Paired altered-self MHC I recognition receptors enhance NK cell immunity to acute viral infection

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Abstract

Natural killer (NK) cells are essential mediators of host defense against murine cytomegalovirus (MCMV) infection. We have shown that licensed Ly49R$^+$ Ly49G2$^+$ NK cells mediate MHC I D^k-dependent control of MCMV in C57BL/6 (B6) mice bearing a C57L-derived NK gene complex (NKC). However, precise roles for the Ly49R activation receptor and the Ly49G2 inhibitory receptor in D^k-dependent MCMV control are not defined. Furthermore, a basis for D^k-dependent NK cell sensing of MCMV-infected targets and control of MCMV infection remains unclear.

To delineate a role for Ly49G2 in MHC I D^k resistance, we generated *Ly49g2*^{C57L} mutant mice via CRISPR/Cas9 gene-editing. A single cytosine insertion resulted in Ly49G2 truncation and undetectable NK cell surface expression. Ly49G2-deficient mice exhibited increased mortality due to abrogated MCMV control by NK cells, despite an otherwise unmodified Ly49 repertoire and normal NK cell development. Our findings thus demonstrate for the first time a vital role for an inhibitory receptor in NK cell-mediated antiviral immunity.

The MCMV immunoevasin m04/gp34 escorts MHC class I (MHC I) molecules to the surface of infected cells where these complexes bind Ly49 inhibitory receptors and prevent NK cell attack. Nonetheless, we have found that Ly49G2 promotes robust Ly49R$^+$ NK cell expansion and antiviral immunity during MCMV infection.

We thus examined a role for gp34 in Ly4R$^+$ Ly49G2$^+$ NK cell sensing of MCMV infection. We discovered that Ly49R was selectively triggered during MCMV infection on antiviral NK cells licensed by the Ly49G2 IR. We further found that Ly49R recognition of MCMV-infected targets was dependent on MHC I D^k and MCMV gp34 expression. Remarkably, although Ly49R was critical for Ly49G2-dependent antiviral immunity, blockade of the activation receptor in Ly49G2-deficient mice had no impact on virus control, suggesting that paired Ly49G2 MCMV-sensing might enable Ly49R$^+$ NK cells to better engage viral targets. Indeed, MCMV gp34 facilitated Ly49G2 binding to infected cells, and the IR was required to counter gp34-mediated immune evasion. A specific requirement for Ly49G2 in antiviral immunity was further explained by its capacity to license cytokine receptor signaling pathways and enhance Ly49R$^+$ NK cell proliferation during infection. These findings advance our understanding of the molecular basis for functionally disparate self-receptor enhancement of antiviral NK cell immunity.

Dedication

This dissertation is dedicated to my family. Their love, support, and encouragement strengthen my resolve and add color to my life on a daily basis. I am fortunate to have parents that instilled in me a passion for science during my early childhood; from trips to the Virginia Discovery Museum and summer workbooks with my Mom to adventures in the creeks of Sugar Hollow with my Dad, I am sincerely thankful for their commitment to fostering my intellectual growth and for pushing me to pursue an education in science. I am grateful to my sisters Natalie and Erika for never hesitating to join me for walks in the woods or fishing on the lake. Special thanks are due to my grandparents for their genuine interest in my immunology research and for showing me how to take time for rest and relaxation. Finally, this thesis is dedicated to my wife Karolina. She has been my rock on this intellectual journey with her unwavering faith, unconditional love, and drive to have fun.

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Abbreviations

β-gal	Beta-galactosidase
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
AS	Altered-self
ATCC	American type culture collection
B2m	Beta-2-microglobulin
B6	C57BL/6
BM	Bone marrow
bp	Base pair
CDS	Coding sequence
CFSE	CellTrace™ carboxyfluorescein succinimidyl ester
CPD	Cell proliferation dye
CRISPR	Clustered regularly interspaced short palindromic repeats
cRNP	CRISPR-Cas9 ribonucleoprotein
crRNA	CRISPR RNA
CTV	CellTrace™ violet
DC	Dendritic cell
DL	Detection limit
DMEM	Dulbecco's modified eagle medium
Dox	Doxycycline hyclate
EBV	Epstein-Barr virus

FBS	Fetal bovine serum
$G2^-$	Endogenous $Ly49G2^-$
$G2^+$	Endogenous $Ly49G2^+$
$G2^{null}$	CRISPR-targeted $Ly49G2^{null}$
gDNA	Genomic DNA
GM-CSF	Granulocyte macrophage colony stimulating factor
GO1	Ly49G2-deficient NKC^L mice
gp	Glycoprotein
GZMB	Granzyme B
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hpi	Hours post-infection
HRM	High-resolution melting
HSCT	Hematopoietic stem cell transplant
HSV	Herpes simplex virus
IFN	Interferon
IFNGR	IFN-γ receptor
iKIR	Inhibitory KIR
IL	Interleukin
ILC	Innate lymphoid cell

IMDM	Iscove's modified dulbecco's medium
i.p.	Intraperitoneal
IR	Inhibitory receptor
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
i.v.	Intravenous
J7	Jurkat CD3ζ reporter engineered to produce β-gal
KIR	Killer cell immunoglobulin-like receptor
LRC	Leukocyte receptor complex
mAb	Monoclonal antibody
MCMV	Murine cytomegalovirus
MFI	Mean fluorescence intensity
MHC I	Major histocompatibility complex class I
MOI	Multiplicity of infection
NK	Natural killer
NKC	Natural killer gene complex
NKC^{L}	Congenic B6 mice possessing C57L-derived NKC
NKR	Natural killer cell receptor
NO	Nitric oxide
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PFU	Plaque forming units
Poly(I:C)	Polyinosinic-polycytidylic acid
Prf1$^{-/-}$	Perforin 1-deficient
R^{-}	Ly49R^{-}
R^{+}	Ly49R^{+}
R^{null}	CRISPR-targeted Ly49R^{null}
ROV^{-}	Ly49R^{-}Ly49O^{-}Ly49V^{-}
ROV^{+}	Ly49R^{+}Ly49O^{+}Ly49V^{+}
RPMI	Roswell park memorial institute
SCID	Severe combined immunodeficiency
sgRNA	Single-guide RNA
SGV	Salivary gland virus
SNP	Single nucleotide polymorphism
SOT	Solid organ transplant
STED	Stimulated emission depletion
TC	Tissue culture
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
VZV	Varicella zoster virus
WT	Wild-type

Chapter I

Preface

NK cells are cytotoxic lymphocytes that are essential mediators of the innate immune response to viruses and tumors (1, 2). They belong to the recently characterized and growing family of innate lymphoid cells (ILCs), and differentiate and mature in the bone marrow (BM), lymph nodes, spleen, tonsils, and thymus (3, 4). NK cells are distinct from other ILC subsets in that they can be recruited to infected tissues where they rapidly limit viral spread by directly lysing infected or malignant target cells (5). Further highlighting the essential role of these cytolytic innate immune effectors, patients with NK cell deficiency (NKD) frequently succumb to fatal viral infections before adulthood and are particularly susceptible to herpesviruses (6).

Equipped with the capacity to kill, NK cells necessitate a highly specific mechanism to distinguish self from non-self. Indeed, their effector activities are regulated by signals derived from a diverse array of germline-encoded cell surface activating and inhibitory NK cell receptors (NKRs) (1). Whereas activating NKRs bind stress-induced or pathogen-derived molecules on target cells to drive cellular activation pathways, inhibitory NKRs bind to self or self-like molecules resulting in NK cell tolerance (7, 8). As NKR – ligand interactions are essential to NK cell sensing of non-self molecules, the identification of NKR/ligand pairs that are critical

for NK cell recognition of viral targets has been the subject of intense research over the last several decades.

To address this gap in knowledge, investigators have used genetic strategies to identify host and viral genes required for NK cell resistance to viral infection. In particular, murine cytomegalovirus (MCMV) has proven useful for studying the complex interface between viral immunoevasins and the host NK cell response. Among the first identified NKR/ligand pairs shown to be critical for MCMV control was that between the Ly49H activation receptor expressed on the surface of NK cells in B6 mice, and the MCMV-encoded MHC I mimic m157 expressed on the surface of virus-infected targets (9, 10). More recently, our laboratory has shown that blockade of the Ly49R^{C57L} (Ly49R allele from C57L mice) activation receptor abrogates MHC I D^{k}-dependent NK cell immunity to MCMV (11). However, the molecular basis for Ly49R-mediated MCMV recognition remains to be elucidated.

Although the biological significance of NK cell activating receptors in viral infection has long been appreciated, the role of inhibitory receptors (IRs) has been more difficult to unravel due to their dual roles in effector inhibition and NK cell licensing (discussed herein). Indeed, discrete IR/MHC I gene pairs are associated with improved disease outcomes in several human chronic viral infections, including human immunodeficiency virus (HIV), hepatitis C virus (HCV), and HCMV (12–19). Relatedly, our laboratory has shown that interactions between the Ly49G2 IR expressed on NK cells in C57L or MA/My mice, and host expression of

MHC I D^k, are linked with MHC I-dependent MCMV control (20–23). In order to elucidate the role of NK cell IRs in human disease, it is essential to understand whether IRs are specifically required for NK cell-mediated viral resistance *in vivo.*

NK cell effector functions during viral infection

NK cells are so named for their ability to kill viral and tumor targets without prior antigen exposure, in contrast to their cytotoxic T lymphocyte counterparts. They primarily achieve target cell destruction by selectively delivering cytotoxic granules across a point of contact with the malignant target cell known as the lytic synapse (24). The most prominent lytic proteins found within cytotoxic granules are perforin and granzyme B (GZMB) (25). Besides lytic granule-mediated cell death, NK cells can also utilize CD95L/FasL or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) to engage CD95/Fas or TRAIL-R1-R2 death receptors, respectively, to kill viral or tumor targets (26). However, NK cells preferentially and rapidly kill targets with lytic granules, and only after granule depletion do they switch to death receptor-mediated killing (27). Indeed, both perforin and GZMB are critical for NK cell-mediated control of MCMV infection (28, 29), and patients with genetic mutations that impede perforin or GZMB expression experience recurrent herpesvirus infections (30). Thus, lytic granule-driven killing is an essential feature of NK cell-mediated resistance to viral infection.

Virus-activated NK cells can also produce an array of inflammation-associated effector cytokines and chemokines which can regulate the activities of nearby NK cells, or other innate and adaptive immune cells. These cytokines include interferon (IFN)-γ, TNF-α, and granulocyte macrophage stimulating factor (GM-CSF), and chemokines CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CCL8 (IL-8) (1). In particular, NK cells and tissue-resident type 1 ILCs are the major IFN-γ-producing cells 24-36 hours post-infection (hpi) with MCMV, and IFN-γ is critical for restriction of acute viral spread and protection against lethality during MCMV infection (28, 31). Relatedly, patients with genetic mutations impeding IFN-γ receptor (IFNGR) signaling can exhibit susceptibility to several herpesvirus infections, including HCMV, varicella zoster virus (VZV), and herpes simplex virus (HSV) (32). The IFNGR is expressed in all nucleated cells and its signaling can induce intracellular antiviral programs, including induction of nitric oxide (NO) production and protein kinase R (PKR) expression, both of which are strong inhibitors of viral replication (33–35). Beyond direct antiviral effects, IFN-γ can improve the quality and efficiency of MHC I and MHC II antigen processing and presentation by facilitating antigen presenting cell (APC) maturation, enhancing expression of costimulatory molecules on APCs, and upregulating the genes involved in the MHC I/II antigen processing and presentation pathway (36–38). NK cell-derived TNF-α may also be important for antiviral immunity, as TNF-α limits viral replication, and neutralization of TNF-α results in 1) greater susceptibility to acute MCMV and 2) decreased NK cell IFN-

γ production during infection (39, 40). Furthermore, although a specific role for NK cell GM-CSF during MCMV infection has not been elucidated, GM-CSF-derived APCs can directly modulate NK cell antiviral activities (41, 42). Thus, NK cell-derived inflammatory mediators play important roles in modulation of innate and adaptive immune outcomes during viral infection.

In addition to their lytic or pro-inflammatory antiviral effects, NK cells can also act as regulators of early virus-specific T cell responses. In the context of acute MCMV infection, NK cell-derived IL-10 can restrict liver immunopathology, in part by regulating the activation status of $CD4^+$ and $CD8^+$ T cells (43). Furthermore, our laboratory has shown that MHC I D^k-dependent MCMV control by NK cells increases retention and recovery of splenic dendritic cells (DCs) and enhances early $CD8^+$ T cell immunity (44). Likewise, in B6 mice, $Ly49H^+$ NK cell-mediated elimination of MCMV-infected cells during acute infection is associated with a dampened type I IFN response, greater DC numbers, and augmented early priming of $CD8^+$ T cells (45). By contrast, others have shown that rapid lysis of MCMV-infected DCs by $Ly49H^+$ NK cells limits the longevity and magnitude of the antiviral T cell response to MCMV (46). Therefore, NK cell effector activities can have both direct and indirect immunoregulatory effects on early antiviral T cell responses.

Regulation of NK cell activation

NK cell activation is in large part regulated by a broad repertoire of germline-encoded cell surface activating and inhibitory NKRs (1). Activating NKRs lack intrinsic signaling motifs and therefore must associate with transmembrane immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor molecules (e.g. DAP10, DAP12, CD3ζ, or FcεR1γ) to activate downstream signaling cascades (7). By contrast, IRs possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails which can recruit, activate, and associate with SH2 domain-containing phosphatases (e.g., SHP-1 and SHP-2) to antagonize activating NKR signaling pathways (8). Ultimately, the combined signaling output resulting from NKR – ligand interactions can instruct the formation of an inhibitory or lytic immune synapse (47), the latter of which facilitates NK cell-mediated cytotoxic activities and cytokine release.

Most NKRs are expressed in a variegated manner and the majority are encoded by genes clustered in the natural killer gene complex (NKC) or leukocyte receptor complex (LRC) (48). Moreover, whereas mouse NK cells express polymorphic NKC-encoded Ly49 C-type lectin-like receptors, human NK cells express functionally orthologous LRC-encoded killer Ig-like receptors (KIR) (49). Importantly, murine Ly49 and human KIR *inhibitory* NKRs specifically bind to MHC I or MHC I-like molecules. By contrast, Ly49 and KIR *activating* NKRs bind self-MHC I and MHC I-like molecules, as well as molecules associated with cellular

stress or viral infection (7). Although structurally distinct, convergent evolution of polymorphic Ly49 and KIR gene families has likely been similarly influenced by 1) the polymorphic nature of their cognate MHC I ligands, 2) pathogen manipulation of MHC I molecules to evade the immune system, and 3) the influence of KIR/Ly49 – MHC I gene pairs on reproductive fitness (50). While an individual NK cell typically expresses one to several Ly49 or KIR, there is considerable skewing of the NKR repertoire on the basis of MHC I engagement during NK cell development (51). NK cell heterogeneity is further influenced by allelic diversity and the number of genes within a given Ly49/KIR haplotype (52). Thus, NK cells are poised to detect alterations in self-MHC I molecules with receptors that trigger opposing signaling pathways, and are tasked with maintaining tolerance to "self", while simultaneously eradicating "non-self."

Functional studies of murine Ly49 receptors have yielded numerous important insights into how KIR regulate human NK cell biology, and vice versa. Studies on how inhibitory KIR and Ly49 receptors regulate NK cell immune synapse formation are a salient example of this. When Ly49 or KIR IRs engage self-MHC I, the IRs form tight clusters, resulting in ITIM phosphorylation, which drives SHP-1 phosphatase recruitment and activation (8). ITIM-activated SHP-1 dominantly impedes actin-dependent activating receptor clustering and stimulation by directly dephosphorylating downstream signaling adaptors Vav, SLP-76, LAT, and PLC-γ **(Figure 1)** (53–55). On the other hand, there is also evidence that inhibitory KIR (iKIR) can *promote* phosphorylation events. For instance, SHP-2

can facilitate MAPK signaling (56) and in the absence of MHC I ligands, SHP-2 is bound by iKIR in $CD4^+$ T cells, resulting in greater T cell receptor (TCR)-dependent IL-2 responses **(Figure 1)** (57). iKIR ligation can also drive Crk phosphorylation, resulting in its binding to tyrosine kinase c-Abl, which contributes to NK cell inhibition by preventing F-actin network formation and limiting activating receptor translocation **(Figure 1)** (58, 59). Thus, MHC I-binding NKRs are essential regulators of NK cell homeostasis and impart striking functional diversity to NK cells at the population level.

