO1}-D^{k} recipients (**Figures 14B, 14C**). Moreover, R^{+}G2^{+} NK cells displayed selective upregulation of CD25 and KLRG1 expression, especially in NKC^{GO1}-D^{k} recipients, in comparison to either R^{+}G2^{-} or R^{+}G2^{null} NK cells (**Figure 14D**). Together, these data demonstrate a cell intrinsic role for Ly49G2 in promoting specific Ly49R^{+} NK cell responses to MCMV infection.

To confirm that R^{+}G2^{+} NK cells are responsible for enhanced virus control, we enriched R^{+}G2^{-} and R^{+}G2^{+} NK subsets and separately transferred them into B6.D^{k} (i.e. NKC^{B6}) recipients. Since NKC^{L}-derived NK cells are resistant to PK136 (anti-NK1.1) depletion (Dighe et al., 2005; Xie et al., 2009), this system allowed us to ablate endogenous NKC^{B6} NK cells in recipients prior to transfer. Thus, any effects on virus control stem from the transferred NK cells (**Figure 14E**). While R^{+}G2^{-} NK cells had no impact on virus control, recipients of R^{+}G2^{+} NK cells exhibited lower viral burden and greater accumulation of NK cells in spleen than recipients of R^{+}G2^{-} NK cells (**Figures 14F, 14G**), thus confirming that licensed R^{+}G2^{+} NK cells provide essential MCMV control.

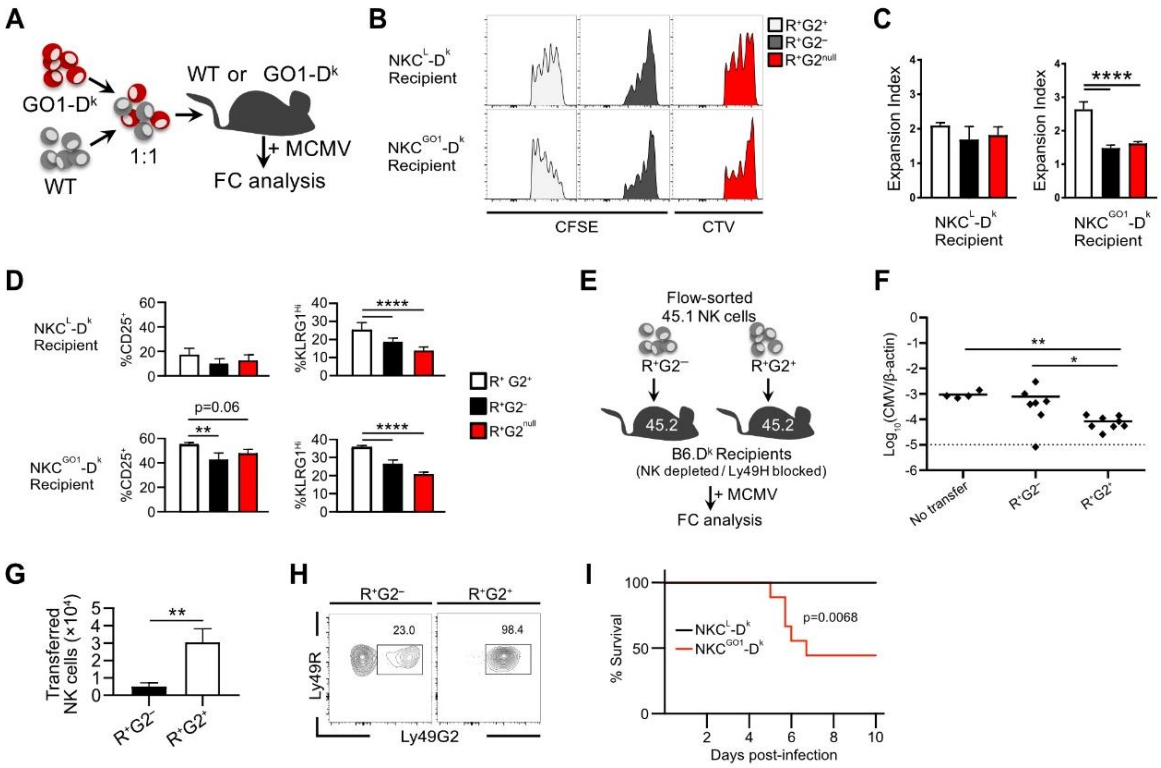

Figure 14. Ly49G2 receptor licensing enables Ly49R⁺ NK cell activation, virus control and host survival during MCMV infection. (A) Diagram illustrating the adoptive transfer of NKC^{L}-D^{k} (CFSE-labeled) and NKC^{GO1}-D^{k} (CTV-labeled) donor splenic NK cells (mixed 1:1) into NKC^{L}-D^{k} or NKC^{GO1}-D^{k} recipients 24hr prior to MCMV infection. (B) CFSE and CTV dilution profiles of enriched donor splenic NK cells from NKC^{L}-D^{k} or NKC^{GO1}-D^{k} mice. (C) Quantification of expansion indexes of data in (B). (D) Frequency of CD25^{+} or KLRG1^{hi} donor NK cells in (B) on day 4 p.i. (E) Diagram illustrating NKC^{L}-D^{k} NK enrichment and flow sorting into two major subpopulations. Sorted cells (~3x10^{5}) were adoptively transferred into NKC^{B6}-D^{k}-CD45.1 mice pretreated with α-Ly49H (3D10) and α-NK1.1(PK136) mAbs. (F) Quantification of viral genomes in the spleens of recipient mice day 4 p.i. (G) Accumulation of transferred NK cells in the spleen day 4 p.i. (H)

Ly49G2 positivity day 4 p.i. after cell sorting. (I) Host survival curves for NKC^{L}-D^{k} or NKC^{GO1}-D^{k} mice following infection with $1x10^{6}$ PFU MCMV. **(**A – G) Mice were infected with $2.5x10^{4}$ PFU MCMV. Data are representative of 3-4 independent experiments with 3-4 mice per group. Error bars indicate mean ± SD. In E, statistical significance determined by post-hoc Dunn's test (*p<0.05, **p <0.01, ***p< 0.001, ****p< 0.0001). In H, data are from a single experiment with 9-10 mice per group. Log ranked Mantle-Cox test was used to determine statistical significance p = .0068.

Ly49g2^{129} gene activation has been shown to occur in mature NK cells in vitro in the presence of IL-2 (Makrigiannis et al., 2004). Additionally, Ly49G2$^+$ NK cells in B6 mice expand nonspecifically following bone marrow transplantation and MCMV infection (Barao et al., 2011). Whether due to clonal expansion or de novo *Ly49g2* expression in Ly49G2$^-$ NK cells remains uncertain, but it may be upregulated in activated NK cells. Whereas most adoptively transferred R$^+$G2$^-$ NK cells remained so during infection, a minor fraction clearly expressed Ly49G2 receptors (**Figure 14H**). This could be the result of *Ly49g2* gene activation, or possibly clonal expansion of a rare population of residual R$^+$G2$^+$ cells remaining following flow sorting prior to adoptive transfer. Nonetheless, G2$^-$ NK cells are not a significant precursor population to G2$^+$ NK cells and R$^+$G2$^+$ NK cells undergo dramatic clonal expansion during MCMV infection.