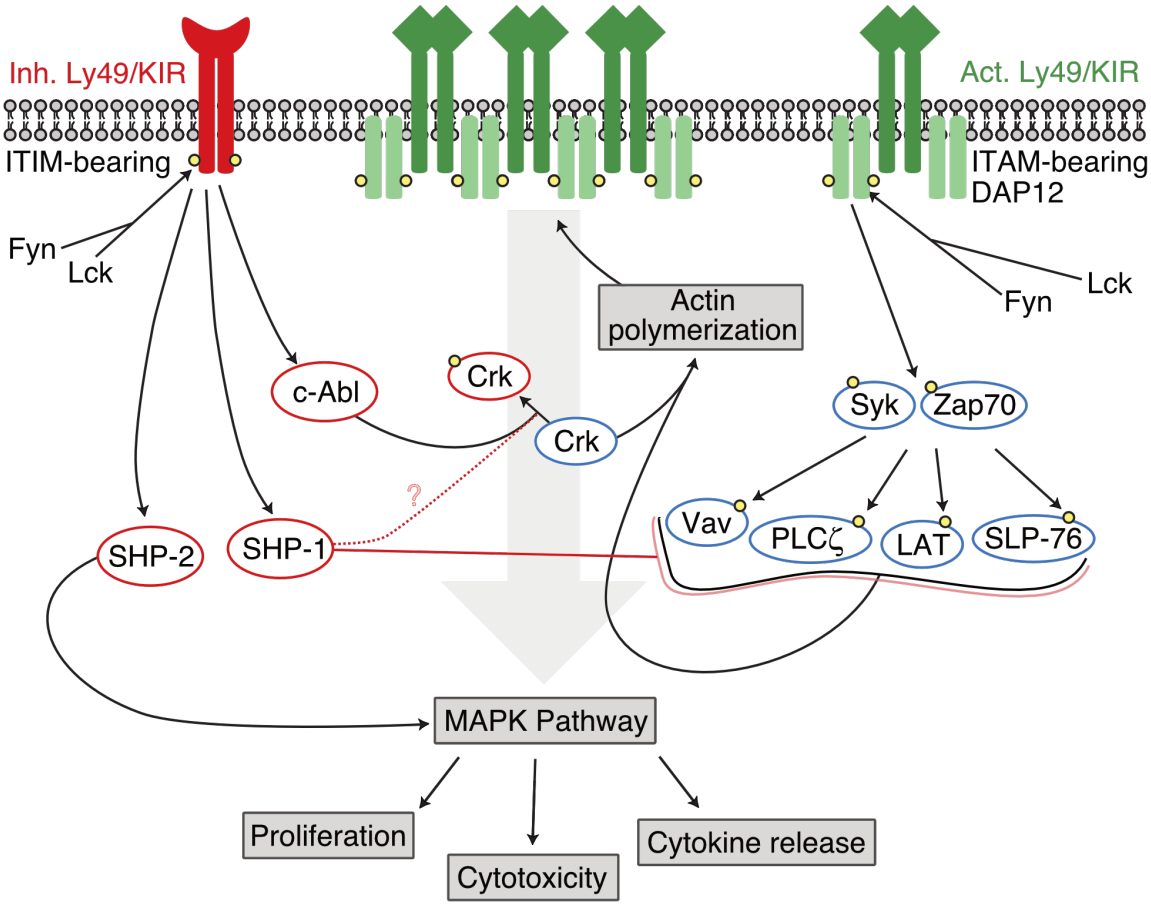


Figure 1. Ly49/KIR signaling pathways.

Figure 1. Ly49/KIR signaling pathways. Upon ligand binding, Ly49/KIR activating receptors (green) recruit DAP12 via a positively charged residue in their transmembrane domain (60). DAP12 phosphorylation by Src-family kinases Lck and Fyn promotes downstream signaling through Syk and Zap70, which activate Vav, PLCγ, LAT, and SLP-76 (61). Activation of this signaling cascade promotes Crk-dependent actin polymerization and activating receptor clustering (59). Downstream activation of the MAPK/ERK pathway has been linked to several NK cell effector activities, including proliferation, cytotoxicity, and cytokine release. Self-MHC I-binding inhibitory Ly49/KIR (red) possess ITIM motifs in their cytoplasmic tails; upon ligand engagement, ITIM phosphorylation by Lck and Fyn promotes recruitment and activation of SH2-domain containing phosphatases SHP-1 and SHP-2 (8). IR clustering is actin-independent, in contrast to Ly49/KIR activating receptors. Downstream substrates for SHP-1 include Vav, PLCγ, LAT, and SLP-76. Given that SHP-1-mediated p-Crk dephosphorylation is critical for immune synapse formation in T cells (62), SHP-1 may act similarly in NK cells. Despite its phosphatase activity, SHP-2 has been shown to positively regulate MAPK signaling and NK cell proliferation (56). IR-mediated Crk phosphorylation prevents actin-dependent activating receptor clustering and appears to be dependent on the activity of tyrosine kinase c-Abl (58, 59), but the basis for IR-dependent c-Abl activation remains unclear.

Aside from stimulation via their activating receptors, NK cells can also be stimulated through cytokine receptors. Indeed, MCMV infection can induce production of IL-12, IL-18, and IFN-α, and IFN-β (IFN-I) by other immune and non-immune cells (63, 64), primarily via toll-like receptor (TLR) stimulation (65). Although these cytokines themselves can directly limit viral replication, they have also been shown to engage NK cell cytokine receptors to stimulate production of IFN-γ (41, 66), which is important for regulating the acute antiviral immune response. Moreover, IL-12 and IFN-I can induce DC production of IL-15, which, when *trans*-presented to NK cells, results in a "priming" event that enhances NK cell-mediated lysis of malignant targets and is further associated with increased NK cell GZMB and perforin protein expression (67). Notably, cytokine priming is likely required for effective target cell lysis by NK cells. Whereas resting NK cells possess pre-formed GZMB and perforin mRNAs, they lowly express GZMB/perforin proteins. IL-15, IL-12, or IFN-I priming can induce GZMB and perforin protein expression in NK cells, which is further correlated with enhanced NK cell-mediated target killing *in vitro* and MCMV control *in vivo* (29, 68). In addition to their direct effects on NK cell IFN-γ production and cytotoxicity, IL-12 and IFN-I have also been shown to induce expression of the high affinity IL-2 receptor, CD25, on NK cells via a STAT4-dependent mechanism (69). As NK cell-intrinsic CD25 expression is required for optimal NK cell proliferation and accumulation during MCMV infection (70), differential sensing of IL-12 and IFN-I by select subsets of NK cells may endow them with a significant growth advantage.

Tolerance mechanisms may likewise be important for regulating NK cell homeostasis; IL-10 neutralization exacerbates activation-induced cell death in NK cells during acute MCMV infection (71), suggesting that IL-10R signaling antagonizes pathways which drive NK cells into a hyperactivated or hyperinflammatory state. Cytokine receptor signaling is thus required for virus-specific NK cell activation, cytotoxicity, and survival.

NK cell licensing and self-tolerance

Self-MHC I-binding Ly49 and KIR IRs were initially defined on the basis that they can inhibit cellular activation. However, they can also enhance basal NK cell responsiveness via a dynamic MHC I-dependent tuning process which is referred to as NK cell 'licensing' or 'education' (72, 73). An individual NK cell is considered licensed if it possesses an IR receptor capable of binding self-MHC I ligand(s) expressed by the host **(Figure 2A)** (72–74). Compared to their unlicensed counterparts, licensed NK cells degranulate to a greater extent, produce more IFN-γ, and can better sense and lyse viral or tumor targets which downregulate MHC I (i.e., missing-self rejection) **(Figure 2B-C)** (1, 8). Enhanced responsiveness of licensed NK cells is explained by their 1) increased basal mTOR/Akt pathway activation (75), 2) heightened glycolytic rate (76), 3) greater mobilization and accumulation of GZMB-filled lytic granules (77), 4) increased adhesion to target cells due to elevated expression of costimulatory/adhesion receptors LFA-1 and

DNAM-1 (78–80), and 5) faster actin-dependent movement of activating receptors due to their specialized compartmentalization in the plasma membrane (81, 82). Taken together, these findings indicate that self-specific IRs can modulate the responsiveness of a variety of dynamic cellular processes, ultimately resulting in a lower threshold for activation in licensed NK cells.

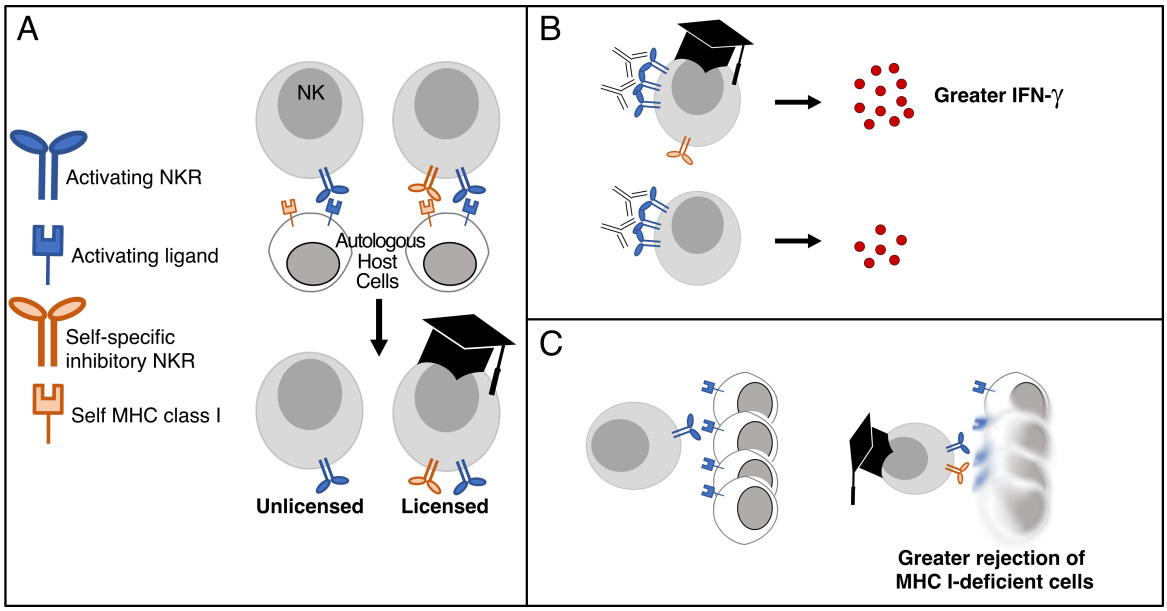


Figure 2. Licensing NK cell reactivity through IR/self-MHC I interactions.

Figure 2. Licensing NK cell reactivity through IR/self-MHC I interactions. (A) NK cells which possess a self-specific NK cell IR (e.g., Ly49 and KIR) can engage self-MHC I molecules expressed by autologous host cells, resulting in IR/MHC I-dependent enhancement of NK cell reactivity also known as licensing. (B) Licensed NK cells produce greater IFN-γ in response to activating receptor crosslinking relative to unlicensed NK cells. (C) In contrast to unlicensed NK cells, licensed NK cells can detect loss of MHC I expression on malignant target cells using their self-MHC I-specific IRs. Licensed NK cells thus exhibit greater rejection of MHC-deficient cells.

Although several diverse biological impacts of NK cell licensing have been documented, how IR signaling affects these changes has long been debated (83). The “licensing” model proposes that NK cells are unlicensed or hyporesponsive at steady-state **(Figure 2)**; self-MHC I-specific IR-engagement of cognate ligand sends a positive signal, resulting in the acquisition of heightened reactivity (i.e., licensing) and the ability to reject MHC I-deficient targets. By contrast, IR^+ NK cells which do not interact with their cognate MHC I ligands remain unlicensed, and non-responsive **(Figure 2)**. On the other hand, the “arming/disarming” model proposes that NK cells with self-specific IRs become armed when these signals are counterbalanced by low-level signaling through activating NKRs. Accordingly, NK cells lacking self-specific IRs undergo disarming via persistent low-level signaling through activating NKRs as unimpeded stimulation of these pathways is predicted to drive a negative signal, resulting in functional anergy of NK cells and failure to eradicate cells on the basis of loss of MHC I expression.

To date, it remains unclear whether NK cell IRs engender functional reactivity via licensing or by preventing NK cell disarming. Nonetheless, recent studies have shed light on how NK cells acquire and maintain their responsiveness. NK cell reactivity is quantitatively tuned to the amount of IR signaling received by a particular IR/self-MHC I pair (84, 85). IR/MHC I pairs which propagate stronger inhibitory signals give rise to NK cells with heightened reactivity to activation receptor stimulation. In this context, IR/MHC I-dependent tuning might facilitate a greater positive “licensing” signal, or alternatively, might better

prevent “disarming” via activating NKRs. Separate studies have demonstrated that NK cell functional reactivity is reversible; when transferred into a host environment with altered MHC I expression, mature NK cells adjust their responsiveness in line with their IR signaling input **(Figure 3)** (86, 87). In these settings **(Figure 3)**, it is likewise impossible to delineate whether an IR-dependent positive signal is responsible for 1) maintaining NK cell licensing in these settings, or 2) preventing activating NKR-induced disarming. Notwithstanding, IR-mediated self-MHC I sensing continuously and quantitatively tunes NK cell activating receptor signaling pathways, endowing educated NK cells with greater functionality while also ensuring tolerance to self.

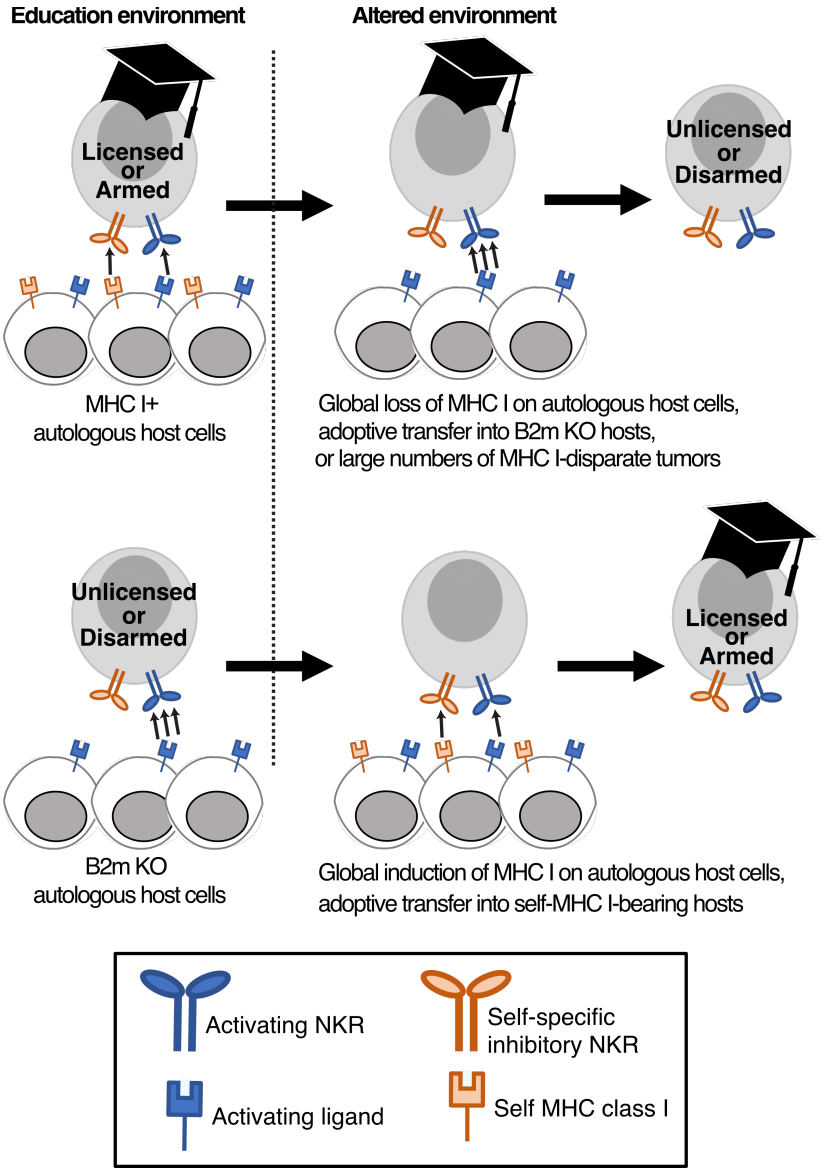


Figure 3. MHC I-dependent rewiring of mature NK cell responsiveness.

Figure 3. MHC I-dependent rewiring of mature NK cell responsiveness. (Top) Adoptive transfer of mature licensed or armed NK cells into an MHC I-deficient environment results in unlicensed or disarmed NK cells which are tolerized to missing-self targets. Likewise, large numbers of MHC I-deficient tumor cells and global inducible deletion of MHC I from autologous host cells can reset licensed NK cell responsiveness. (Bottom) Adoptive transfer of mature unlicensed or disarmed NK cells into a self-MHC I ligand-bearing environment enhances NK cell activating receptor signaling pathways and resets NK cell reactivity to missing-self targets.

More recently, mutation of a critical tyrosine residue in the IR ITIM domain revealed that ITIM phosphorylation is required to recruit and activate SH2 domain-containing phosphatases (88), and to maintain NK cell reactivity (72, 89). Still, it is unknown 1) if the same self-specific IR signals which license NK cell reactivity are required for rejection of missing-self targets and 2) how induction of tolerance is achieved. Understanding how NK cells modulate SHP-1 activity is likely key to uncovering how licensed NK cells achieve and maintain their reactive state. NK cells which develop without SHP-1 are hyporesponsive to activating receptor stimulation and fail to eradicate tumors lacking MHC I expression (75, 90). On the other hand, ablation of SHP-1 from mature NK cells (i.e., NK cells that developed with SHP-1 activity) renders them more sensitive to activating receptor ligation and promotes NK cell-mediated tumor lysis (91, 92). Recent studies indicate that SHP-1 protein abundance is lower in licensed NK cells, which directly correlates with their enhanced reactivity (92). Taken together with previous observations showing that NK cells quantitatively adjust their responsiveness in line with MHC I engagement, these findings suggest multiple roles for SHP-1 in licensed NK cells: 1) SHP-1 is required during development for NK cells to become licensed, 2) SHP-1 abundance in licensed NK cells determines the likelihood of responding to activating receptor stimulation, and 3) SHP-1 abundance or activity is tuned by IR-dependent MHC I engagement. Whereas the basis for lower SHP-1 abundance in licensed NK cells remains unclear, these studies clearly indicate that ITIM signaling and SHP-1 are major regulators of NK cell responsiveness.