Having verified the importance of the Ly49G2 receptor on Ly49R$^+$ NK cell-mediated MCMV control in the spleen, we assessed their role in host survival by administering a sublethal dose of MCMV to Ly49G2 WT and GO mice. All mice with WT Ly49G2$^+$ NK cells survived the infection, whereas >50% of GO mice succumbed (**Figure 14H**). The Ly49G2 inhibitory self-receptor thus is essential in MHC I-dependent virus immunity and host survival when co-expressed on NK cells with its functionally discordant Ly49R self-receptor counterpart.

Discussion

While a widely held paradigm suggests licensed NK cells primarily thwart NK-mediated virus control, here we demonstrate the inhibitory Ly49G2 NK cell receptor is required to specifically augment host defenses, including NK cell differentiation and proliferation, and limit virus spread during MCMV infection. A role for an inhibitory receptor in virus control may seem paradoxical since several studies show NK-mediated antiviral activities are diminished in their presence (Mahmoud et al., 2016; Orr et al., 2010; Rahim et al., 2015, 2016). Moreover, NK cells can mediate MCMV control in MHC I-deficient animals lacking self-receptor ligands (Tay et al., 1995; Polić et al., 1996; Orr et al., 2010). MCMV m157-specific Ly49H^{+} NK cells, however, display MHC I-independent MCMV control (Arase et al., 2002; Smith et al., 2002). Hence, licensed NK cells may be dispensable if virus-specific recognition by NK activation receptors is adequate to overcome tolerance. Nonetheless, we found that licensed R^{+}G2^{+} NK cells are essential to mediate vigorous MHC I-dependent host immunity during WT MCMV infection. Moreover, licensed NK cells disarmed by exposure to MHC I^{lo} host cells can regain the capacity to mediate missing-self responses after MCMV-induced activation (Bern et al., 2019; Sun and Lanier, 2008b). Licensed NK cells thus may be uniquely poised to overcome self-tolerance during MCMV infection.

Though seemingly counterintuitive, inhibitory receptors have been shown to augment lymphocyte effector functions. In T cells, the inhibitory NKG2A receptor was shown to increase control of ectromelia virus infection by promoting NKG2A^{+} CD8 T cell

survival (Rapaport et al., 2015). Related to this, human inhibitory KIRs enhanced murine CD8 T cell proliferation ex vivo in response to stimulation by dendritic cells bearing transgenic cognate HLA molecules (Ugolini et al., 2001). Additionally, expression of self-specific inhibitory KIRs was found to coincide with increased CD8 T cell survival and better overall virus control in patients infected with HIV, HCV, or HTLV-1 (Boelen et al., 2018).

Self-MHC I-specific inhibitory receptors which license developing NK cells also increase the extent of activation receptor stimulation (Anfossi et al., 2006; Fernandez et al., 2005; Kim et al., 2005). Licensed NK cells that are educated on self-MHC I undergo expansion and differentiation in response to MCMV infection (Prince et al., 2013; Sungur et al., 2013; Wei et al., 2014; Zamora et al., 2017), and in HCMV-infected individuals (Béziat et al., 2013). Memory NK cells expressing self-specific inhibitory Ly49 receptors in hapten-sensitized mice likewise display enhanced recall responses (O'Leary et al., 2006; Wight et al., 2018). Despite that licensed NK cells expand in these varied contexts, a basis for this response is poorly understood. We envision several possibilities may account for selective expansion in response to viral infection: 1) licensing could increase activation receptor signals in response to virus or virus-induced antigens via altering activation signal transduction cascades. This explanation predicts that both licensing and activation receptors can specifically recognize and respond to virus-infected target cells. 2) Sustained binding of the inhibitory receptor could promote NK cell synapse formation and conjugation to infected target cells. 3) The licensing receptor could modify the activation receptor ligand so that activation signals are increased. Ongoing studies are

focused on determining how the Ly49G2 receptor enables NK cells to specifically recognize and respond to MCMV infection.

Although the Ly49G2 receptor was found to be essential, virus control was abolished when the Ly49R self-receptor was neutralized. Two additional activation receptors implicated in H-2^k-dependent MCMV resistance include Ly49L and Ly49P which are potential allele variants that both bind MCMV gp34–D^k complexes (Desrosiers et al., 2005; Fodil-Cornu et al., 2011; Pyzik et al., 2011). Indeed, adult Ly49L$^+$ NK cells protected BALB.K neonates upon transfer and subsequent MCMV challenge. A role for Ly49P$^+$ NK cells in vivo remains elusive since there is no available serologic or genetic tool to selectively ablate this subset. Still, we found that MCMV resistance in NKC^L-D^k mice is abolished either by serologic or genetic depletion of Ly49G2$^+$ NK cells. Moreover, the Ly49R-monospecific mAb sufficed to abrogate MCMV control to a similar extent as immunodepletion of Ly49G2$^+$ NK cells. A role for Ly49P in MHC I-dependent MCMV resistance thus is unclear.

Our data instead demonstrated expression of both Ly49G2 and Ly49R receptors in individual NK cells is required to elicit MCMV control. Although H-2D^k tetramers were previously shown to bind Ly49R, they were folded with human β-microglobulin which could have affected the interaction (Makrigiannis et al., 2001). Consistent with the prior study, we found Ly49R reporter cells were specifically stimulated by D^k-bearing YB20-D^k rat lymphoma cells and MCMV-infected M2-10B4 bone marrow stromal cells. Curiously, Ly49R reporters did not respond to uninfected or IFN-β-stimulated M2-10B4 cells with high D^k. We speculate D^k conformational differences in the different cell lines may

underlie disparate Ly49R responses. Nonetheless, MCMV infected M2-10B4 cells consistently triggered Ly49R signaling which was abrogated by IFN-β treatment. We additionally found that Ly49R expression on mouse NK cells is regulated by host cell D^k expression, similar to Ly49D downregulation in the presence of its ligand, D^d (George et al., 1999a). Altogether these data suggest the Ly49R self-receptor is sensitive to variations in D^k expression, especially during MCMV infection.

Since both Ly49R and Ly49G2 self-receptors bind the same ligand, a qualitative change in H-2D^k on infected target cells might result in a loss of Ly49G2-dependent self-control, increased Ly49R-mediated recognition, or a combination of these effects leading to increased NK cell activity, proliferation and virus control. This might occur through NK self-receptor-dependent recognition of viral peptide ligands or virus-induced modification of host MHC I. In human, select peptides can nullify stimulation of KIR inhibitory receptors by their cognate MHC I ligands (Borhis et al., 2013; Fadda et al., 2010). In contrast, KIR2DS2, a human NK cell activating receptor, exhibits a strong affinity for highly conserved flavivirus peptide motifs presented by HLA-C*0102 (Naiyer et al., 2017), which suggests MHC I-specific NK activation receptors can specifically recognize viral antigens presented by MHC molecules. Although Ly49 receptors interface with MHC I molecules beneath the peptide binding groove, they can also display peptide selectivity (Deng et al., 2008; Su et al., 1999). It is possible that Ly49 activation receptors might display similar specificity for virus peptide-modified host MHC I (Brown et al., 2019).

Specific proliferation is a salient feature of antigen-dependent effector NK cell responses during MCMV infection. Splenic NK cells generally expand and become activated in an antigen-independent manner via cytokine stimulation (Biron and Tarrio, 2015), whereas Ly49H^{+} NK cells exhibit DAP12-dependent proliferation in B6 mice (French et al., 2006). Selective expansion of the R^{+}G2^{+} subset in H-2D^{k} mice is reminiscent of that seen with MCMV m157-specific Ly49H^{+} NK cells. In addition to increased proliferation, these NK cells also become KLRG1^{hi} CD62L^{lo} CD25^{+} (Dokun et al., 2001; Fogel et al., 2013; Sun et al., 2009). Our data thus are consistent with increased CD25 observed for NK cells responding to MCMV (Lee et al., 2012). We additionally observed non-selective CD25 upregulation on NK cells in infected mice lacking Ly49G2 or D^{k}, consistent with the hypothesis that CD25 is regulated independent of Ly49 activation receptors (Lee et al., 2012). It is possible that licensed virus-specific NK cells are more sensitive, or have better access to IL-12 during MCMV infection. Altogether, our data suggest R^{+}G2^{+} NK cells undergo antigen-specific stimulation which promotes their differentiation and effector functions.

Ly49R signals in isolation are inadequate since Ly49G2 co-expression is required for optimal NK effector function. scRNA-Seq analysis revealed that extrinsic and intrinsic factors affected NK cells expressing discordant self-receptors to increase genes for cell cycle regulation and proliferation during MCMV infection. In comparison, NK cells from mice lacking the Ly49G2 self-receptor or its cognate ligand skewed gene expression towards inflammatory response pathways. We infer that NK proliferation and differentiation is dependent on a balance of inhibitory and activation receptor signaling

pathways in R^+G2^+ NK cells which shifts to overcome self-tolerance upon recognition of infected targets. We further posit that R^+G2^- NK cells are unable to overcome disarming in the absence of licensing receptor-enhanced recognition of target cells.

In conclusion, our data uncovers an underappreciated role for inhibitory self-receptors in promoting activation and expansion of NK cells in response to viral infection. This involves a novel mechanism of NK cell detection of viral infection that is reliant upon a receptor pair with discordant functions. We predict these self-receptors working in tandem may be much more sensitive to subtle variations in MHC I ligands (i.e. altered-self) so as to trigger highly aggressive NK cell effector activities and increased proliferation. This intricate host-pathogen interaction may be an important immune strategy in nature which underscores the need for further research to determine if similar receptor pairings are present in humans. A better understanding of such inhibitory and activation receptor pairs will further the development of new strategies to augment host immunity and improve clinical outcomes in the context of viral infections, tissue transplant, and cancer.

Chapter III: A Case for the Importance of Paired Self-MHC I Receptors in MHC class I-dependent NK cell immunity during virus infection

INTRODUCTION

The MHC I receptors and their ligands are incredibly polymorphic and their genes are independently segregated on separate chromosomes. This results in individuals that have the potential to express a mix of orphan and functional MHC I receptors. NK cells have a wide vista of MHC I receptors to choose from which may be beneficial to host immunity against pathogens in the presence of their cognate ligands. Whether MHC I receptors encoded from separate NKC haplotypes can interact with one another in a biologically meaningful way to promote NK cell responsiveness to viral infection has not been thoroughly interrogated. In this chapter, data are presented in support of the notion that Ly49 receptors encoded from independent NKCs may cooperate in control of viral infection. This chapter also includes further discussion on the implications and a presumed mechanistic basis for variable paired receptor-mediated NK cell recognition and control of viral infection.

Allelic Polymorphism Impacts NK cell Responsiveness and Control of Viral Infection

Viral control in NKC^{L}-D^{k} mice is dependent upon both D^{k} and licensed-$G2^{L+}$ NK cells. Whereas viral control in NKC^{b6} mice is MHC I independent. Interestingly, NKC^{b6} encodes for the $Ly49A^{b6}$ inhibitory receptor which has been shown to selectively bind D^{k}, whereas $G2^{b6}$ does not (Silver et al., 2002). Whether $Ly49A^{b6}$ or $G2^{b6}$ license NK cells on D^{k} has not been shown. We assessed the licensing status of both NK cell subsets to determine whether either subset licenses on D^{k}. We found that D^{k} primarily affected IFN-γ production by $Ly49A^{b6+}$ NK cells, but not of $G2^{b6+}$ NK cells (**Figure 15A,B**). These data are consistent with the notion that inhibitory receptors license NK cells in the presence of their cognate ligands. Additionally, these data suggest Ly49 receptor polymorphism results in functionally diverse receptor allotypes with different ligand binding profiles. Since we previously showed that licensed-$G2^{L+}$ NK cells specifically proliferate in the context of MCMV infection, we examined whether unlicensed-$G2^{b6+}$ selectively proliferate in the presence of D^{k}. Selective proliferation of $G2^{b6+}$ NK cells did not occur in the presence nor absence of D^{k} during MCMV infection (**Figure 16**). We also assessed whether $Ly49A^{b6+}$ NK cell accumulation occurs in the presence of D^{k}, since $Ly49A^{b6}$ is licensed by D^{k}. In order to circumvent the potentially confounding factor of $Ly49H^{+}$ NK cells specifically responding to MCMV infection, we blocked Ly49H signaling using a neutralizing antibody. In the absence of m157-specific Ly49H+ NK-mediated MCMV immunity, $Ly49A^{+}$ NK cells selectively accumulated in the presence of

D^k (**Figure 17**). These data suggest that licensed-Ly49A$^+$ NK cells are specifically responding to MCMV infection in the presence of D^k. However, D^k does not confer a benefit to viral control in NKC^{b6} mice (**Figure 4**). This is suggestive that NKC^{b6} lacks key additional D^k-dependent factor(s), potentially R^L, which are required for MCMV resistance. Thus, we tested whether NKC^L encodes for factors that can complement NKC^{b6} to provide D^k-dependent MCMV resistance. NKC^{het} and NKC^{het}-D^k mice were infected with MCMV with or without depletion of $G2^{L+}$ NK cells using the allotype-specific mAb AT8. Interestingly, D^k dependent resistance was preserved after depletion of $G2^{L+}$ NK cells (**Figure 18**). These data suggest that the NKC^{b6} may encode a factor(s) that promotes MCMV resistance in an NKC^L and D^k-dependent manner. Altogether, these data provide evidence for genetic epistasis between multiple NKC haplotypes and MHC I.

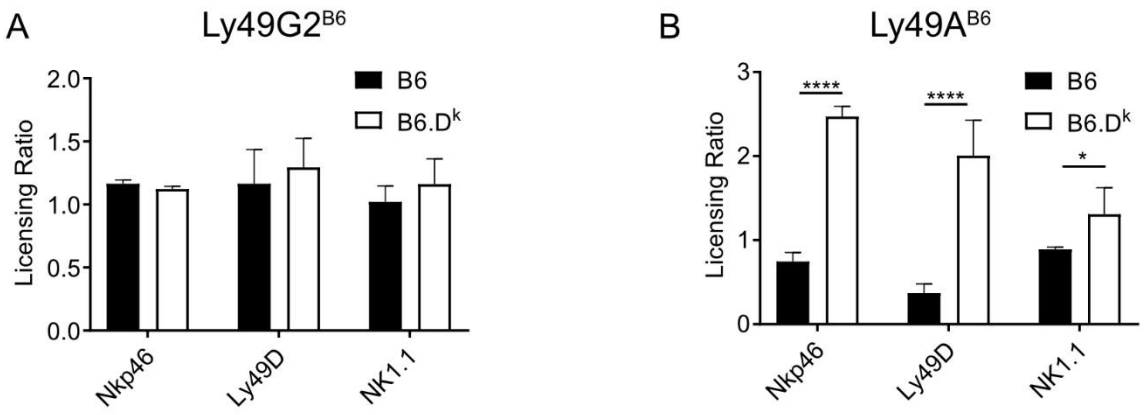

Figure 15. Differential licensing patterns of A^{b6} and $G2^{b6}$ in the presence of D^{k}.