MHC I recognition by NK cells: *in trans* and *cis*

Single nucleotide polymorphisms (SNPs) in NKRs have given rise to unique allotypes **(Table 1)** with disparate MHC I binding strengths, specificities, and cell surface expression levels (93, 94). Allelic variation in the ligand-binding domain of self-MHC I-binding IRs can alter MHC I binding preferences (94–96), impacting on NK cell-mediated cytotoxic functions. For example, SNPs in the natural killer receptor domain (NKD) of Ly49 receptors alter their MHC I binding specificity (96). The NKDs of IRs Ly49G2^{Balb} and Ly49G2^{B6} differ by only a single amino acid (Asp/Gly^{246}); whereas Ly49G2^{Balb} binds MHC I D^{k} and D^{d}, Ly49G2^{B6} binds only D^{d} (95, 96). As a result, a rat NK cell line engineered to express Ly49G2^{Balb} receptors is tolerized by MHC I D^{k}-expressing target cells **(Figure 4A)**. By contrast, those bearing Ly49G2^{B6} receptors kill D^{k}-expressing targets **(Figure 4B)** (95). It follows that the highly polymorphic nature of MHC I molecules themselves likewise impacts NKR/MHC I interactions (97). Genetic diversity in NKR/MHC I pairs thus directly influence NK cell recognition and effector functions.

Function	Human	Mouse strain		
		B6	BALB	C57L, MA/My, 129
Activating	KR2DL4	Ly49H	Ly49L	Ly49R
	KIR2DS1	Ly49D		Ly49P
	KIR2DS2			Ly49U
	KIR2DS3			
	KIR2DS4			
	KIR2DS5			
	KIR3DS1			
Inhibitory	KIR2DL1	Ly49A	Ly49A	Ly49B
	KIR2DL2	Ly49B	Ly49B	Ly49E
	KIR2DL3	Ly49C	Ly49C	Ly49EC
	KIR2DL5	Ly49E	Ly49E	Ly49G2
	KIR3DL1	Ly49F	Ly49G2	Ly49I
	KIR3DL2	Ly49G2	Ly49I	Ly49O
		Ly49I	Ly49Q	Ly49Q
		Ly49J		Ly49S
		Ly49Q		Ly49T
				Ly49V

Table 1. Activating and inhibitory MHC I-binding NKRs in human and mouse (adapted from (98)).

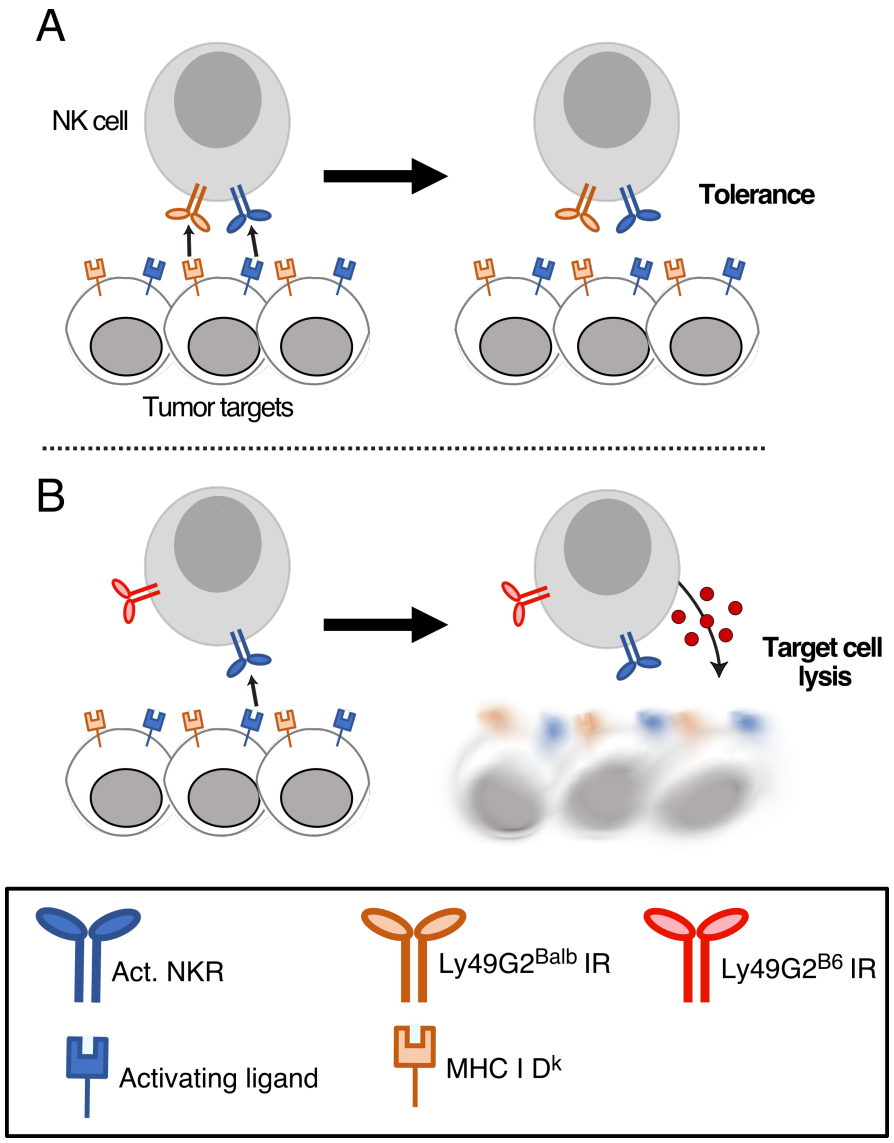


Figure 4. NKR allelic variation influences IR-dependent MHC I recognition and NK cell-mediated effector functions.

Figure 4. NKR allelic variation influences IR-dependent MHC I recognition and NK cell-mediated effector functions. (A) A rat NK cell line engineered to express Ly49G2^{Balb} IRs is tolerized to MHC I D^{k}-bearing targets due to inhibitory signals propagated from Ly49G2^{Balb}/D^{k} interactions. (B) Ly49G2^{B6}-bearing rat NK cells lyse D^{k}-bearing targets because Ly49G2^{B6} does not bind D^{k}.

In addition to sensing MHC I molecules on neighboring cells (*in trans*), self-MHC I-binding Ly49 IRs can also interact with MHC I molecules expressed on the same NK cell (*in cis*) (99). Both *trans*- and *cis*-MHC I molecules compete for the same MHC I-binding site on the IR, due to the flexible nature of the stalk which allows for backfolding, and consequently, an equilibrium (i.e., *cis* $\rightleftharpoons$ *trans*) describing IR— self-MHC I interactions exists **(Figure 5)** (100). Importantly, *cis*-MHC I/Ly49 IR interactions have been shown to restrict IR recruitment to the immune synapse *in trans* (101), resulting in less inhibitory signaling (99) and more efficient NK cell-mediated lysis of MHC I-deficient tumor targets (102). These findings indicate that the balance between *trans*- and *cis*-MHC I sensing modulates IR signaling input during NK cell recognition of target cells.

Whether NK cell-intrinsic MHC I expression is required for licensing is still unclear. Whereas studies of the Ly49C^{B6} IR suggest that *cis*-MHC I interactions are dispensable for NK cell licensing (103), mutation of the stalk of the Ly49A^{Balb} IR, which allows for *trans*- but not *cis*-MHC I binding, indicates that *trans*-MHC I interactions are sufficient to inhibit but insufficient to license NK cells (102). A mechanistic basis for these discordant findings has not been elucidated but may be explained by the differences in the experimental approaches used to evaluate the contribution of *cis*-MHC I, or alternatively, explained by differences in how Ly49C vs Ly49A engage MHC I molecules. In further support of a role for *cis*-MHC I in NK cell licensing, Ly49A^{+} NK cells require cell-intrinsic MHC I expression to maintain their responsiveness to MHC I-mismatched tumor cells (104). Although

human iKIR have not yet been shown to similarly engage *cis*-MHC I molecules, recent work suggests that human NK cell-intrinsic MHC I expression licenses NK cells and maintains their reactivity (105). Taken together, and given the importance of MHC I engagement for NK cell licensing, it is likely that *cis* ⇌ *trans* interactions are key for tuning the responsiveness of NK cells to environmental changes in MHC I expression.

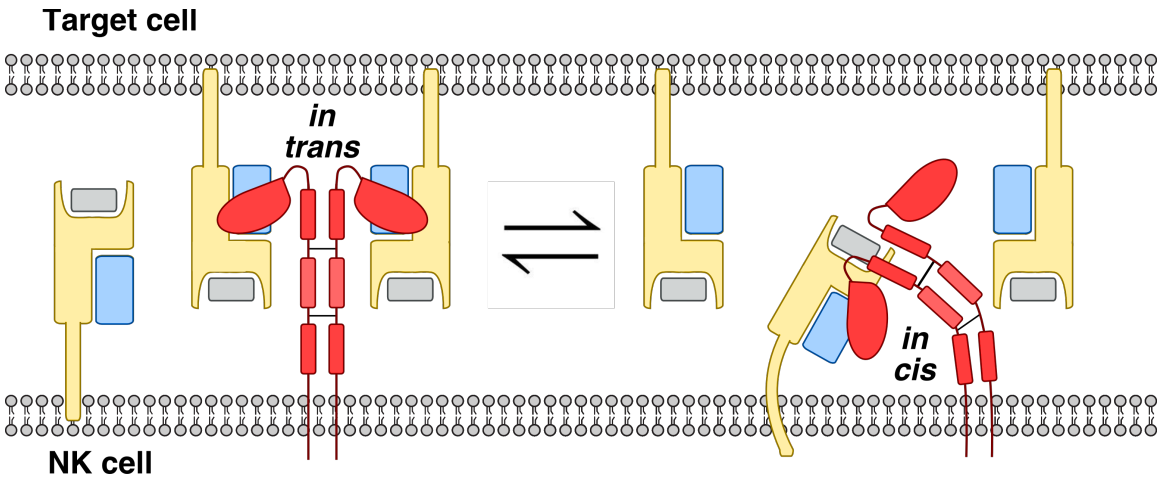


Figure 5. *Trans* vs *cis*-recognition of self-MHC I by NK cell IRs.

Figure 5. *Trans* vs *cis*-recognition of self-MHC I by NK cell IRs. Self-MHC I chains (yellow) associating with beta-2-microglobulin (B2m) (blue) and peptide (grey) can be presented *in trans* by target cells, or *in cis* on the NK cell itself, and bound by self-MHC I-binding Ly49 IRs (red). Whereas Ly49 IRs can bind two self-MHC I molecules *in trans*, they bind a single self-MHC I molecule *in cis* (106). An equilibrium between *trans* and *cis* bound Ly49 IRs thus exists.

Self-IR and MHC I associations in human viral disease

iKIR/MHC I-dependent licensing of NK cell reactivity is likely to have significant impacts on human viral disease outcomes. Considering the effects of iKIR/MHC I polymorphism on NK cell licensing, genetic diversification of these loci might further affect human NK cell-mediated antiviral immunity. Indeed, human genetic association studies have linked particular iKIR/human leukocyte antigen (HLA) gene pairs with disease outcomes in patients infected with HIV, HCV, and HCMV (107, 108).

A role for licensed NK cells in HIV control is supported by studies showing that particular subsets of iKIR$^+$ NK cells can detect loss of MHC I expression on HIV-infected cells. To evade TCR recognition, HIV Nef facilitates downregulation of HLA-A and HLA-B (109), and HIV Vpu mediates downregulation of HLA-C (110). Thus, licensed iKIR$^+$ NK cells can identify and kill HIV-infected CD4 T cells lacking self-HLA class I expression (i.e., missing-self recognition) (111). The extent to which a given licensed NK cell subset facilitates killing of HIV-infected cells is in part influenced by genetic variation among HIV strains, which can impact Nef- or Vpu-mediated downregulation of HLA molecules (110, 112–114). Likewise, allelic variation in iKIRs and their MHC I ligands also affects HIV-specific NK cell cytotoxic activities. For instance, licensed NK cells expressing *KIR3DL1* and *HLA-B* alleles which impart high cell surface expression levels exhibit greater killing of HIV-infected CD4$^+$ T cells in comparison to licensed NK cells expressing *KIR3DL1* and

HLA-B alleles which impart low cell surface expression levels (115). Higher iKIR/HLA expression levels at steady-state may give rise to licensed NK cells with heightened reactivity to HIV-infected missing-self targets (111), akin to how Ly49/MHC I interactions shape NK cell responsiveness in mice (85). iKIR allelic variation can likewise impact receptor clustering or HLA binding preferences. Indeed, KIR3DL1 polymorphism can alter the receptor's binding affinity and specificity for HLA-B (94). Related to this, select allotypes of *KIR3DL1* and *HLA-B* together strongly correlate with delayed progression to acquired immunodeficiency syndrome (AIDS) (12). Moreover, a single variant encoding valine at position 47 of *KIR3DL1* is significantly associated with elite HIV control in patients who also encode for *HLA-B*57* allotypes (13). 'Elite controllers' of HIV exhibit <50 copies HIVRNA/mL, maintain their $CD4^+$ T cell counts, and do not progress to AIDS, without antiretroviral therapy (116). Control of HIV infection by licensed NK cells may help to prevent HIV-induced loss of $CD4^+$ T cells (116) and further viral spread, allowing for durable, long-term antiviral immunity to HIV.

There is likewise strong, albeit more limited evidence suggesting that *iKIR/HLA* genes pairs influence control of HCV infection by licensed NK cells. The combination of homozygosity for select allotypes of *KIR2DL3* and its cognate ligand *HLA-C1*, or *KIR3DL1* and its ligand *HLA-Bw4*, are linked to enhanced HCV clearance (14–16). Although a basis for these genetic associations remains unclear, an intriguing possibility is that KIR2DL3/HLA-C1 and KIR3DL1/HLA-Bw4 gene pairs enable licensed NK cells to detect altered MHC I expression on HCV-

infected targets. This hypothesis is supported by a recent study showing that the HCV core protein interferes with maturation of MHC I molecules and facilitates their degradation (117). Moreover, both KIR2DL3 and KIR3DL1 recognize their MHC I ligands in a peptide selective manner (118, 119), which directly influences the strength of the inhibitory signal propagated by a given iKIR. Relatedly, certain HCV core-derived peptides presented by HLA-C1 weakly bind to KIR2DL3 and fail to inhibit NK cell degranulation (120). Thus, select subsets of licensed NK cells may readily recognize HCV-mediated immune-evasion, influencing HCV infection outcomes and disease progression.

All classes of herpesviruses, including HCMV, can facilitate downregulation of MHC I from the surface of infected cells (121) to escape TCR recognition. However, this can elicit licensed NK cell reactivity on the basis of iKIR-mediated recognition of altered MHC I expression levels. The robustness of iKIR-driven HCMV immunity is hinted at by a study showing that $iKIR^+$ NK cells from a severe combined immunodeficiency (SCID) patient expanded and controlled HCMV infection in the absence of T cells (122). Additionally, HCMV seropositive individuals, as well as hematopoietic stem cell transplant (HSCT) recipients experiencing HCMV reactivation, exhibit selective clonal-like expansion of licensed NK cells (17, 18), which display enhanced functionality relative to their unlicensed counterparts. In further support of a role for licensed NK cells in control of HCMV, *iKIR*/*HLA* gene pairs are strongly associated with increased time to HCMV viremia in in solid organ transplant (SOT) recipients (19). Thus, licensed NK cells may

help to identify and eliminate HCMV-infected cells, which may be especially important in immunocompromised individuals.

Collectively, these studies strongly implicate licensed NK cells and their self-specific IRs in detection of HIV, HCV, or HCMV infection **(Table 2)**. However, whether a given licensed NK cell subset is beneficial or detrimental to disease control likely depends on genetic variation in iKIRs, their MHC I ligands, and MHC I-targeting viral immunoevasins. Further mechanistic investigation is thus required to understand 1) the specific disease contexts in which licensed NK cells can be harnessed, 2) which *iKIR/HLA* gene pairs are most beneficial for antiviral immunity, 3) the functional importance of IRs for virus recognition by NK cells, and in overall disease outcome, and 4) the molecular basis for IR-mediated detection of virus-infected targets.

Virus	Inh. KIR allotype	HLA allotype	Associated outcome	Refs.
HIV	*3DL1*h*	*Bw4-80I*	Protection	12
	3DL1(I47V)	*B*57:01*	Elite control	13
HCV	*2DL3/2DL3*	*C1/C1*	Protection	14, 15
	3DL1	*Bw4-80T*	Protection	16
	3DL1	*Bw4/Bw4*	Protection	16
HCMV	*2DL2, 2DL3*	--	Protection	122
	2DL3	*C1/C1*	Subset expansion	17
	2DL1	*C2/C2*	Subset expansion	17
	2DL3/2DL3	*C1*	Subset expansion	18
	2DL3	*C1*	Protection	19

Table 2. iKIR/HLA allotypes associated with protection from viral infection. *KIR/KIR* or *HLA/HLA* denotes two copies of a given allotype. Each implicated *HLA* allotype is a cognate ligand for the implicated *iKIR*.

Linking natural killing, licensing, and antiviral immunity

Foundational studies of host resistance to MCMV infection in mice have provided a basis for understanding the significance of licensed NK cells in human viral disease. Indeed, several decades ago, the MHC I H-2^k haplotype was linked to protection from lethal MCMV infection in genetically disparate inbred mouse strains, including MA/My, CBA, C3H/HeJ, and BALB.K (123–125). Subsequent studies by our laboratory and others revealed that NK cells are required for H-2^k-dependent MCMV resistance in MA/My mice (23, 125), despite lacking the Ly49H activation receptor known to be critical for MHC I-independent MCMV control in B6 mice (9, 23). Further genetic mapping of the H-2^k haplotype uncovered MHC I D^k as a dominant and critical MCMV resistance factor (20, 21), and notably, we found that antiviral immunity is abrogated by depletion of NK cells expressing the Ly49G2 IR in D^k-bearing MA/My or C57L-derived mouse strains (20, 21, 126, 127). Moreover, Ly49G2$^{MA/My}$ or Ly49G2^{C57L} allotypes bind MHC I D^k and license NK cells, which is associated with D^k-dependent expansion of Ly49G2$^+$ NK cells during MCMV infection (11, 20, 128). Ly49G2 was predicted to benefit the NK cell response to MCMV by licensing NK cell functionality and by recognizing altered levels of D^k molecules on the surface of MCMV-infected targets (20–22), as MCMV is known to facilitate D^k downregulation via glycoprotein (gp) immunoevasins gp40 and gp48 (129). Thus, akin to iKIR/HLA associations in human, these studies

strongly suggest that Ly49G2/D^k interactions are important for licensed NK cell-mediated detection and control of viral infection.

The significance of licensed NK cells in antiviral immunity is still debated, however. Studies in B6 mice (H-2^b) have suggested that MHC I K^b-binding IRs Ly49C or Ly49I can restrict proliferation, expansion, and virus control mediated by NK cells coexpressing the Ly49H activation receptor (130). Yet, why Ly49C/I impede Ly49H-mediated MCMV resistance is unclear, as Ly49C/I interactions with K^b license NK cells and enhance their reactivity to missing-self tumor targets (75, 131), and MCMV gp40 and gp48 likewise facilitate downregulation of K^b from the surface of infected cells (22, 132). Notably, the aforementioned studies used the 5E6 mAb, which binds both Ly49C and Ly49I, thus preventing evaluation of the contribution of each individual IR to MCMV control. A more recent study addressed this issue using IR-specific mAbs and found that Ly49C does not in fact limit proliferation of $Ly49H^+$ NK cells, but rather, Ly49I is responsible for the restriction (133). Curiously, despite their proliferative disadvantage, $Ly49H^+Ly49I^+$ NK cells appear more activated following MCMV infection relative to $Ly49H^+Ly49I^-$ NK cells (133). The disparate impacts of Ly49C vs Ly49I on Ly49H-dependent MCMV resistance might be explained by their different capacities to bind K^b molecules *in cis* (134, 135), their MHC I-binding specificities (134, 136), or their relative affinities for K^b (96). Regardless of the mechanism, these findings suggest that certain IRs may be better equipped than others to recognize viral manipulation of MHC I expression.

Since CMVs establish lifelong, opportunistic infections in their hosts, licensed NK cell immunity may be relevant to CMV-infected HSCT recipients, who often experience latent viral reactivation (137). Remarkably, although unlicensed Ly49H$^+$ NK cells are thought to be critical for acute MCMV resistance (130), in syngeneic and allogeneic HSCT recipient B6 mice, licensed Ly49H$^+$ NK cells expand, exhibit heightened reactivity, and impart essential MCMV protection (138, 139). These findings suggest that HSCT can unleash antiviral licensed NK cells from tolerance mechanisms they experience in non-HSCT settings. Given the significance of IR/MHC I-interactions in NK cell tolerance, it will be important to understand how MHC I molecules from different host/donor cell types alter the reactivity of licensed NK cells in HSCT. Relatedly, using radiation BM chimeras, we found that licensed Ly49G2$^+$ NK cells require MHC I D^k expression on both hematopoietic and non-hematopoietic cells to maintain their reactivity to missing-self targets and to control MCMV infection (21, 128). Similarly, separate work in B6 HSCT recipient mice has shown that non-hematopoietic cell-derived MHC I is required for NK cells to reject missing-self targets during MCMV infection (140). Licensed NK cells are thus critical mediators of antiviral immunity in HSCT settings, and their responsiveness is modified by MHC I molecules derived from both hematopoietic and non-hematopoietic cell types.

Loss of inhibition during viral infection and viral evasion mechanisms

Whereas the importance of self-MHC I-binding IRs in enhancement of NK cell reactivity is well-established, how they mechanistically function in the context of viral infection is unclear. Because many viruses facilitate downregulation of MHC I on infected cells to evade T cell immunity (141), a prevailing hypothesis in the field is that self-IRs enable licensed NK cells to better detect viral missing-self targets, which coexpress ligands for NK cell activating receptors.

Inasmuch as this hypothesis assumes a loss of inhibitory signaling occurs during IR-mediated virus recognition, *cis*-MHC I molecules (see 'MHC I recognition by NK cells: *in trans* and *cis*') may regulate the number of free self-IRs available to engage MHC I molecules on infected cells *in trans* (142). Therefore, as cell surface MHC I levels are dynamically reduced on viral targets, IRs which can interact with *cis*-MHC I might stop propagating inhibitory signals more rapidly **(Figure 6).** A shift in *cis* vs *trans* interacting IRs may be regulated by the extent to which a given IR engages *cis*- vs *trans*-MHC I. This may be further influenced by 1) the number of IRs on the surface of the NK cell, 2) the level of NK cell MHC I expression, 3) the intrinsic affinity of a given IR for *cis*- vs *trans*-MHC I molecules, and 4) IR polymorphism. Thus, *cis*-MHC I sensing may help to release licensed NK cells from inhibition during recognition of virus-modified MHC I.

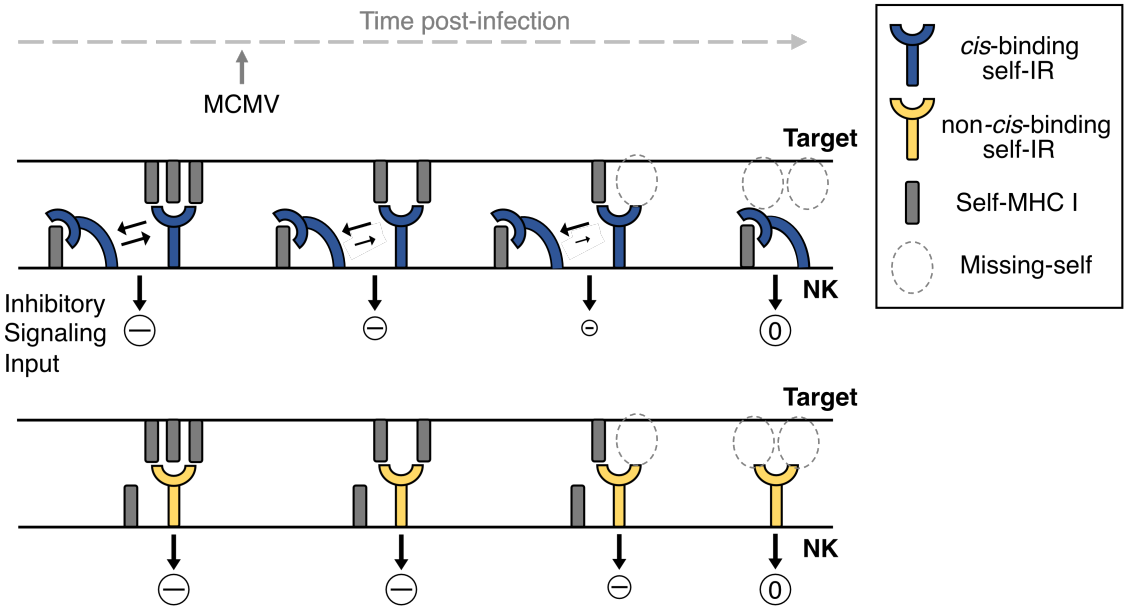


Figure 6. A model for IR-mediated *cis*-MHC I sensing during viral infection.[1]

[1] Pathogens (2021) Vol. 10:908, Copyright © 2021, the authors. Adapted.

Figure 6. A model for IR-mediated *cis*-MHC I sensing during viral infection. An equilibrium exists between the number of *trans*- vs *cis*-bound Ly49 IRs under steady-state conditions. Dominant IR-mediated inhibitory signals maintain NK cells in a tolerant state. During infection, viruses facilitate loss of self-MHC I expression on the surface of infected cells (i.e., missing-self). Self-specific IRs which can engage MHC I molecules *in cis* may diminish their negative signals more readily than self-specific IRs which poorly engage *cis*-MHC I, allowing for more efficient licensed NK cell-mediated detection of viral targets during stimulation via virus-specific activating NKRs. Adapted from (142).

Loss of inhibition could also be achieved if self-IRs fail to bind certain MHC I-peptide complexes during viral infection. Indeed, the structure of an MHC I-bound peptide is highly sensitive to peptide sequence variations (143). Moreover, self-specific NK cell IRs have been shown to bind their MHC I molecules in a peptide-selective manner. For example, the strength of the inhibitory signal propagated by human KIR3DL1, KIR2DL2, or KIR2DL3 IRs is modified by the peptide presented by their respective cognate MHC I ligand (118, 119). Likewise, murine Ly49C and Ly49I IRs bind their MHC I ligands in a peptide-selective manner, resulting in differences in IR signaling input (136, 144). As the balance of "non-self" vs "self" MHC I peptide content is rapidly shifted in favor of "non-self" peptides during viral infection (145, 146), recognition of virus-induced or -encoded peptides may release licensed NK cells from inhibition during recognition of infected targets.

MCMV promotes downregulation of MHC I from the surface of infected cells via immunoevasins m06/gp48 and m152/gp40. Whereas gp40 retains fully formed MHC I molecules in the ER (147), gp48 redirects them to the lysosome for degradation **(Figure 7)** (148). We thus predicted that Ly49G2-mediated sensing of missing-self viral targets might explain its role in MCMV resistance. However, MCMV also encodes the immunoevasin m04/gp34 which binds MHC I molecules in the ER and escorts them to the cell surface **(Figure 7)** (149). Moreover, a role for self-specific IRs in missing-self recognition is challenged by work from the Jonjić group showing that surface gp34/MHC I complexes can engage certain NK

cell IRs (Ly49A^{B6} and Ly49A^{BALB}) to evade mediated missing-self detection (129, 150). Thus, we hypothesized that select self-specific IRs (i.e., Ly49G2) might poorly bind altered-self (AS) gp34/MHC I complexes on viral targets **(Figure 8)**, unleashing signaling via virus-specific activating NKRs (142).

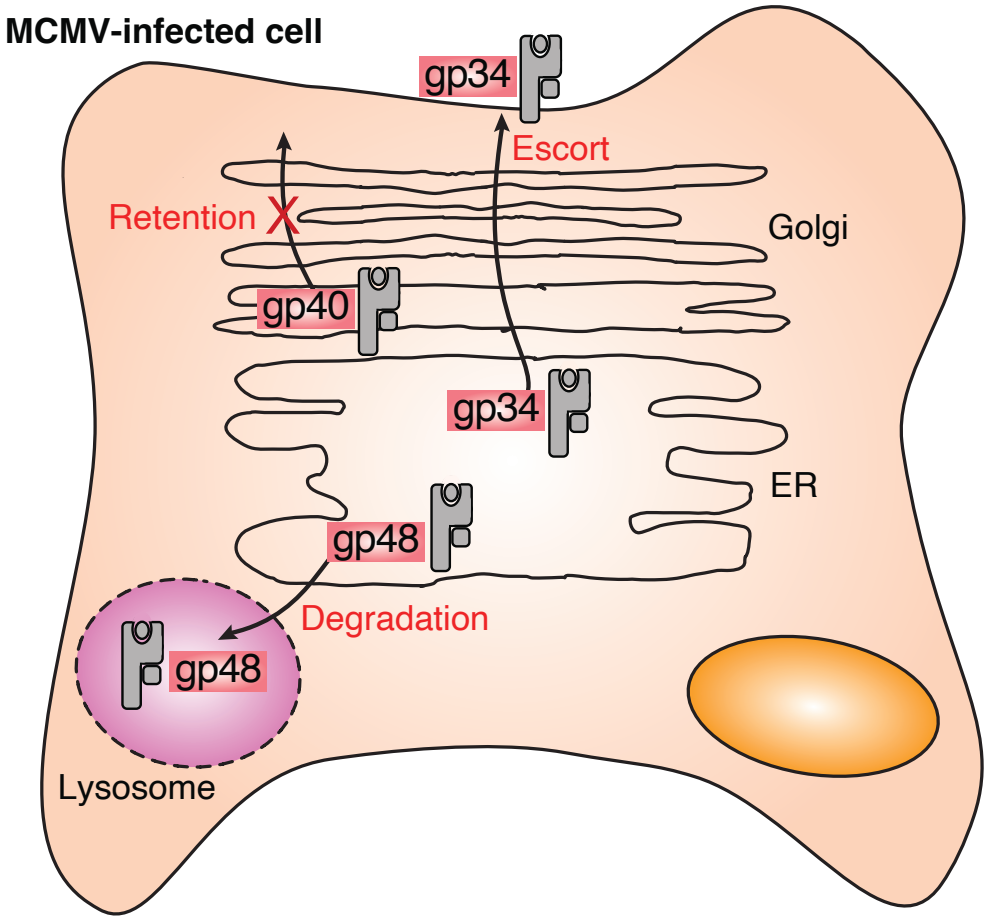


Figure 7. MCMV immunoevasins modify host MHC I expression and affect NK cell recognition of viral target cells.

Figure 7. MCMV immunoevasins modify host MHC I expression and affect NK cell recognition of viral target cells. MCMV facilitates downregulation of MHC I from the surface of infected cells via immunoevasins m06/gp48 and m152/gp40. Whereas gp40 retains fully formed MHC I molecules in the ER (147), gp48 redirects them to the lysosome for degradation (148). MCMV also encodes the immunoevasin m04/gp34 which binds MHC I molecules in the ER and escorts them to the cell surface (149). Engagement of surface gp34/MHC I complexes by NK cell IRs is predicted to facilitate MCMV-evasion of missing-self detection (129, 150).

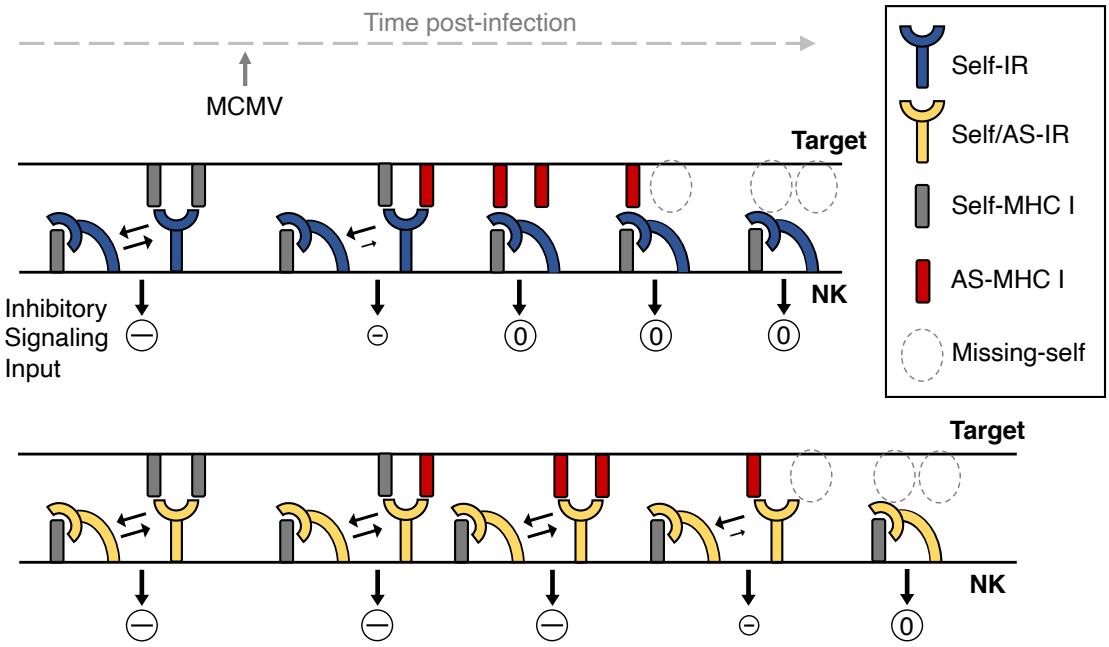


Figure 8. A model for altered-self recognition by NK cell IRs.[2]

[2] Pathogens (2021) Vol. 10:908, Copyright © 2021, the authors.