(A and B) Mouse splenocytes from the indicated mouse strains were cultured for 6 hours with the plate-bound antibodies to the indicated activation receptors. Licensing ratios calculated for $G2^{b6}$(A) and $Ly49A^{b6}$(B). Data is representative of 2-3 independent experiments with 3-4 mice per group.

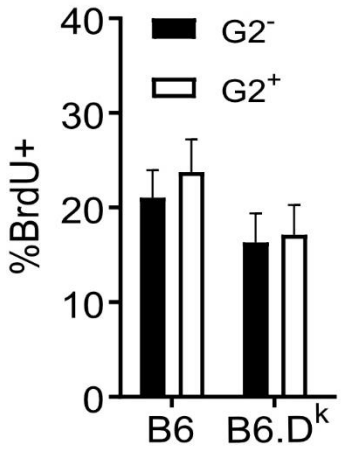

Figure 16. G2^{b6+} does not selectively proliferate in response to MCMV infection.

BrdU incorporation of NK cell subpopulations within the indicated mouse strains 90 hours post MCMV infection. Data is representative of 2 independent experiments with 3-5 mice per group.

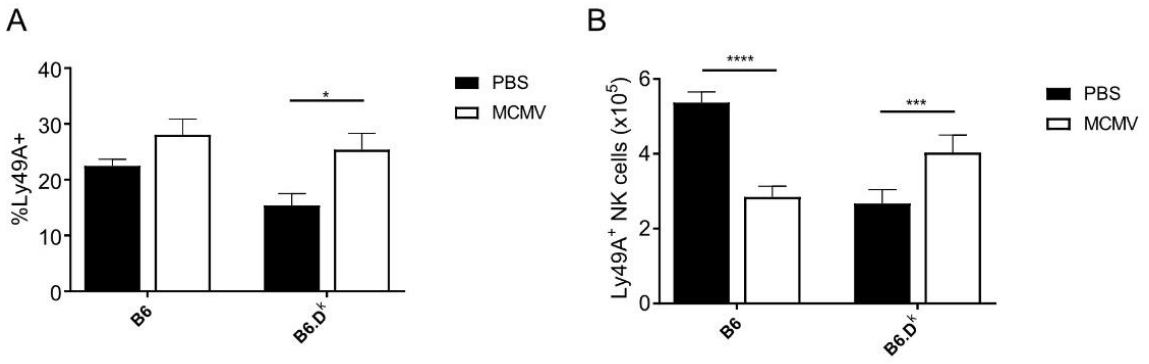

Figure 17. Accumulation of A^{b6} in the presence of D^k.

The indicated mouse strains were pretreated with mAb (3D10) specific to H^{b6}. Frequency(A) and cell numbers(B) for splenic A^{b6+} NK cells 90 hours post MCMV infection. Data is representative of 1-2 independent experiments with 3-4 mice per group.

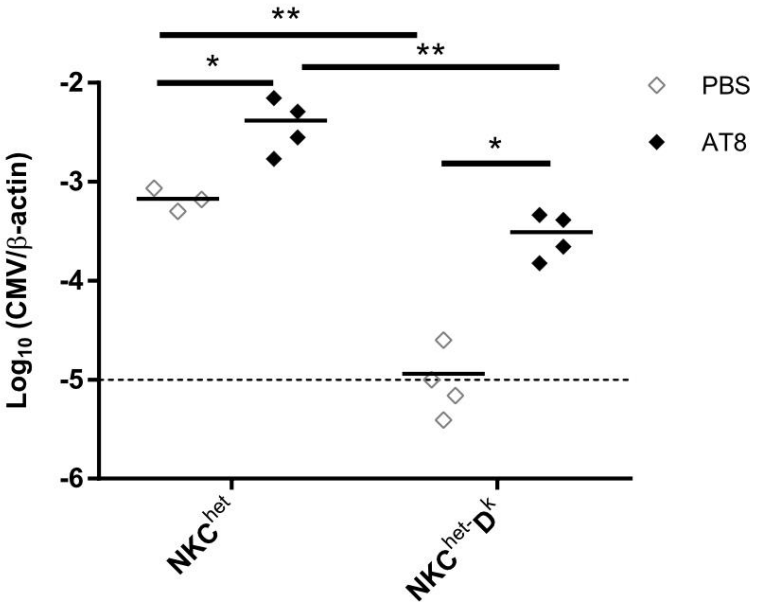

Figure 18. NKC^{het}-D^{k} mice are resistant to MCMV in a D^{k}-dependent manner in the absence of G2^{l} NK cells.

Quantification of viral genomes in the spleens of the indicated mouse strains. Mice were treated with either PBS or anti-G2 monoclonal ab (AT8) prior to infection. Mice were infected i.p. with 5x10^{4} pfu Smith strain MCMV and evaluated for spleen virus levels 90hr p.i. Data is representative of 3 independent experiments with 2-4 mice per group.

DISCUSSION

NKC^{het} and NKC^{het}-D^{k} G2^{c57l}-depleted mice differ in their ability to control MCMV infection in the spleen suggesting that there are NKC^{b6} and NKC^{c57l} factors working together to promote MCMV resistance in a D^{k} dependent manner. It is important to note that MCMV resistance in the NKC^{het} setting occurred in the presence of Ly49H NK cells which may be a confounding factor. However, Ly49H^{+} NK cell proliferation and responsiveness is limited by inhibitory MHC I receptors in the presence of self-MHC I (Forbes et al., 2016; Orr et al., 2010). Ly49H^{+} NK cells also control MCMV in an MHC I independent manner (Sun and Lanier, 2008b). Thus, if Ly49H contributes to D^{k}-dependent MCMV resistance in NKC^{het}-D^{k} G2^{L}-depleted mice it must cooperate with another NKC^{L} factor(s). We favor a model in which redundancy between Ly49 receptors that are able to license on D^{k} – in this case Ly49A^{b6} and G2^{L} – facilitates their ability to augment R^{L+} NK cell function and recognition of MCMV infection. This would suggest that in NKC^{het}-D^{k} mice Ly49A^{b6} educates R^{L+} NK cells which then limit the spread of MCMV in a D^{k}-dependent manner. Thus, infected target cell recognition via paired self-MHC I receptors expressed on licensed NK cells may be a general mechanism to provide highly specific and efficient NK cell immunity during virus infection.

Ly49D^{b6} reporter cells were shown to not be stimulated by D^{k+} target cells nor stained by D^{k} multimers in two independent studies (Hanke et al., 1999; Scarpellino et al., 2007). Additionally, primary Ly49D^{b6+} NK cells are not stimulated H-2^{k} target cells (George et al., 1999b; Nakamura et al., 1999). It is possible that Ly49D^{b6} does not recognize D^{k} in the

context of MCMV infection. Whereas reporter cells bearing the highly related R^L has been shown to recognize D^k expressing cells (**Figure 6B**), D^k tetramers (Makrigiannis et al., 2001), and virus infected stromal cells (**Figure 6G**). Additional studies focusing on interrogating the binding profiles of Ly49D and other activation Ly49s to virus-modified MHC I molecules are warranted.