Figure 8. A model for altered-self recognition by NK cell IRs. An equilibrium exists between the number of *trans*- vs *cis*-bound Ly49 IRs under steady-state conditions (described in Figure 6). Over the course of infection, altered-self (AS)-MHC I molecules begin to appear on the surface of infected cells, followed by missing-self. Self-specific IRs with higher affinity for self-MHC I vs AS-MHC I may more readily adopt a *cis*-bound conformation, rapidly diminishing inhibitory signaling and unleashing signaling via virus-specific activating NKRs. By contrast, IRs which bind self/AS-MHC I with equal affinity may continue to propagate inhibitory signals until missing-self is achieved. Thus, IRs which fail to distinguish self from AS may facilitate viral evasion of NK cell immunity.

Selective activation of licensed NK cells during CMV infection

The signal-driving activating NKRs required in MHC I-dependent licensed NK cell antiviral immunity are less well defined. NK cell activation and target cytolysis is tightly regulated, and typically requires synergistic stimulation through multiple co-activating, adhesion, and cytokine receptors (8). Herein, we highlight select examples of CMV recognition by activating NKRs and their impacts on licensed NK cell-mediated CMV control.

In H-2^b B6 mice, MCMV resistance is mediated by NK cells expressing the Ly49H activating NKR, which recognizes the surface glycoprotein MCMV m157 expressed on viral targets (9, 10). Importantly, m157 is an MHC I mimic and does not require B2m or the transporter associated with antigen processing for efficient surface expression in MCMV-infected cells (151). Moreover, transgenic expression of Ly49H is sufficient to confer MCMV resistance to susceptible mice, irrespective of their H-2 haplotype (152). Thus, recognition of m157 by Ly49H and control of MCMV infection in NKC^{B6} mice are MHC I-independent.

On the other hand, genetic mapping of MA/My mice by the Vidal group revealed that a gene within the *Ly49* cluster, and another gene in the major histocompatibility complex, are together associated with MCMV resistance (153). Further analysis indicated that the $Ly49P^{MA/My}$ activating NKR directly recognizes target cells *in vitro* in a manner dependent on surface MHC I D^k and MCMV m04/gp34 expression (153, 154). Notably, gp34 binds MHC I molecules and

escorts them to the surface of infected cells **(Figure 7)**; thus, Ly49P recognizes surface gp34/D^k complexes. Additionally, broad-neutralization of Ly49P using a depletion antibody that binds Ly49P, Ly49V, and Ly49T rendered MA/My mice highly susceptible to MCMV infection (153). Thus, Ly49P was predicted to mediate D^k-dependent MCMV control in MA/My mice. However, genetic mapping did not specifically implicate Ly49P, but rather broadly identified the NKC as an MCMV resistance factor. Furthermore, lacking a specific, non-depleting antibody against Ly49P, the significance of $Ly49P^{MA/My}$ on NK cell-mediated virus control *in vivo* is unclear.

More recently, our laboratory discovered that specific neutralization of the $Ly49R^{C57L}$ activating NKR abrogates licensed $Ly49G2^+$ NK cell-mediated MCMV resistance (11) in congenic MHC I D^k-bearing B6 mice possessing a C57L-derived NKC (i.e., NKC^L-D^k and see **Table 1**). As Ly49R binds D^k tetramers (155), we hypothesized that like Ly49P, Ly49R might also bind D^k-gp34 complexes on MCMV-infected cells (11, 142). Taken together with our observations that endogenous $Ly49R^+$ $Ly49G2^+$ (R^+ $G2^+$), but not $Ly49R^+Ly49G2^-$ (R^+ $G2^-$) NK cells exhibit selective activation, accumulation the spleen, and expansion by percentage during MCMV infection in a D^k-dependent manner (11), and depletion of $Ly49G2^+$ NK cells abrogates D^k-dependent antiviral immunity (11, 20, 21), we posited that Ly49G2-mediated loss of inhibition might synergize with viral ligand recognition by Ly49R to lessen the requirement for licensed NK cell activation during viral infection **(Figure 8)** (142).

Activating NKR have likewise been implicated in NK cell-mediated detection of HCMV. For instance, KIR2DS1 selectively binds HLA-C2 molecules expressed on HCMV-infected (but not uninfected) target cells (156). Furthermore, licensed iKIR$^+$ NK cells coexpressing the heterodimeric CD94/NKC2C activating receptor preferentially expand in patients infected with HCMV (17). And recently, iKIR$^+$ NKG2C$^+$ NK cells were shown to selectively proliferate *in vitro* in response to certain HCMV-encoded UL40 peptides presented by HLA-E (157). Therefore, akin to Ly49R or Ly49P in mice, the KIR2DS1 or NKG2C activating receptors may contribute to licensed NK cell-mediated detection of HCMV-infected targets.

Rationale and Specific Aims

MCMV resistance is abrogated by depletion of NK cells expressing the Ly49G2 IR in D^k-bearing MA/My or C57L-derived mouse strains (20, 21, 126, 127). More recently, we found that neutralization of the Ly49R activating NKR abolishes D^k-dependent MCMV control (11). We further discovered that Ly49R$^+$ Ly49G2$^+$ NK cells exhibit greater expansion and accumulation during MCMV infection in comparison to *endogenous* Ly49R$^+$ Ly49G2$^-$ NK cells (11). Additionally, we found that adoptive transfer of Ly49R$^+$ Ly49G2$^+$ NK cells, but not *endogenous* Ly49R$^+$ Ly49G2$^-$ NK cells confers protection against MCMV infection (11). *However, lacking genetic knockouts of Ly49G2 and Ly49R, we can only infer the functional role of each MHC I D^k-binding receptor during MCMV infection*. Nonetheless, our prior findings suggest that Ly49G2 and Ly49R are important for viral target detection. Relatedly, the MCMV-encoded gp34, gp40, and gp48 immunoevasins regulate the levels of surface D^k expression on infected cells **(Figure 7)**. Whereas gp40 and gp48 facilitate D^k downregulation, gp34 delivers D^k to the cell surface and gp34-D^k complexes are found in surface immunoprecipitates from MCMV-infected cells (Xeufang Xie, unpublished data). We thus hypothesized that 1) Ly49R and Ly49G2 together are required in D^k-dependent MCMV control and 2) parallel sensing of gp34 via Ly49R and Ly49G2 is essential to enhance NK cell responsiveness during MCMV infection.

Specific Aim 1: Define whether Ly49G2 and Ly49R are required in MHC I D^k-dependent MCMV resistance. NK cells coexpressing Ly49G2 and Ly49R selectively expand during MCMV infection. A genetic requirement for either receptor in MCMV control has not been established. Thus, we generated Ly49G2-deficient mice using CRISPR/Cas9 gene-editing and tested the functional contribution of Ly49G2 to virus control, NK cell responsiveness, and $Ly49R^+$ NK cell expansion during MCMV infection (Chapter II). We also used Ly49R- and Ly49G2-specific CRISPR/Cas9 ribonucleoprotein complexes to ablate the individual receptors from primary NK cells and assessed the impact of receptor deficiency on MCMV-specific NK cell activation and proliferation in infected D^k-bearing host mice (Chapter III).

Specific Aim 2: Define the molecular basis of paired Ly49 receptor interactions with MHC I D^k molecules at steady-state and during MCMV infection. MCMV gp34-D^k complexes are found on the surface of viral targets during MCMV infection. Accumulation of $Ly49R^+$ $Ly49G2^+$ NK cells during MCMV infection is D^k-dependent, and both receptors have been shown to specifically recognize D^k. It remains unclear whether Ly49R- or Ly49G2-mediated recognition of MHC I D^k is modified by gp34 expression. To test this, we engineered reporter cell lines expressing chimeric Ly49R-CD3ζ or Ly49G2-CD3ζ receptors. We also generated gp34-deficient MCMV using CRISPR/Cas9 gene-editing. We then tested the impact of gp34 on 1) stimulation of Ly49R or Ly49G2 reporter cell lines and 2) D^k-dependent NK cell-mediated MCMV control (Chapter III).

Chapter II

The Ly49G2 inhibitory receptor is essential to MHC I-dependent NK cell control of murine cytomegalovirus infection[3]

[3] Proceedings of the National Academy of Sciences (2019) Vol. 116:26768-26778, Adapted.

This work (11) was performed in collaboration with Awndre Gamache, who discovered a novel role for the Ly49R activating receptor in D^k-dependent MCMV control.

My major contributions to the work included generation of the Ly49G2-deficient mouse strain and evaluation of Ly49G2 and Ly49R binding to MCMV-infected targets. Together, we characterized the phenotypic impacts of Ly49G2 deficiency on $Ly49R^+$ NK cell functionality during MCMV infection.

Introduction

Natural killer (NK) cells are innate lymphocytes that play a nonredundant role in sustaining host immunity to virus infections (158, 159). They respond to environmental cues by integrating signals from diverse arrays of activating and inhibitory receptors (IRs), including structurally unrelated killer Ig-like receptor (KIR) or Ly49 receptors expressed in different species. Both human KIR and rodent Ly49 families include germline-encoded inhibitory and activating receptors that 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 (160, 161).

Both KIR and Ly49 inhibitory self-receptors help tune NK cells during interaction with host MHC I (8, 85). "Self-aware" NK cells that have been tuned are said to be educated or licensed, as evidenced by enhanced effector function following activating receptor stimulation, and the ability to kill missing-self (MHC I^{-}) target cells (72–74). Licensed NK cells may improve clinical outcomes in human patients with chronic virus infections, including hepatitis C virus (HCV) or HIV (12, 14, 162). Indeed, licensed NK cells have been found to respond and accumulate during several different human virus infections, including Hantavirus, Chikungunya virus, hepatitis B virus, HCV, HIV, and cytomegalovirus (CMV) (17, 163–166).

The MHC I D^{k} molecule promotes NK cell-mediated control of murine CMV (MCMV) infection in different mouse strains, including MA/My and C57L.D^{k} (20,

23, 167, 168). Host resistance in these strains was found to correspond with the Ly49G2 inhibitory receptor encoded in most Ly49 haplotypes so far studied (20, 52, 169). Importantly, Ly49G2 self-receptor allotypes expressed in MA/My and C57L.D^k mice can license NK cells via D^k, whereas others (e.g., Ly49G2^{B6}) cannot (20, 95, 128). This licensing effect correlates with D^k–dependent virus control and is abolished by specifically depleting Ly49G2$^+$ NK cells prior to MCMV infection (20, 21, 126, 128, 170).

More recently, we discovered that D^k-dependent MCMV control requires Ly49R signaling, as blocking the activating receptor prior to infection resulted in rapid MCMV spread (11). Coincident with MCMV control, we observed selective expansion of Ly49R$^+$ Ly49G2$^+$ (R^+ $G2^+$) NK cells during MCMV infection (11). Moreover, R^+ $G2^+$ NK cells appeared phenotypically more activated than their endogenous Ly49R$^+$ Ly49G2$^-$ (R^+ $G2^-$) counterparts, and this effect was D^k-dependent (11). R^+G2^+ NK cells likewise transferred MCMV control to NK cell-deficient hosts, in contrast to R^+G2^- NK cells (11). NK cell subset activation and proliferation differences suggested impaired virus control in the absence of Ly49G2 might result from inept target cell sensing or poor NK cell expansion.

Nonetheless the importance of inhibitory self-receptors for MHC I in virus immunity is still debated, and a precise role for the Ly49G2 IR in D^k-dependent MCMV control is not defined. Thus, we generated *Ly49g2*^{C57L} mutant mice via CRISPR/Cas9 gene-editing. A single cytosine insertion resulted in Ly49G2 truncation and undetectable NK cell surface expression. Ly49G2-deficient mice exhibited increased mortality due to abrogated MCMV control by NK cells, despite

an otherwise unmodified Ly49 repertoire and normal NK cell development. Furthermore, NK cells derived from Ly49G2-deficient animals exhibited poor responsiveness to activating receptor crosslinking, less accumulation in the spleen during MCMV infection, and impaired virus-specific activation and proliferation. Our findings identify a vital role for self-specific IRs in antiviral NK cell effector activities.

Results

Generation of *Ly49g2*-Deficient Mice.

Ly49G2^{+} NK cells were previously shown to mediate MHC I D^{k}-dependent MCMV resistance in MA/My, C57L.D^{k}, and B6.NKC^{C57L}-D^{k} mice (20, 170). A specific role of Ly49G2 in virus control, however, remained to be elucidated. Thus, we used CRISPR/Cas9 genome editing to initially generate B6.NKC^{C57L} (NKC^{L}) mice deficient in Ly49G2 expression. The C57L allele of *Ly49g2*^{C57L} exon 4 was selectively targeted in NKC$^{B6/L}$ heterozygous embryos, which aided in genotypic and allotypic screening for mutant founders **(Figures 9A and 10A-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 10B).**

Ly49G2 allotype-specific staining showed that NK cells from GO founder offspring had reduced cell surface Ly49G2^{L} expression **(Figure 10C)**. 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 9B-C)**. 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 9D)**. 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 9E)**. Whole-genome exome sequencing confirmed *Ly49g2* cytosine insertions in both GO strains. Moreover, only 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 3-5)**. Highly specific Ly49 gene-editing thus selectively abolished Ly49G2 surface expression on GO NK cells.

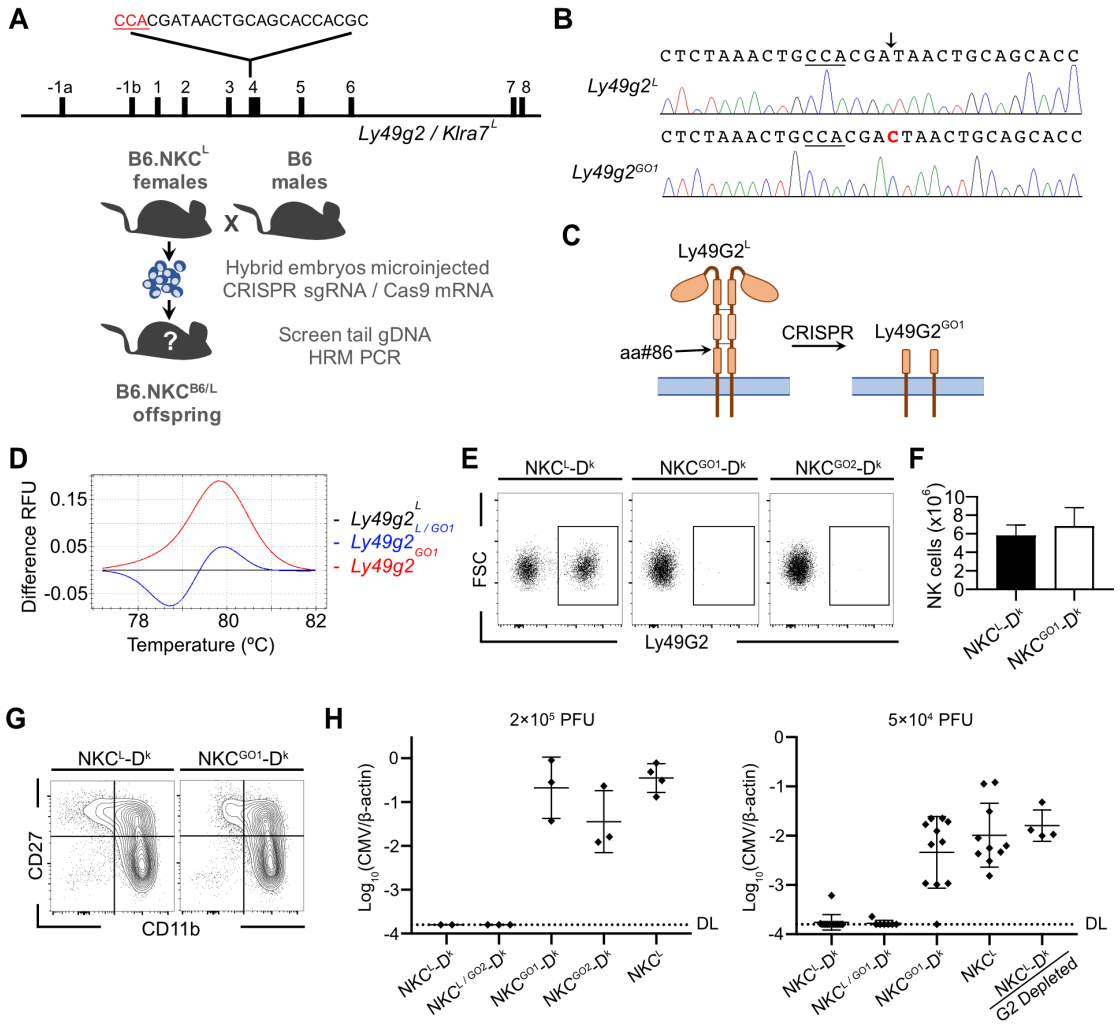


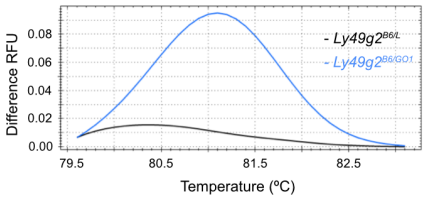
Figure 9. NK cells develop normally in *Ly49g2*-deficient GO mice but fail to control MCMV infection.