The "missing self" hypothesis was first described by Klass Karre in 1986 (Kärre et al., 1986). Simply put, NK cells are uniquely poised to recognize a loss or aberrant expression of MHC I on abnormal cells which leads to their destruction. Missing-self recognition is a strategy employed by other immune cells and immune cell pathways. For example, macrophages employ the Sirp receptor family which have activating and inhibitory variants that signal through DAP12 or ITIMs, respectively, similar to Ly49 (Barclay and Brown, 2006). Sirp-α is an inhibitory receptor that recognizes CD47 on host cells. Downregulation (missing) of CD47 (self) occurs on aged and diseased cells which allows for phagocytosis by macrophages (Oldenborg et al., 2000). Botryllus Schlosseri is a tunicate that utilizes an allorecognition system that controls fusion dynamics with nearby colonies. This is dependent upon the major histocompatibility factor, BHF, which acts as a self-signal that inhibits cytotoxicity between colonies (Rosental et al., 2018). Whereas colonies that share BHF alleles tolerant to one another (Voskoboynik et al., 2013). Thus, "missing self" is both a broadly present and primitive mechanism of cell recognition.

A consistent theme among the above examples of "missing self" is that inhibitory receptor mediated inhibition dominates over parallel activation receptor signaling.

Ultimately, a loss of inhibition is a key step in recognition of missing-self targets. The variety of these paired-receptor systems is suggestive that a "missing-self" mechanism may not fully explain how selective forces (e.g. improved health and longevity associated with reproductive or immune functional effects) might have shaped evolutionary patterns of *genetic variation* amongst the genes for NK receptors and their MHC I ligands. An intriguing possibility is that paired-receptors for self-ligands rather evolved to discriminate endogenous cognate ligands, including those altered by post-translation modification or peptide-modified isoforms (herein referred to as "altered-self").

The polymorphic MHC I receptors have likely primarily evolved to recognize microbial patterns in the context of MHC I. This notion is gaining momentum based off of recent reports demonstrating that NK associated MHC I receptors engage with MHC I molecules bearing specific microbial peptides. For example, the human KIR2DS4 and KIR2DS2 recognize specific bacterial RecA and flavivirus RNA helicase peptides in the context of HLA-C, respectively (Naiyer et al., 2017; Sim et al., 2019). The crystal structure of mouse Ly49 shows that the ligand binding domain does not interact with the peptide binding groove of mouse MHC I (Tormo et al., 1999). Despite this, it is known that select peptides can influence MHC I binding to Ly49 receptors (Lemieux et al.; Marquez and Kane, 2015; Su et al., 1999). Peptides have also been shown to regulate binding of nonclassical MHC I (class Ib) molecules to their respective receptors. NKG2 family receptors are another family of paired receptors with discordant functions. NKG2A recognizes the class Ib molecule, $Qa1^b$, in the mouse. When ERAAP is absent or

functionally inert an endogenous self-peptide, FL9, displaces the normal Qdm-peptide on $Qa1^b$ molecules (Nagarajan et al., 2012). This FL9- $Qa1^b$ complex has a reduced affinity for NKG2A which leads to altered-self recognition. This suggests that peptides are key modulators of MHC I affinity for both KIR and Ly49 MHC I receptors can act as an essential component of "altered-self" recognition of microbially infected cells by paired receptors **(Figure 5)**.

Individuals that are seropositive for HIV yet fail to succumb to AIDS and maintain low viral burdens in the absence of antiretroviral therapy are known as elite controllers (Deeks and Walker, 2007). A significant proportion of elite controllers have been found to carry a specific allotype of KIR3DL1 that is associated with protection in the presence of its cognate ligand, HLA-B*57 (Martin et al., 2018). Interestingly, KIR3DS1, an activation KIR that is highly related to KIR3DL1 (and its paired receptor counterpart), has also been shown to be associated with delayed progression to AIDS in the context of HLA-B*57 (Martin et al., 2002). Moreover, KIR3DS1 has been shown to bind HLA-B*57 in the context of select HIV-derived peptide epitopes (O'Connor et al., 2015). As KIR3DL1 and KIR3DS1 are paired-receptors it is possible that they also work in tandem to recognize viral signatures in the context of HLA-B*57, not unlike G2 and R in the context of D^k. Thus, paired-receptor recognition of viral infection may extend beyond Ly49 receptors to the KIR receptors in human.

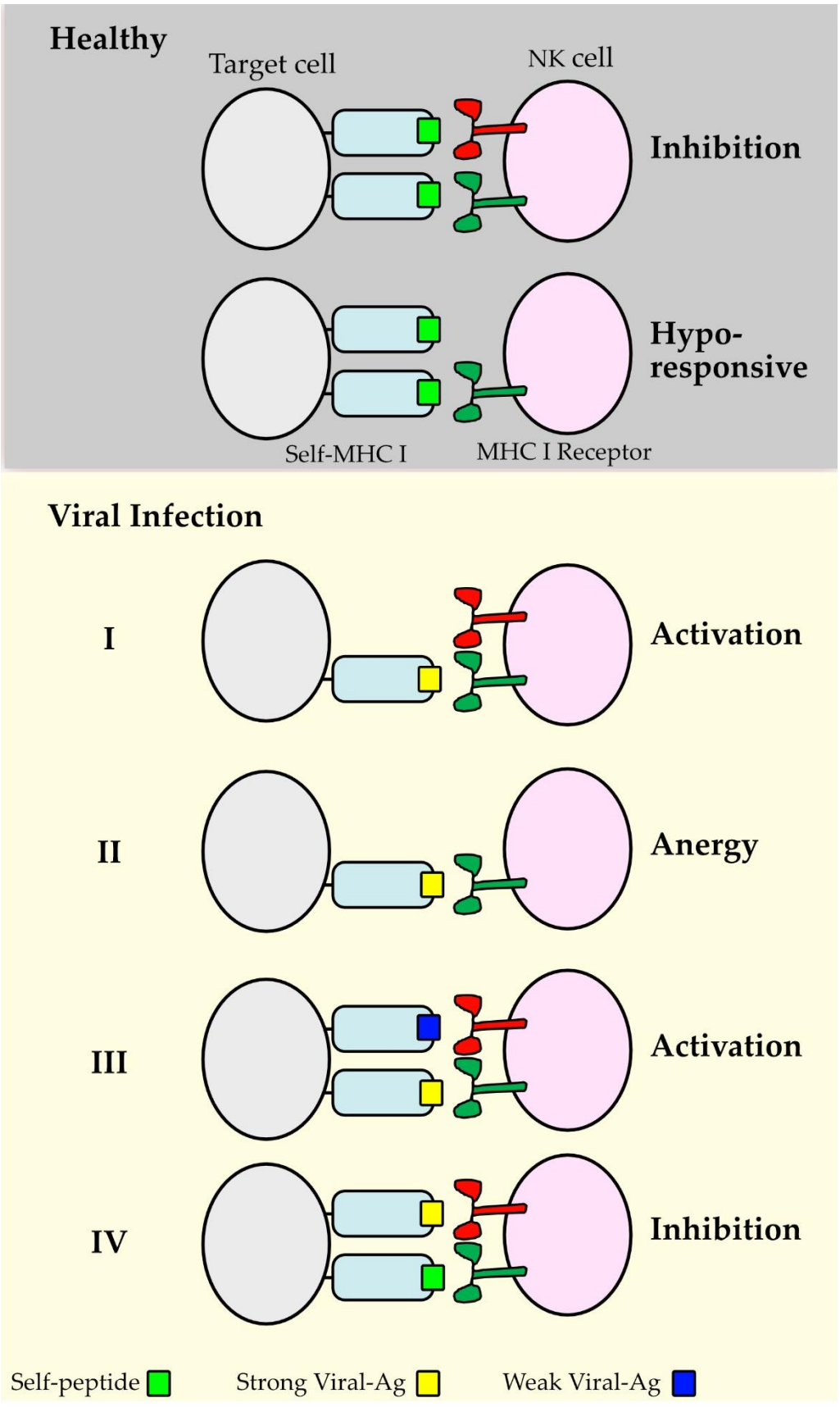
Healthy
Target cell
NK cell
Inhibition
Hypo-
responsive
Self-MHC I
MHC I Receptor
Viral Infection
I
Activation
II
Anergy
III
Activation
IV
Inhibition
Self-peptide
Strong Viral-Ag
Weak Viral-Ag

Figure 19. Model of paired receptor recognition of virus modulated/modified MHC I

MHC I inhibitory receptors are shown in red and activation receptors are shown in green. Peptides/antigens are denoted by colored squares. Under healthy conditions NK cells with self-MHC I inhibitory receptors license NK cells and limit self-MHC I activation receptor signaling. Whereas NK cells without self-MHC I inhibitory receptors are relatively hyporesponsive. Potential scenarios during viral infection include: (I) Downregulation of MHC I and display of virus-modified MHC that is bias for activation receptors, (II) same scenario with an unlicensed NK cell which cannot sense a change in MHC I, (III) Expression of virus-modified MHC I that binds weakly to self-MHC I inhibitory receptors but strongly to self-MHC I activation receptors, (IV) Virus-modified MHC I binds strongly to self-MHC I inhibitory receptors promoting inhibition.

Chapter IV: Future Studies

Future Directions

Class Ib molecules are currently heavily understudied. Given the fact that the majority of the CD8 T cell repertoire are sensitive to classical MHC I, it was thought that class Ib must not be playing a major role in mammalian immunity. In human, there are 16 different class Ib, compared to the 3 classical MHC I. This differs from the mouse, which harbors as many as 30 class Ib genes, which currently do not have any known homologues in humans, with the exception of Qa-1 and HLA-E (Goodall et al., 2018). Generally, the class Ib are highly conserved and less polymorphic than their classical MHC I counterparts. They have a much more fastidious peptide repertoire, many of which bind peptides that are highly conserved across mammalian species. Furthermore, class Ib are dynamically regulated and have restricted expression profiles compared to the ubiquitously expressed classical MHC I. Growing evidence suggests that there are robust NK and T cell responses to viral pathogens and tumors in the context of class Ib thus greater attention must be applied in this area.

Separating the effects of licensing and disarming has proven difficult. A major question remaining in the field is whether the inhibitory receptor actively promotes education independent of merely limiting activation receptor signaling. To my knowledge, no experiment to date has sufficiently addressed this question. A major challenge includes the lack of any models which allow for studying NK cells in the absence of activation receptor signaling. A mouse model devoid of NK cell associated activation receptors

with the exception of select activation receptor(s) expression driven by an inducible promoter would be useful for addressing this question. An alternative strategy would be to license other lymphocyte populations that are not thought to receive chronic activation signals like NK cells. Transducing CD8 T cells with self-specific MHC I inhibitory receptors may be a viable approach in this case. If CD8 T cells could be shown to exhibit a licensed phenotype when bearing self-specific inhibitory receptor(s) it would suggest that education is a general phenomenon that can be extended to other lymphocyte populations and licensing promotes responsiveness via TCR. Caveats to this approach include the assumptions that TCR signaling can benefit from licensing and that CD8 T cells do not express activation receptors that disarm them under steady state conditions. Nonetheless, these experiments might help to determine whether licensing phenotypes can manifest in the absence of disarming-activation receptors.

Our coculture experiments in chapter II show that as MHC I expression goes down during in vitro MCMV infection of stromal cells, R reporter cell activity increases. This is suggestive that a specific viral signature may be recognized by R, and since R^+ NK cells only respond in the presence of D^k, R is likely recognizing this signature in the context of D^k expression. Since we know that MCMV manipulates infected host cells to escort MHC I to their surface, I hypothesize that the quality of these select MHC I molecules allow for higher affinity interactions with R. Future experiments should focus on characterizing the constituent peptides and post-translational modifications of putative MCMV-modified MHC I molecules. This can be done by capturing MHC I from infected fibroblasts using fc-R chimeric proteins which can then be processed for

mass spectrometric analysis. Alternatively, D^k tetramers bearing peptides derived from MCMV ORFs might be used to screen R receptive MHC I pairings.

It is thought that activation MHC I receptors are evolutionarily derived from the ancestral MHC O inhibitory receptors by mutation (Abi-Rached and Parham, 2005). The activation receptor studied in this thesis, R, is thought to be closely related to Ly49A. In fact, the monoclonal antibodies used to character R in this thesis, 12A8 and 4E5, are both capable of binding a common epitope on A, whereas neither are reactive with G2. This suggests that Ly49A and R may have arisen from a common ancestral gene. This brings into question whether G2, Ly49A, and R bind D^k in a similar manner. X-ray crystallographic analysis will go a long way to determine how and where these different Ly49 receptors interface with MHC I.

Ly49 activation receptors are known to signal through the transmembrane adaptor protein DAP12 (Lanier et al., 1998; Smith et al., 1998). DAP12 contains ITAM motifs which facilitate the recruitment and activation of tyrosine kinases such as Syk and Zap70. Syk and Zap70 downstream signaling cascades include the Vav family proteins. Vav signaling proteins are at a critical intersection of activation and inhibitory signals derived from MHC I receptors. Activation of NK cytolytic activity via DAP12 has been shown to be Vav1-independent and Vav2 and Vav3 dependent (Cella et al., 2004). Alternatively, Ly49 inhibitory receptors are known to signal through their ITIM motifs which activate phosphatases (such as SHP-1, SHP-2 and SHIP1) that contain Src homology 2 (SH2) domains. Vav1 is a known substrate of SHP-1 and Vav1

dephosphorylation is thought to play a major role in inhibition of NK cell activation (Stebbins et al., 2003). Additionally, Vav1 is known to promote the formation of functional immune synapses in T cells (Tybulewicz, 2005). Loss of inhibition via G2 may allow for effective Vav1 signaling driven by NK activation receptors. This signaling cascade can then be supported by Vav2 and Vav3 signaling derived from R activation. Careful characterization of the phosphorylation states of the Vav family proteins during primary NK cell coculture with MCMV infected target cells is warranted to help clarify how signaling via G2 and R ultimately promote NK cells activation.

Proliferation is a hallmark of effector killer-lymphocyte response to viral infection. CD25 was shown to be selectively upregulated on $R^{+}G2^{+}$ NK cells in the NKC^{l}-D^{k} mice (**Figure 9C, 9D**). These data suggest that Ly49 can influence CD25 expression in the context of MCMV infection. Although CD25 was shown to be strongly upregulated in a Ly49-independent and IL-12-dependent manner (Lee et al., 2012), our data are not in contrast to the findings in this study. For instance, the authors characterize total NK cells in Ly49 signaling deficient mice rather than the MCMV-specific NK cell population within WT animals. Thus, it is worth interrogating whether signaling via CD25 is necessary to promote MCMV-specific NK cell expansion. To test this, the nondepleting, and receptor neutralizing, α-CD25 mAb (Nihei et al., 2014) could be administered during MCMV infection to see if there is an impact on effector NK cell expansion.