Figure 9. 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 intraperitoneally with 2×10^{5} PFU (Left) or 5×10^{4} PFU (Right) MCMV and evaluated for spleen virus levels 90 hours post-infection (hpi). 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 3 independent experiments with 3 to 4 mice per group. (H, Left) Two to 4 mice per group. (Right) Combined data from 3 separate experiments with 3 to 5 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

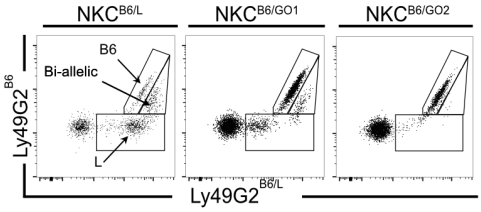


Figure 10. CRISPR/Cas9-mediated editing of *Ly49g2*^{L}.

Figure 10. 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.

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

Table 3. Details for GO1 *Ly49* exome consensus sequences overlapped at the CRISPR target site.

[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.

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

Table 4. Details for GO2 *Ly49* exome consensus sequences overlapped at the CRISPR target site.

[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 5. GO *Ly49* exome consensus sequences detailed in Tables 3 and 4.

>Ly49q1/Klra17

AAAAACATGAACTGCAGGAAACTCTAAACTGCCACCATAACTGTAGCACCATGCAAAATGAC
ATCAAC
GCAAAGGAAGAAATGCTGAGAAATATGCCTCTAGAGTGTAGTACAGGAGATGATCTTCTGAA
ATCCC TCAACAGAGAACAGAAGAGATGGTACAGTGAA

>Ly49q1/q2-related *(97.7%)*
CAAGAAAAACATGAACTGAGGGAAACTCTAAACTGCCACCATAACTGTAGCACCATGCAAAG
TGACA
TCAACGCAAAGGAAGAAATGCCGAGAAATATGCCTCTAGAGTGTAGTACAGGTGATGATCTT
CTAAA ATCCCTCAACAGAGAACAGAAGAGATGGTACAGTGAA

>Ly49e/Klra5

TTTTTCAGTATAGTCAACACAAACAAGAAATACACGAAACTCTAAACCACAACCATAACTGCA
GCAAC
ATGCAAAGTGACATCAAATTAAAGGAAGAAATGTTGAGAAATAAGTCTATAGATTGCAGTCCA
GGTGA
GGAACTTCTGGAATCCCTCAACAGAGAACAGAACAGATGGTACAGTGAAACCAAGACA

>Ly49v/Klra22

AAACTGCAGGAAATTCTAAACCACCACAATAACTGCAGCAACATGCAAAGTGACATCAACTT
GAAGG
ATGAACAGCTGAAAAATAAGTCTATAGAGTGTAATCTTCTGGAATCCCTCAACAGGGATAAGA
ACAGA TTGTATAATAAAACCAAGACTGTTTTAGATTCCTTGCAGCACA

>Ly49s/Klra19

TCAACACAAACAAGAAATCCATGAACCTCTAAACTACCACCATAACTTCAGCAACATGCAAAG
TGATT
TCAACTTAAAGGAAGAAATGTTGACAAATAAGTCTATAGACTCTAGGTCAGGCAATGAACTTC
TGGAA TCCCTCAACAGAGAACAGAACAGAGGGTACAGTGAAACTAAGACA

>Ly49t/Klr20

CAACAAAAACATGAACTGCAAGAAACTCTAAACTGCAACGATAACTGCAGCACCACGCAAAG
TGACA
TCAACTTAATGGATGAACTGCTGAGAAATAAGTCTATAGAATGTAGGCCAGGCAATGATCTTC
TGGAA TCCCTCAACAAGGAACAGAGCAGATGGTACAGTGAAACCAAGACTCTTTTAGATT

>Ly49r/Klra18 (100%)

TCAACAAAAACATGAACTGCAGGAATTTCTAAAACACCACAATAACTGCAGCATCATGCAAAG
TGACA
TCAACTTGAAGGATGAACTGCTGAAAAATAAGTCTATAGAGTGTAATCTTCTGGAATCCATCA
ACAGG GATCAGAACAGATTGTATAATAAAACCAAGACTGTTTTAGATTCCTTACAGC

>*Ly49r*-related_a *(97.4%)*
GTCAACAAAAACATGAACTGCAGGAATTTCTAAAACACCACAATAACTGCAGCATCATGCAAA
GTGAC
ATCAACTTGAAGGATGAACAGCTGAAAAATAAGTCTCTAGAGTGTAATCTTCTGGAATCCCTC
AACAG GGATCAGAACAGATTGTATAATAAAACCAGGACTGTTTCAGATTCCTTACAGCACAC

>*Ly49r*-related_b *(89.7%)*
TTTTTCAGTGTAGTCAACAAAAGAATGAACTGCAGGAAATTCTAAACCGCCACCATAACTGCA
GCATC
ATGCAAAGTGACATCAGCTTAAAGGAAGAACTGCTGAGAAATAAGTCTATAGTGTGTA

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

TTTTTCAGCATATTCAACAAAAACATGAACTACAGGAAACTCTAAACTGCCACGACTAACTGC
AGCAC
CACGCAAAGTGACGTCAACTTGAAGGATGAACTGCTGAGAAATAAGTCTATAGAGTGTAGGC
CAGGC
AATGATCTTCTGGAATCCCTCAACAGGGATCAGAAAAGATGGTACAGTGAAACTAAGACTTTT
TCAG

>*Ly49p/d*

TCACCAAAAACATGAACTGCAGGAATTTCTAAAACACCACAATAACTGCAGCATCATGCAAAG
AGACA
TCAACTTGAAGGATGAACTGCTGAAAAATAAGTCTATAGAGTGTAATCTTCTGGAAACCCTCA
ACAGG GATCAG

>*Ly49p/Klra16*

TTTTTCAGTATGGTCAACAAAAACATGAACTGCAGGAATTTCTAAACCACCACAATAACTGCA
GCATC
ATGCAAAGTGACATCAAATTGAAGAATGAACTGCTGAAAAAGAAGTCTATAGAGTGTAATCTT
CTGGA ATCCCTCAACAGGGATCAGAACAGATTGTATA

>*Ly49o/Klra15*

TCAACAAAAAGAACTGCAGGAAATTCTAAACCACCACAATAACTGCAGCAACATGCAAAGTG
ACATCA
ACTTGAAGGATGAACTGCTGAAAAATAAGTCCATAGAGTGTGATCTTCTGGAATCCCTCAAC
AGGGA TCAGAACAGATTGTATAATAAAACCAAGACTGTTTTAGATTCCTTAAAGCAC

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 9F-G)**. 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 90 hours post-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 9H)**. Thus, the Ly49G2 inhibitory self-receptor is required for NK cells to mediate MHC I-dependent MCMV control.

Ly49G2 licenses stimulation through activating NK cell receptors.

Since licensed NK cells display greater responsiveness to activating receptor stimulation than their unlicensed counterparts (72–74), 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 percentage of responsive $Ly49R^+$ NK cells from mice that express the licensing receptor and its cognate ligand (**Figures 11A-B**). A small fraction of responsive NK cells (2 to 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.

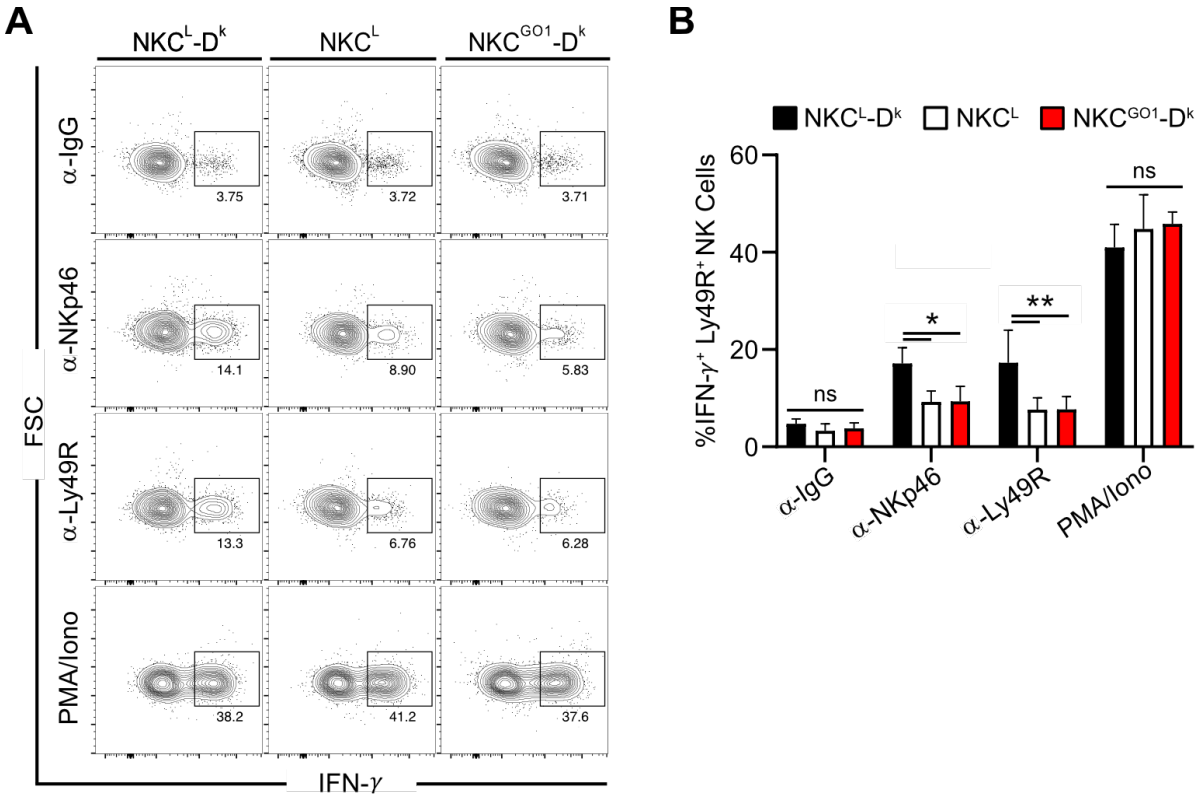


Figure 11. Ly49G2-licensed NK cells exhibit greater IFN-γ production following activating receptor stimulation.

Figure 11. Ly49G2-licensed NK cells exhibit greater IFN-γ production following activating receptor stimulation. (A) 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. (B) Percentages of splenic $Ly49R^{+}$ NK cells that express IFN-γ from A. Data in A and B are representative of 2 independent experiments with 4 mice per group. Error bars indicate mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Ly49G2 and Ly49R Receptors Engage MHC I D^k molecules on MCMV-infected Cells.

Prior work showed that both Ly49G2 and Ly49R receptors can bind soluble D^k tetramers (155). 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 (171) to examine whether MA/My- or C57L-derived self-receptor allotypes bind D^k. In comparison to control J7 cells, reporter cells expressing chimeric $G2^M$-CD3ζ, $G2^L$-CD3ζ, or R-CD3ζ receptors were selectively stained and stimulated with Ly49-specific mAbs 4D11 or 12A8 **(Figures 12A-B)**. Moreover, each of these reporter cell lines specifically responded when cocultured with YB2/0-D^k cells, but not YB2/0 cells **(Figure 12B)**. Thus, Ly49G2 and Ly49R self-receptors both bind MHC I D^k.

We next tested whether Ly49G2 and Ly49R self-receptors can recognize MCMV-infected M2-10B4 targets bearing D^k ligands. Despite IFN-β–induced D^k surface expression on M2-10B4 cells **(Figure 12C)**, only the Ly49G2 reporters significantly responded **(Figure 12D)**. Additionally, despite both Ly49G2 and Ly49R reporters responding to targets infected with MCMV for 24 to 72 h **(Figure 12D),** Ly49G2 signals diminished as MCMV infection progressed and D^k cell surface expression declined, whereas Ly49R signaling was maintained throughout. Intriguingly, IFN-β treatment of MCMV-infected targets prevented Ly49R-specific recognition, whereas Ly49G2 reporters were undeterred.

Together, these data demonstrate that while both self-receptors recognize D^k, their binding affinities and MCMV response patterns differ.

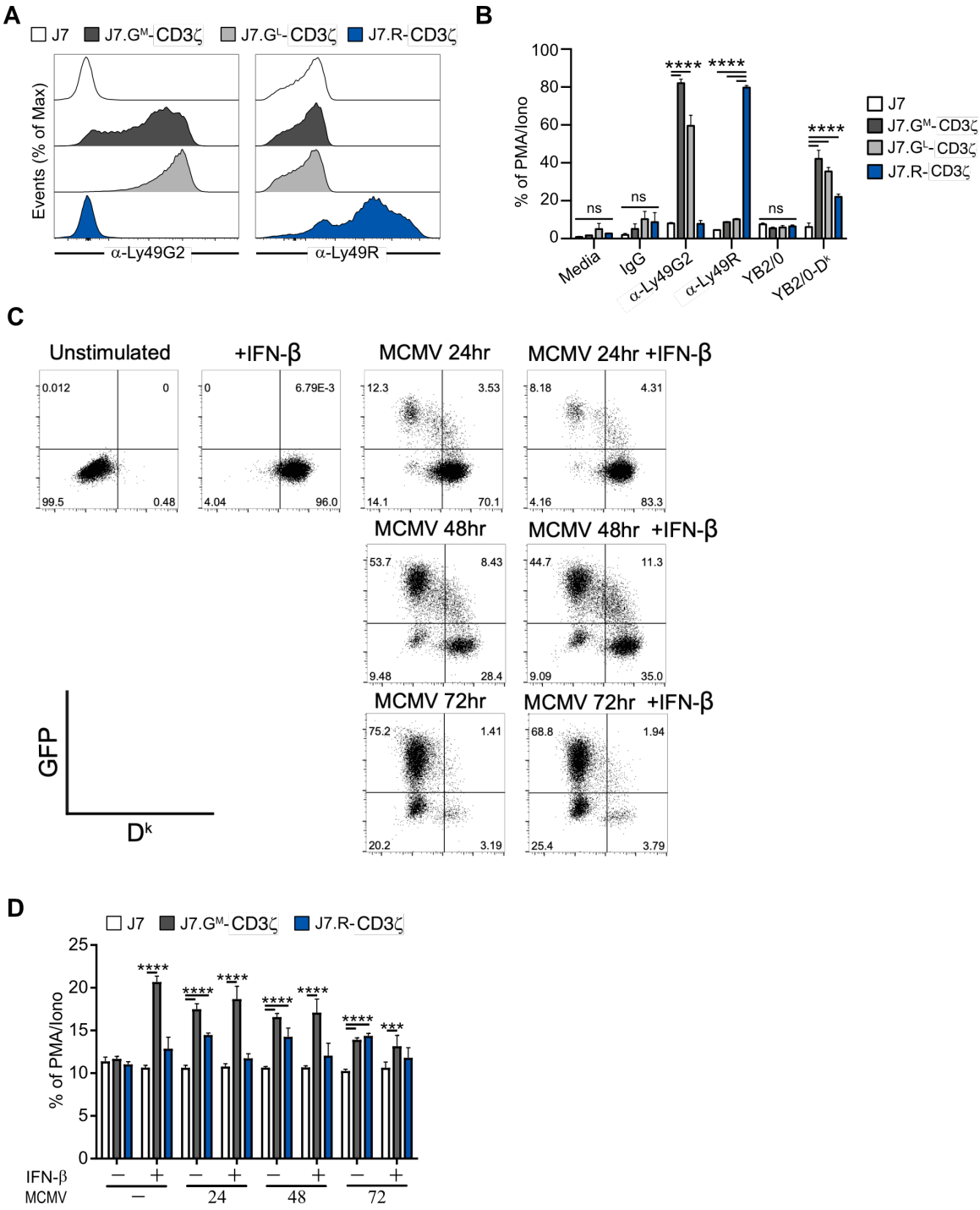


Figure 12. Ly49G2 and Ly49R receptors differently engage MHC I D^k molecules on MCMV-infected targets.

Figure 12. Ly49G2 and Ly49R receptors differently engage MHC I D^k molecules on MCMV-infected targets. (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.G^M-CD3ζ, J7.G^L-CD3ζ, or J7.R-CD3ζ reporter cells. (B) Reporter cells were stimulated with plate-bound mAbs or target cells. (C) Representative flow plots are shown for M2-10B4 cells infected with MCMV-GFP ((172), 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). (D) Reporter cells were cocultured for 12 h with infected M2-10B4 cells pretreated with IFN- β 16 h before coculture, as indicated. Target cells were infected for the indicated times prior to coculture with reporter cells. Data in A and B are representative of 3 to 5 independent experiments with 2 to 5 samples per group. Data in C are representative of 2-3 experiments with 2-6 samples per group. Data in D is representative of 2 experiments with 3 to 6 samples per group. Error bars indicate mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Ly49G2 Promotes Specific Activation and Terminal Differentiation of Ly49R$^+$ NK Cells during MCMV Infection.

Because NK cells can become activated in response to virus-induced inflammation (173, 174), we examined the impact of Ly49G2-deficiency on Ly49R$^+$ NK cell responses during MCMV infection. First, we assessed KLRG1 expression since virus-specific Ly49H$^+$ NK cells were shown to acquire and maintain KLRG1^{hi} expression during MCMV infection (173, 175). We observed that a significantly higher fraction of NKC^L-D^k R$^+$G2$^+$ NK cells exhibited KLRG1 terminal differentiation than their counterpart R$^+$G2^{null} NK cells, and this difference was D^k-dependent as both subsets displayed similar KLRG1^{hi} frequencies in NKC^L mice **(Figures 13A-B)**.

CD25 up-regulation on NK cells also occurs during MCMV infection, largely due to virus-induced IL-12 (69, 176). In mice lacking Ly49G2 or D^k, CD25 increased on Ly49R$^+$ NK cells to a greater extent than what was seen in NKC^L-D^k mice **(Figures 13C-D)**. This is likely due to the sustained inflammatory environment in mice lacking Ly49G2 or D^k caused by unfettered viral spread **(Figure 9H)** (177). In NKC^L-D^k mice, however, CD25 selectively increased on R$^+$G2$^+$ cells. Together, these data suggest that D^k-licensed R$^+$G2$^+$ NK cells experienced virus-specific activation, as opposed to more general, cytokine-mediated stimulation resulting from virus-induced inflammation.

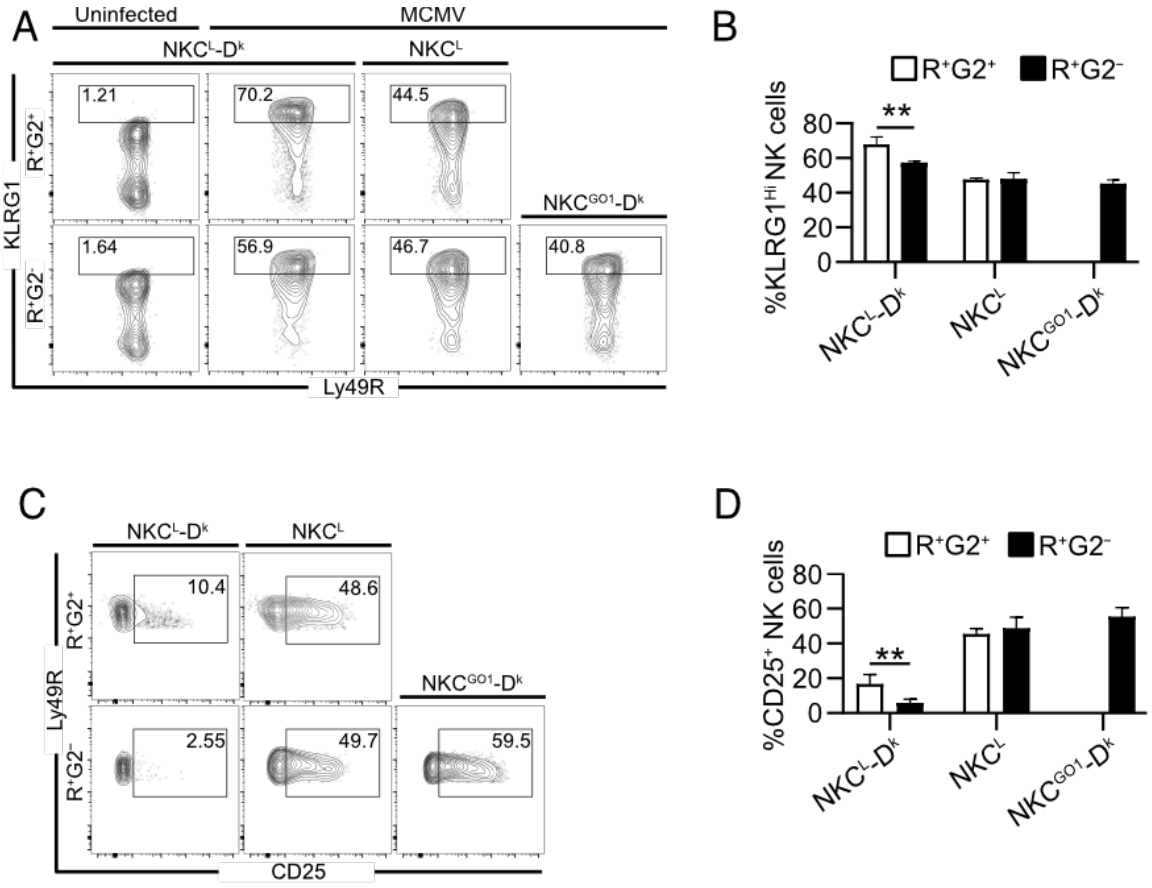


Figure 13. Ly49G2 promotes virus-specific activation and differentiation of Ly49R$^+$ NK cells during MCMV infection.

Figure 13. Ly49G2 promotes virus-specific activation and differentiation of Ly49R$^+$ NK cells during MCMV infection. (A and B) Flow plots and quantification of KLRG1^{hi} splenic NK cell subsets from uninfected or mice 90 hpi. (C and D) Flow plots and quantification of CD25$^+$ splenic NK cell subsets from mice 90 hpi. (Mice were infected with 5 × 10^4 PFU MCMV. All data are representative of 2 to 3 independent experiments with 3 to 4 mice per group. Error bars indicate mean ± SD. *$P < 0.05$, **$P < 0.01$.

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 (126, 127). We thus examined whether Ly49G2 governs this expansion. Whereas R$^+$ NK cells significantly increased in NKC^L-D^k spleens by 90 hpi, there was a notable decrease in mice lacking either the self-receptor or its cognate ligand **(Figures 14A-B)**. This finding is consistent with previous work showing that high viral burden induces splenic lymphopenia and lymphoid architecture collapse (47). These results suggest that Ly49G2 and D^k together enhance the accumulation of Ly49R$^+$ NK cells during MCMV infection.

We next measured NK cell incorporation of BrdU to gauge whether increased proliferation accounts for selectively expanded R$^+$G2$^+$ NK cells. We observed that a greater proportion of NKC^L-D^k R$^+$ G2$^+$ NK cells incorporated BrdU during infection than their R$^+$ Ly49G2-deficient counterparts **(Figures 14C-D)**. Variation in subset proliferation was not seen in NKC^L or GO1-D^k mice, suggesting that D^k-licensed R$^+$ G2$^+$ NK cells selectively increased proliferation during infection. Taken together, these data demonstrate that the selective accumulation of R$^+$ NK cells resulted from Ly49G2-mediated enhancement of proliferation in response to MCMV infection.

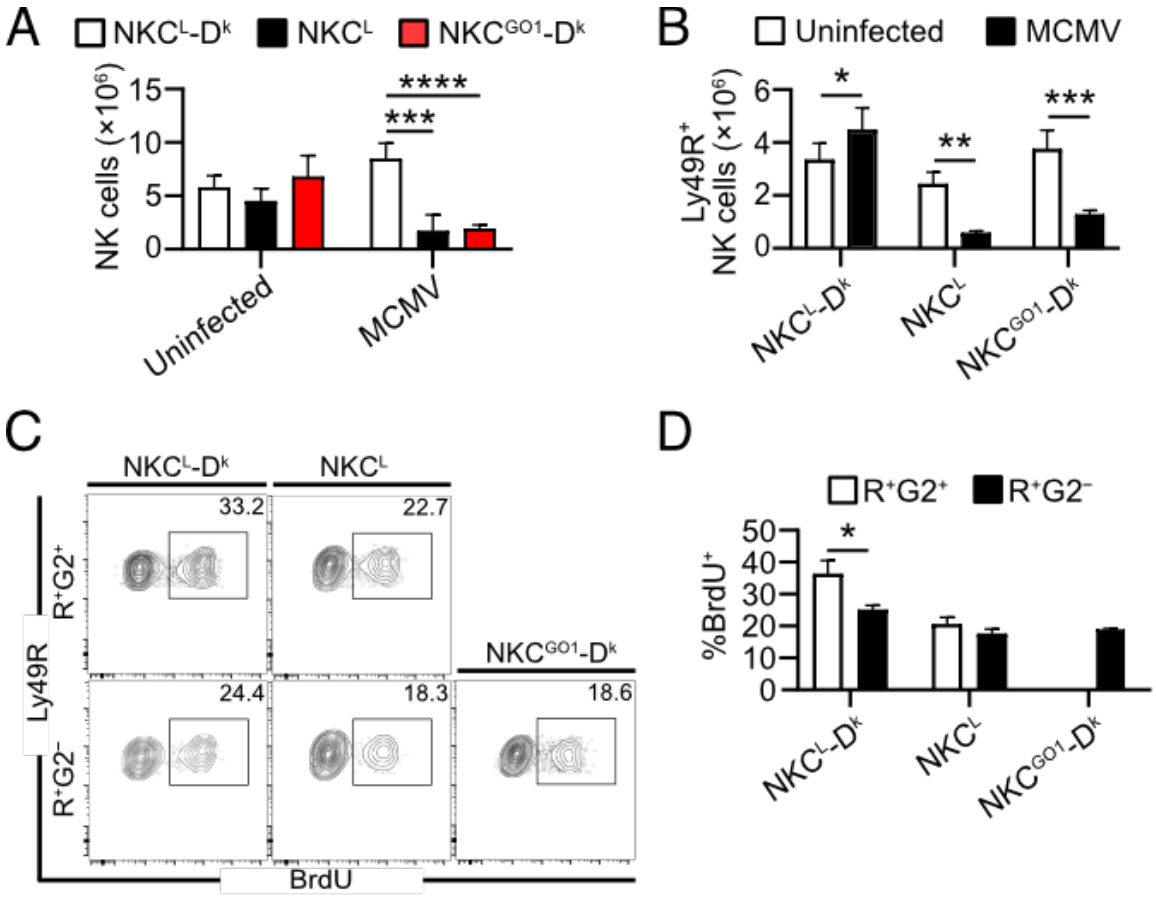


Figure 14. Ly49G2 promotes Ly49R^{+} NK cell accumulation and proliferation during MCMV infection.

Figure 14. Ly49G2 promotes Ly49R$^+$ NK cell accumulation and proliferation during MCMV infection. (A and B) Total NK cells and Ly49R$^+$ NK cells from the spleens of uninfected mice and mice 90 hpi. (C and D) BrdU incorporation after 3-h pulse BrdU treatment 4 dpi. In A–D, mice were infected with 5×10^4 PFU MCMV. (H) Selected hallmark genes and corresponding gene enrichment analysis of NKCL-Dk splenic NK cells. Data are representative of 3 to 6 independent experiments with 3 to 4 mice per group. Error bars indicate mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Ly49G2 is vital for host survival during MCMV infection.

Having verified the importance of the Ly49G2 receptor on NK cell-mediated MCMV control in the spleen, we assessed its 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 15)**. Thus, the Ly49G2 inhibitory self-receptor is essential in MHC I-dependent virus immunity and host survival when coexpressed on NK cells with its functionally discordant Ly49R self-receptor counterpart.

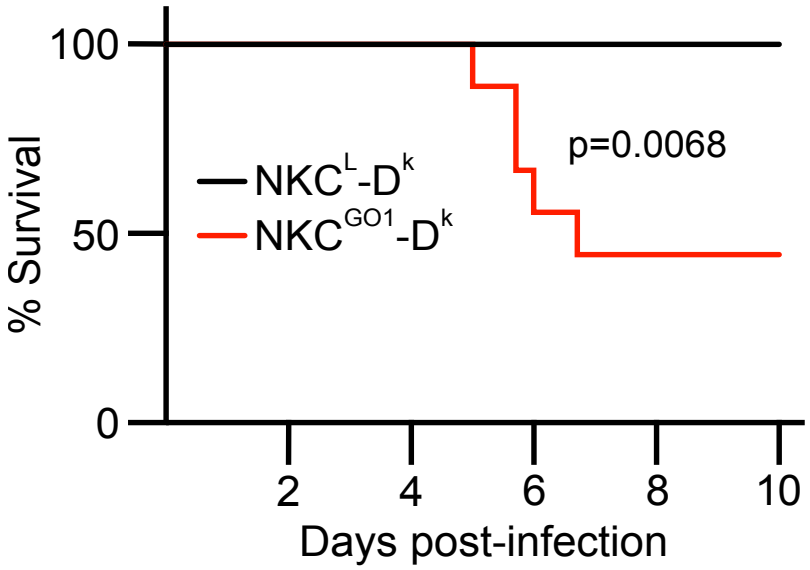


Figure 15. Ly49G2 is critical for protection against sublethal MCMV infection.

Figure 15. Ly49G2 is critical for protection against sublethal MCMV infection. Host survival curves for NKC^{L}-D^{k} or NKC^{GO1}-D^{k} mice following infection with 1×10^{6} PFU MCMV. Data are from a single experiment with 9 to 10 mice per group. Log-ranked Mantle–Cox test was used to determine statistical significance. $P = 0.0068$.

Discussion

While a widely held paradigm suggests licensed NK cells primarily thwart NK-mediated virus control, here we demonstrate that the inhibitory Ly49G2 NK cell receptor is required to specifically augment host defenses, including NK cell activation, differentiation, and proliferation, and to limit virus spread during MCMV infection. A role for an IR in virus control may seem paradoxical since several studies show NK-mediated antiviral activities are diminished in their presence (130, 178–180). Moreover, NK cells can mediate MCMV control in MHC I-deficient animals lacking self-receptor ligands (130, 181, 182). However, MCMV m157-specific Ly49H$^+$ NK cells display MHC I-independent MCMV control (10, 183). Hence, licensed NK cells may be dispensable if virus-specific recognition by NK activating receptors is adequate to overcome tolerance. Nonetheless, we found that Ly49G2 is essential for Ly49R$^+$ NK cell effector responses 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 (184, 185). Licensed NK cells thus may be uniquely poised to overcome self-tolerance during MCMV infection.

Although seemingly counterintuitive, IRs 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 (186). Related to this, human inhibitory KIRs (iKIRs) enhanced

murine $CD8^+$ T cell proliferation ex vivo in response to stimulation by dendritic cells bearing transgenic cognate HLA molecules (187). Additionally, expression of self-specific iKIRs was found to coincide with increased CD8 T cell survival and better overall virus control in patients infected with HIV, HCV, or human T cell leukemia virus type 1 (188).

Self-MHC I-specific IRs that license developing NK cells also increase the extent of activating receptor stimulation (72–74). Licensed NK cells that are educated on self-MHC I undergo expansion and differentiation in response to MCMV infection (126, 128, 139, 189), and in HCMV-infected individuals (17). Memory NK cells expressing self-specific Ly49 IRs in hapten-sensitized mice likewise display enhanced recall responses (190, 191). 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 their selective expansion in response to viral infection. 1) Licensing could increase activating receptor signals in response to virus or virus-induced antigens via altering activation signal transduction cascades. This explanation predicts that both licensing and activating receptors can specifically recognize and respond to virus-infected target cells. 2) Sustained binding of the IR could promote NK cell synapse formation and conjugation to infected target cells. 3) The IR could modify the activating receptor ligand so that activating signals are increased. Ongoing studies are focused on determining how the Ly49G2 IR enables NK cells to specifically recognize and respond to MCMV infection.

Our data additionally provide mechanistic insight into how Ly49R contributes to MCMV sensing. Although MHC I D^k tetramers were previously shown to bind Ly49R, they were folded with human B2m, which could have affected the interaction (155). 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 that 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. 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 MHC I D^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 iKIRs by their cognate MHC I ligands (118, 192). In contrast, KIR2DS2, a human NK cell-activating receptor, exhibits a strong affinity for highly conserved flavivirus peptide motifs presented by HLA-C*0102 (193), which suggests MHC I-specific NK activating 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 (96, 194). It is possible that Ly49 activating receptors might display similar specificity for virus peptide-modified host MHC I (195).

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 (196), whereas Ly49H$^+$ NK cells exhibit DAP12-dependent proliferation in B6 mice (197). Specific proliferation of the R$^+$ G2$^+$ subset in D^k-bearing 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} CD25$^+$ (69, 173, 174, 198). Our data are thus consistent with increased CD25 observed for NK cells responding to MCMV. We additionally observed nonselective CD25 upregulation on NK cells in infected mice lacking Ly49G2 or D^k, consistent with the hypothesis that CD25 is regulated independent of Ly49 activating receptors (69). 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 that Ly49G2 enhances antigen-specific NK cell stimulation, which promotes their differentiation and effector functions.

In conclusion, our data uncover an underappreciated role for self-specific IRs in promoting activation and expansion of NK cells in response to viral infection. We predict that Ly49G2 and Ly49R 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 activating 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

Altered-self MHC class I sensing counters murine CMV gp34-mediated immune evasion

Introduction

Self-specific recognition receptors provide essential regulation of innate immunity at steady-state and in malignancy (199). This is exemplified in the case of germline-encoded self-MHC class I (self-MHC I)-specific cell surface receptors expressed by natural killer (NK) cells. Activating murine Ly49 or human killer Ig-like receptors (KIRs) bind MHC I or MHC I-like molecules and associate with immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor molecules. Ligation of NK cell activating receptors can drive NK cell proliferation, cytokine release, and degranulation (7, 200). In contrast, immunoreceptor tyrosine-based inhibition motif (ITIM)-bearing Ly49/KIR drive recruitment and activation of phosphatases during recognition of self-MHC I (8). Interactions between these signaling-disparate receptors and their cognate MHC I ligands are essential regulators of NK cell activation and self-tolerance.