CD62L is highly expressed on NK cells and is required for efficient responses to viral infections (Peng et al., 2013). The most commonly known function of CD62L is to

prevent lymphocyte egress from lymphoid tissues. However, although CD62L is downregulated after TCR stimulation by lymph node CD8 T cells (Chao et al., 1997), they are capable of full re-expression upon egress into the bloodstream (Mohammed et al., 2016). CD62L crosslinking can also promote lymphocyte proliferation and lytic activity (Nishijima et al., 2005; Seth et al., 1991). More recently, cleavage of surface CD62L by the metalloprotease, ADAM17, has been shown to be a critical step in lymphocyte clonal expansion in response to TCR stimulation by Ag-specific target cells (Mohammed et al., 2019). Interestingly, scRNA-Seq analysis suggests that ADAM17 transcripts are associated with R^+G2^+ NK cells (data not shown) during MCMV infection. Cleavage of CD62L is strongly associated with R^+G2^+ NK cells in NKC^l-D^k mice during MCMV infection and may be critical for their expansion. To test this, mice with cleavage-resistant CD62L (Mohammed et al., 2019) could be crossed to NKC^l-D^k mice to assess the role of CD62L cleavage in effector NK cell expansion.

Closing Remarks

Millions of years of evolution and the NK cells are still employing the same old trick, albeit continually refined. Over 50 years of study in the modern era of immunology and we still don't know how the magician is pulling the rabbit from the hat. The identification of a dual requirement for functionally discordant paired-receptors dependent upon a common cognate ligand to drive NK cell surveillance of viral

infection is an exciting new mechanism. Specifically, determining how shared MHC I ligands of Ly49 and KIR are modified to selectively bind one or more paired receptors and whether this is a general mechanism employed by paired-receptor systems will surely keep us occupied for a number of years. NK cells, bestowed with their plentiful MHC I receptors of incredible diversity, and the subtle nuances of their activation, flourish the complexity and flexibility which define them as essential effectors of the immune system.

Materials and Methods

Ethics Statement. Mouse experiments were performed in accordance with the Animal Welfare Act and approved by the UVA IACUC.

Mice. B6.NKC^{C57L}-D^{k} (NKC^{L}-D^{k}), B6.NKC^{C57L} (NKC^{C57L}), and B6.D^{k} mice (Teoh et al., 2016), as well as B6.NKC^{GO1} (GO1) and B6.NKC^{GO1}-D^{k} (GO1-D^{k}) mice were generated and maintained at UVA under specific pathogen free conditions. B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ mice (Jackson Laboratory) were crossed with B6.D^{k} mice (Teoh et al., 2016) to generate B6.D^{k}-CD45.1 mice.

MCMV. Salivary gland passaged MCMV (Smith Strain; ATCC) was titered on NIH-3T3 or M2-10B4 and i.p. injected at stated doses as described (Rodriguez et al., 2004)(Xie et al., 2007). Mice were injected i.p. with 200 μg PK136, 4D11 or AT8 48 hr before infection to deplete NK cells. Ly49R was neutralized using 200μg mAb 12A8 (a gift from John Ortaldo) given i.p. 72 and 24 hr before infection (George et al., 1999b; Makrigiannis et al., 2001; Mason et al., 1996). Ly49H, NKp46 or NKG2D were respectively neutralized using 200μg mAbs 3D10 , 29A1.4 or C7 given i.p. 24 hr before infection (Brown et al., 2001b; Narni-Mancinelli et al., 2012; Ho et al., 2002). Infected mouse spleen DNA was measured for MCMV genomes via quantitative PCR as described (Wheat et al., 2003).

Antibodies and Flow Cytometry. Flow cytometry (FC) was performed using BD FACS Canto II, CytoFLEX, or Aurora Northern Lights flow cytometers. Data were respectively collected using FACSDiva, CytExpert, or Spectroflo software and analyzed using FlowJo (versions 9.7.2 and 10.1-10.4). Fluorescent mAbs were purchased from BioLegend, BD Biosciences, and eBioscience. The 2.4G2, PK136, 3D10, AT8, and 4D11 mAbs were purified from spent supernatants by the UVA Lymphocyte Culture Center. The 12A8 mAb was kindly provided by John Ortaldo.

Fluorescent mAbs from BioLegend, BD Biosciences, and eBioscience were titrated for optimal resolution and used to stain CD3 (145-2C11), CD19 (6D5), NK1.1 (PK136), CD49b (DX5), NKp46 (29A1.4), Ly49G2 (4D11), Ly49R (12A8), Ly49ROV (4E5), CD27 (LG.7F9), CD11b (M1/70), DNAM1 (10E5), KLRG1 (2F1), Ki67 (16A8), BrdU (BU20a), IFN-γ (XMG1.2), GZMB (NGZB), or CD62L (MEL-14). LIVE/DEAD fixable dyes (Thermo Fisher Scientific) were used to assess cell viability.

Adoptive Transfers. B6.D^{k}-CD45.1 mice were pretreated with NK depleting (PK136) and Ly49H neutralizing (3D10) mAbs 48 hr before and on the day of adoptive transfer. CFSE-labeled spleen NK cells (bulk transfer), or flow-sorted (Influx, UVA FC Core Facility) Ly49^{+} NK subsets (≥99% purity) were i.v. injected into host mice 24 hr before infection. For CFSE staining, 500µl of 10µM CFSE (made fresh in complete RPMI) was added dropwise to enriched NK cells in 500µl with vortexing and then incubated for 5 min at room temperature before quenching in 10mL complete RPMI. 3×10^{5} donor NKC^{L}-D^{k} NK

cells were i.v. injected into host mice 24 hr prior to infection. The expansion index is a measure of the fold expansion of the original population (total divided cells / estimated original starting population).

In Vitro Stimulation and Intracellular Cytokine Staining. Mouse splenocytes cultured in complete RPMI plus IL-2 (200U/ml; Peprotech) were used in *ex vivo* stimulations. Splenocytes (1-2 million) were stimulated with immobilized mAbs 12A8, NKp46 or control IgG (plates coated with 20 μg/ml mAb overnight at 4 °C) or PMA (100 ng/ml) and Ionomycin (1 μg/ml) for 1 hr prior to brefeldin A (BFA) addition, and an additional 4hr with BFA. Stimulated cells were fixed and permeabilized using a kit (Cytofix/Cytoperm; BD Biosciences) followed by staining for intracellular cytokines at 4 °C.

BrdU Incorporation Assay. Mice were i.p. injected with BrdU (1 mg/ 200 μl PBS) 3 hr prior to euthanization. BrdU staining was performed using a kit (BD Biosciences) per manufacturer instructions.

In Vivo Cytotoxicity Assay. In vivo cytotoxicity was performed essentially as described (Oberg et al., 2004; Wei et al., 2014). Briefly, a 1:1 mix of $2x10^6$ D^k and no-D^k bone marrow cells (in 200 μl RPMI) respectively labeled with 5 μM CFSE or CTV were i.v. injected into

host mice. Spleens were harvested 20 hr post-transfer and analyzed for residual donor cells by FC.

Statistical Analysis. Statistical analysis was performed using Graphpad Prism (version 7.04). Significance was assessed using 1- or 2-way ANOVA in conjunction with Tukey or Holms-Sidak post-hoc tests unless otherwise stated (*p<0.05, **p <0.01, ***p< 0.001, ****p< 0.0001). Students T-test or Mann Whitney rank test was used when comparing the means of 2 independent groups.

Design and in vitro transcription of sgRNA. An allele-specific sgRNA (5′-GCG UGG UGC UGC AGU UAU CG-3′) was used to edit *Ly49g2^{L} exon 4* based on available 129 and C57L allele sequences using crispr.mit.edu as described (Ran et al., 2013). The sgRNA was selected to maximize the likelihood of specific *Ly49g2^{L} exon4* editing while minimizing the potential to edit highly related *Ly49* genes. Notably, a 5′ G was appended to the sgRNA to ensure efficient *in-vitro* transcription with T7 polymerase. *Ly49g2^{L}* allele-specific oligonucleotides (Integrated DNA Technologies) cloned in pX330-U6-Chimeric_BB-CBh-hSpCas9 (kindly provided by Feng Zhang; Addgene plasmid #42230) were used to validate gene-editing efficiency in stem cells prior to work with mouse embryos.

The in vitro transcription template was amplified using a high-fidelity DNA polymerase (Phusion, NEB), pX330-*Ly49g2-exon4* vector, and a primer designed to

append the T7 promoter to the *Ly49g2*-sgRNA encoding oligonuceltide (Yang et al., 2014). The template was purified using a kit (Qiagen QIAQuick PCR purification) followed by dialysis against 1X TE. The template was then transcribed and its product purified using Ambion™, MEGAshortscript™, and MEGAclear™ T7 kits.

High Resolution Melting (HRM) PCR Genotyping for Edited *Ly49g2* Alleles. *Ly49g2*^{L} *exon4*-specific primers (For 5′-GAC TAA CTT AGT TTT TCA GC-3′ and Rev 5′-GCA GTT CAT CCT TCA AGT TGA-3′) spanning the sgRNA target site were designed essentially as described (Lundgren et al., 2012). Primers (Integrated DNA Technologies) were optimized and used in HRM PCR as described (Lundgren et al., 2012; Brown et al., 1997, 2001a).

Generation and validation of *Ly49g2* Deficient GO Mice. B6 (NKC^{B6}) males were bred to superovulated B6.NKC^{C57L} (NKC^{L}) females (Jackson Labs) to generate B6.NKC$^{B6/L}$ embryos which were microinjected with *Cas9* protein (PNA Bio or IDT) and *Ly49g2 exon4*-specific sgRNA prior to implantation into foster mothers. Offspring tail DNA was prepared using a kit (Gentraprep) and screened in HRM PCR using *Ly49g2*^{L} *exon4*-specific primers. Five viable offspring carried *Ly49g2*^{L} *exon 4* indels. Two founders transmitted *exon4* indels through the germline and were separately crossed back to NKC^{L} to generate homozygous GO mice, before further crossing to NKC^{L}-D^{k}.

Ly49g2 GO alleles were validated using whole genome exome sequencing of liver DNA which was performed by the Genomic Services Lab at Hudson Alpha essentially as described (Gillespie et al., 2017). Briefly, GO1 and GO2 FastQ files were separately aligned to the *Ly49g2*^{L} reference sequence using BWA-MEM in Sequencher (Gene Codes Corporation). A BWA-MEM-generated BAM file was opened in Tablet (James Hutton Institute) to visualize and identify CRISPR-modified *Ly49* sequences overlapping the target sequence. WT and CRISPR-modified *Ly49* sequences from this alignment were exported and realigned using high stringency parameters (minimum overlap 25 nucleotides, minimum match 97%) in Sequencher. Individual GO *Ly49* contig alignments were reviewed for nucleotide discrepancies and consensus sequences overlapping the *Ly49g2* CRISPR target site are reported in SI Appendix Tables S1-S3.

scRNA-Seq Analysis of NK cells. Single cell cDNA libraries were prepared from negatively enriched (3 rounds NK isolation kit, Miltenyi Biotec) NKC^{L}-D^{k}, NKC^{L}, or NKC^{GO1}-D^{k} spleen NK cells (>80% viability, 90-95% purity) and sequenced by the UVA Genome Analysis and Technology Core (GATC) using a Chromium Controller instrument (10X Genomics) and the Chromium Single Cell 3′ Reagent Kit V3 (10X Genomics) following the manufacturer's protocol. The indexed libraries were sized using the Agilent 4200 TapeStation and pooled into equimolar concentration. Samples were pooled and sequenced in a single run to avoid batch effects. High-throughput sequencing was performed using a NextSeq 500 Sequencer (Illumina) and the High

Output Kit V2.5 (150 cycles) using the following settings for Read 1 (26 cycles), Read 2 (98 cycles) and Read Index (8 cycles). Collected data (.bcl files) were exported for data processing and quality assessment prior to further analysis using Cell Ranger (10X Genomics) data analysis pipelines. Reads were aligned to the transcriptome using the "Count" function in Cell ranger so that expression of selected genes were assigned to single cells via attached barcodes.

Cell Ranger was used to perform t-SNE clustering of single cell data which was further analyzed using the Loupe Cell Browser (10X Genomics). Differentially expressed genes were identified and ranked by statistical significance. Statistically significant genes were further ranked by Log_2 fold change expression differences between clusters and visualized in heatmaps generated in Prism (Graphpad v7.05). Heatmap rank = upregulated genes per cluster (most to least) with a Log_2fold change of at least 1.

Data Availability. All scRNA-Seq data are accessible from the NCBI GEO depository using the following GEO accession number:GSE132394.

Chimeric CD3ζ-Ly49 reporter cells. *Cd3ζ-Ly49g2*^{MAMY}, *Cd3ζ-Ly49g2*^{C57L} and *Cd3ζ-Ly49r* chimeric receptor gene cassettes were generated using PCR essentially as described for *Ly49daz* (Furukawa et al., 2002), though we fused *Cd3z* cytoplasmic tail and *Ly49g*

transmembrane domain coding sequences, followed by coding sequences for *Ly49g* or *Ly49r* ectodomains. Sequence verified constructs were separately subcloned into pMXs-IRES-PURO (kindly provided by K. Iizuka and T. Kitamura). Expression constructs were transfected into 293T cells together with pMD2.G (kindly provided by Didier Trono (Addgene plasmid #12259; http://n2t.net/addgene:12259; RRID:Addgene_12259) and pHIT60 (kindly provided by Alan Kingsman, Oxford University, Oxford, England (Soneoka et al., 1995)) using lipofectamine. Retroviral supernatants were used to transduce J7 cells (a gift from K. Iizuka) and Ly49 receptor-expressing reporter cells were selected in puromycyin-containing media essentially as described (Ito et al., 2009).

CD3ζ-Ly49G2^{C57L} (aka J7.ZG^{L}) reporter cells were flow-sorted for high expression, comparable to CD3ζ-Ly49G2^{MAMY} (aka J7.ZG^{M}) and CD3ζ-Ly49R (aka J7.ZR^{L}). Ly49 reporter cells ($2x10^5$) were stimulated for 8-12hr with plate-bound mAbs, YB20, YB20-D^{k}, or M2-10B4 (± IFN-β or MCMV infection) target cells, or PMA + ionomycin. LacZ activity was determined using the substrate chlorophenol red-D-galactoside (CPRG) as described (Iizuka et al., 2003).

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