Active engagement of self-MHC I by ITIM-bearing receptors (IRs) also serves to increase NK cell functionality via an educational process referred to as licensing (8). Relative to unlicensed NK cells, licensed NK cells are more responsive to activating receptor signaling pathways and readily eradicate viral and tumor targets lacking self-MHC I (i.e., missing-self rejection) (1, 8). Indeed, to evade licensed NK cell immunity, herpesviruses such as murine CMV (MCMV) employ strategic mechanisms to manipulate MHC I and MHC I-like molecules (141, 177). A notable example is the MCMV immunoevasin m04/gp34 which binds

and escorts MHC I to the surface of infected cells, where it can impede both T lymphocyte recognition and missing-self rejection of MCMV targets by licensed NK cells (129, 149, 201). Conversely, MCMV infection studies in distinct mouse models have demonstrated that self-MHC I licensing IRs can also provide essential antiviral NK cell immunity. Indeed, we found that licensed NK cells in MHC I D^k-bearing B6 mice with a C57L-derived *NKC-Ly49* haplotype (i.e. NKC^L-D^k) require the Ly49G2 IR for D^k-dependent licensing, selective expansion, and virus control during MCMV infection (11). Likewise, in MHC I D^d-transgenic B6 mice, interactions between the Ly49A IR and D^d license NK cells and engender essential MCMV protection (202). A mechanistic basis for licensed NK cell-mediated MCMV sensing however remains to be elucidated.

By detecting virus-induced alterations in self-MHC I or MHC I-like molecules during infection, we posited that select pairs of self-specific Ly49 activating and inhibitory receptors may together enable more efficient recognition of infected "altered-self" targets by licensed NK cells (142). Indeed, monoclonal antibody (mAb)-mediated neutralization of the Ly49R activating receptor prior to MCMV infection was shown to abolish licensed NK cell immunity in NKC^L-D^k mice (11), suggesting a role for Ly49R in recognition of MCMV. On the other hand, recognition of MCMV gp34/MHC I complexes by Ly49L or Ly49P activating receptors is correlated with MHC I-dependent MCMV control in BALB.K or MA/My mice, respectively (150, 153, 154, 167). Because the C57L alleles of Ly49G2 and Ly49R bind D^k (11), and MCMV gp34 is required for efficient surface expression

of D^k during infection (129, 150), we predicted that Ly49G2 and Ly49R recognition of MCMV-modified D^k molecules might be an important feature of licensed NK cell sensing of MCMV *in vivo*.

NK cell receptors (NKRs), their MHC I ligands, and viral immunoevasins are thought to have co-evolved in an intricate arms race whereby host NKR/MHC I gene pairs that enable host survival prevail. By contrast, polymorphic viral immunoevasins are likely selected to thwart host immune defenses. Development of next-generation NK cell replacement therapies will undoubtedly benefit from a deeper understanding of the complex biology of NK cells and the NKR-ligand interactions at play during viral infection which might affect NK cell reactivity or self-tolerance. Herein, we demonstrate that Ly49R and Ly49G2 bind to altered-self gp34-D^k complexes on MCMV infected cells. Remarkably, Ly49G2 is required to prevent gp34-mediated evasion of $Ly49R^+$ NK cells during infection. Paired sensing of a shared MHC I ligand by activating and inhibitory Ly49 receptors thus represents an important mechanism of licensed NK cell-mediated viral target detection and host-defense.

Results

Ly49G2^{+} antiviral NK cells downregulate Ly49R in D^{k}-bearing mice.

Prior work in NKC^{L}-D^{k} mice established a key role for the Ly49G2 IR in D^{k}-dependent antiviral immunity mediated by licensed NK cells (11). To further elucidate requirements for licensed NK cell recognition of MCMV, we first compared spleen MCMV titers in immunodeficient NKC^{L}-D^{k} mice depleted of CD4^{+} and CD8^{+} T cells, or lacking perforin (*Prf1*$^{-/-}$-D^{k}), Ly49G2 (GO1-D^{k}), or D^{k} itself (i.e. NKC^{L} mice) **(Figure 16A)**. We observed that NK cells in this mouse model require perforin, host-D^{k} expression, and Ly49G2 to mediate MCMV control at 90 h post-infection (hpi). Moreover, T cell immunity is not essential early during acute infection.

Ly49R neutralization via blocking antibodies was previously shown to abrogate licensed Ly49G2^{+} NK cell antiviral immunity, suggesting it has a key role in MCMV recognition. The Ly49H activating receptor is likewise required for detection of MCMV m157 in B6 mice, which drives clonal expansion of Ly49H^{+} NK cells, especially in *Prf1*-deficient mice (198, 203, 204). We employed a similar strategy to assess the licensed NK cell response to MCMV. We found that expansion of antiviral Ly49R^{+} Ly49G2^{+} (R^{+} G2^{+}) NK cells directly corresponded to decreasing Ly49R cell surface expression in infected *Prf1*$^{-/-}$-D^{k} mice **(Figures 16B-C and Figures 17A-B)**. Ly49R^{+} Ly49G2^{-} (R^{+} G2^{-}) NK cells in contrast declined in

frequency and number and did not alter their expression of Ly49R during infection. This selective decrease of Ly49R in licensed NK cells is analogous to m157-driven Ly49H downregulation in antiviral NK cells during acute MCMV infection in B6 mice (151, 183, 203). These results thus suggest Ly49R engagement of an MCMV-associated ligand gives rise to Ly49R^{Lo} Ly49G2^{+} (R^{Lo} G2^{+}) NK cells during infection.

We next tested if MCMV-induced Ly49R downregulation in *Prf1*$^{-/-}$ mice requires D^{k} expression in host cells. As shown in **Figures 16D-F**, the decrease in cell surface Ly49R expression in antiviral NK cells occurred exclusively in D^{k}-bearing mice. We further investigated whether NK cell Ly49R downregulation corresponded with markers of enhanced NK cell functionality during infection. Antiviral NK cells expanding during MCMV infection have been shown to increase expression of the high-affinity IL-2 receptor CD25 and the maturation marker KLRG1 (11, 69, 173). Intriguingly, we found R^{Lo} G2^{+} NK cells from D^{k} mice significantly upregulated KLRG1^{Hi} and CD25 expression **(Figures 16F-J)**. Antiviral Ly49G2^{+} NK cells thus specifically respond to MCMV-induced changes in host cell D^{k} expression with Ly49R downregulation, increased activation, and maturation.

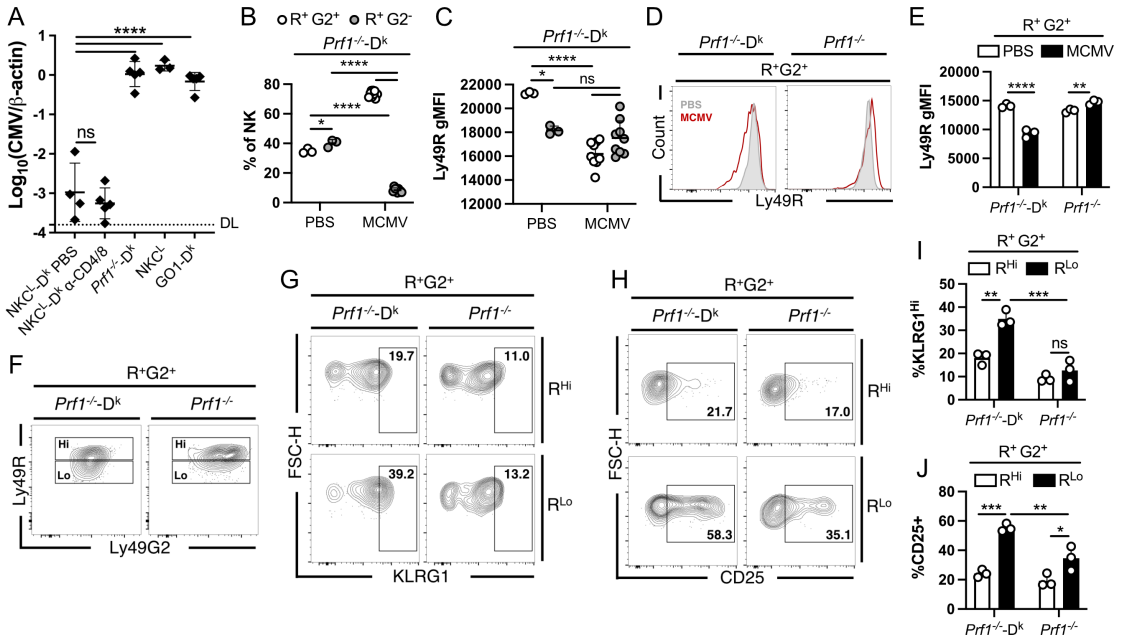


Figure 16. Ly49G2^{+} antiviral NK cells downregulate Ly49R in D^{k}-bearing mice.

Figure 16. Ly49G2^{+} antiviral NK cells downregulate Ly49R in D^{k}-bearing mice. (A) Spleen virus levels (90 hours post-infection (hpi)) for the indicated mice infected with MCMV (2×10^5 PFU). DL, detection limit. (B) Cumulative data showing the percentage of R^{+} G2-disparate spleen NK cells (90 hpi) from control (PBS) or MCMV-infected (2.5×10^4 PFU) *Prf1*$^{-/-}$-D^{k} mice. (C) Ly49R MFI values are shown for R^{+} G2-disparate NK cells. (D and E) Representative histograms (D) and cumulative data (E) show Ly49R expression for splenic R^{+} G2^{+} NK cells from control or MCMV-infected (as for Fig. 1B) D^{k}-disparate *Prf1*$^{-/-}$ mice. (F) Flow plots show the gating strategy for analysis of R^{+} G2^{+} NK cells from infected mice delineated by Ly49R expression. (G-J) Representative flow plots (G and H) or cumulative data (I and J) showing the frequency of KLRG1^{Hi} (G and I) or CD25^{+} (H and J) R^{+} G2^{+} NK cells from infected mice delineated by Ly49R expression. Data in (A-C) are representative of three experiments (3-9 mice per group). Data in (D-I) are representative of two experiments (3 mice per group). For statistical analysis, one-way ANOVA was used in A, two-way ANOVA with post-hoc test in B, C, I, and J, and unpaired two-tailed t test in E. Error bars indicate mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

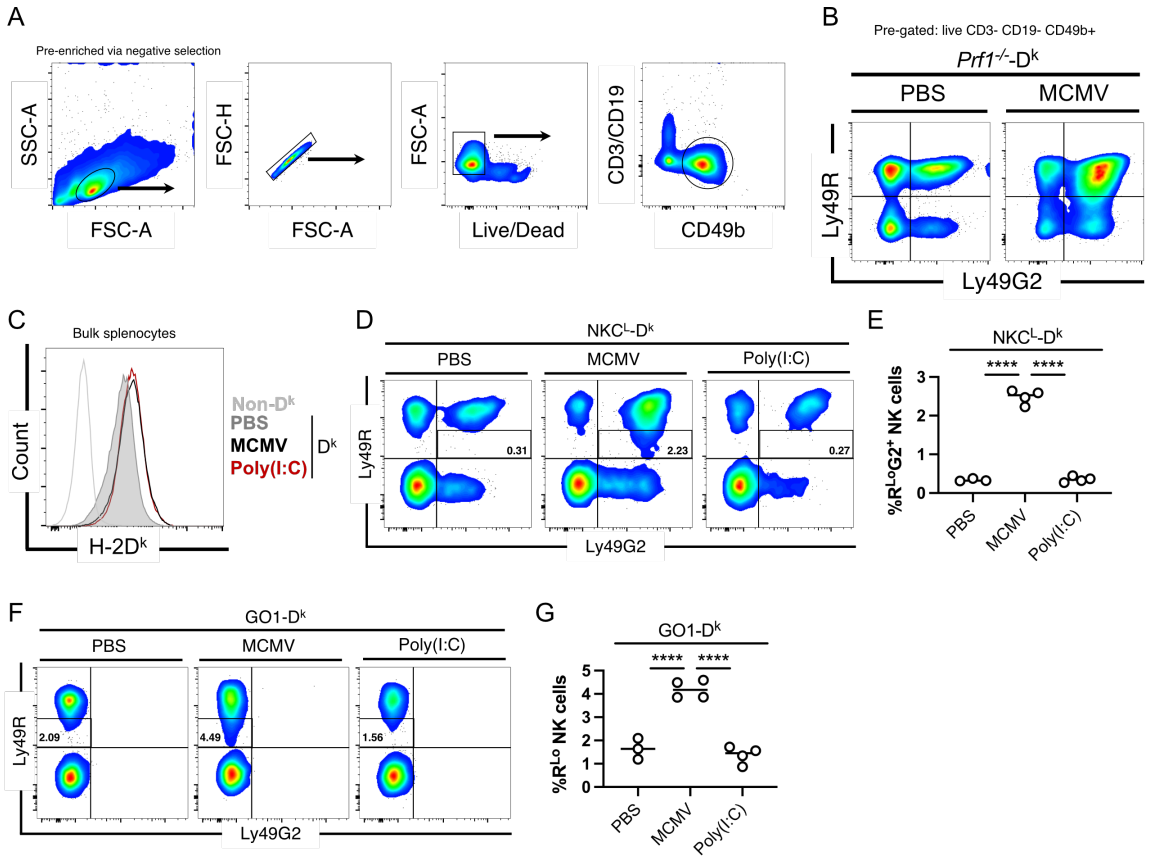


Figure 17. Inflammation-induced MHC I D^k upregulation is insufficient to drive the accumulation of $Ly49R^{Lo}$ NK cells.

Figure 17. Inflammation-induced MHC I D^k upregulation is insufficient to drive the accumulation of Ly49R^{Lo} NK cells. (A) Representative flow plots for pre-enriched splenic mouse NK cells from an uninfected mouse, illustrating the gating strategy for identification of CD49b$^+$ NK cells. (B) Representative Ly49R and Ly49G2 distribution profiles, gated as in (A), from 90 h PBS or 90 h MCMV injected (2.5×10^4 PFU) *Prf1*$^{-/-}$-D^k mice. Data are representative of six experiments. (C) Representative histograms of splenocyte MHC I D^k expression from 90 h PBS, 24 h 200 μg Poly(I:C), or 90 h MCMV injected (5×10^4 PFU) NKC^L-D^k or NKC^L mice. (D and E) Representative splenic NK cell Ly49R and Ly49G2 distribution profiles gated on Ly49R^{Lo}Ly49G2$^+$ subsets (D) and quantification (E) from 90 h PBS, 24 h Poly(I:C), or 90 h MCMV injected NKC^L-D^k mice. (F and G) Representative splenic NK cell Ly49R and Ly49G2 distribution profiles gated on Ly49R^{Lo} Ly49G2$^+$ subsets (F) and quantification (G) from 90 h PBS, 24 h Poly(I:C), or 90 h MCMV injected GO1-D^k mice. Data in A and B are representative of six experiments with three to four mice per group. Data in C-G are representative of two experiments with three to four mice per group. One-way ANOVA with post-hoc test was used for statistical analysis. Error bars indicate mean ± SD. ****$P <$ 0.0001.

Ly49R downregulation in response to MCMV-modified D^k marks proliferating antiviral effectors.

We next sought to elucidate whether Ly49R downregulation is associated with the activation or expansion of virus-responsive Ly49G2$^+$ NK cells. To test this, we transferred D^k-licensed R$^+$ G2$^+$ NK cells into congenically marked Ly49G2-deficient GO1-D^k host mice **(Figure 18A)**. Although donor NK cells homed to the spleens of PBS injected and MCMV-infected mice, we specifically observed proliferation and expansion of donor cells NK cells in response to MCMV infection **(Figures 18B and 18D)**. Intriguingly, whereas R$^+$ G2$^+$ NK cells transferred into PBS injected recipients maintained their Ly49R expression, we observed progressive Ly49R downregulation on Ly49G2$^+$ NK cells proliferating during MCMV infection **(Figures 18E-F)**. Notably, Ly49R downregulation coincided with upregulation of CD25 and KLRG1 on proliferating NK cells **(Figures 18G-H)**. These results showing Ly49R downregulation on proliferating antiviral Ly49G2$^+$ NK cells correspond with those demonstrating selective NK cell activation and maturation during MCMV infection.

We reasoned that Ly49R downregulation might result from NK cell sensing of MCMV-induced alterations in D^k molecules on infected targets. Alternatively, MCMV-induced inflammatory cytokines might facilitate increased host cell D^k expression and subsequent Ly49R downregulation. To address this, we compared the effect of Poly(I:C)- versus MCMV-induced increases in host cell D^k expression

on R^{+} $G2^{+}$ NK cells. We injected NKC^{L}-D^{k} mice with Poly(I:C) or MCMV and measured accumulation of R^{Lo} $G2^{+}$ NK cells. Whereas both treatments similarly increased cell surface D^{k} expression on splenic host cells **(Figure 17C)**, the accumulation of R^{Lo} $G2^{+}$ NK cells was specific to MCMV infection (**Figures 17D-E)**. Because Ly49G2 also binds D^{k}, we reasoned that the IR could have prevented Ly49R engagement of Poly(I:C)-induced D^{k} in NKC^{L}-D^{k} mice. However, Ly49G2-deficient GO1-D^{k} R^{Lo} NK cells likewise accumulated during MCMV infection, but not following Poly(I:C) treatment **(Figures 17F-G)**. Considering that Ly49R downregulation specifically occurred in response to D^{k}-bearing targets **(Figures 16D-F)**, these findings suggest that loss of Ly49R expression during infection results from its engagement of an MCMV-modified D^{k}-ligand, thereby giving rise to experienced R^{Lo} $G2^{+}$ antiviral NK effectors.

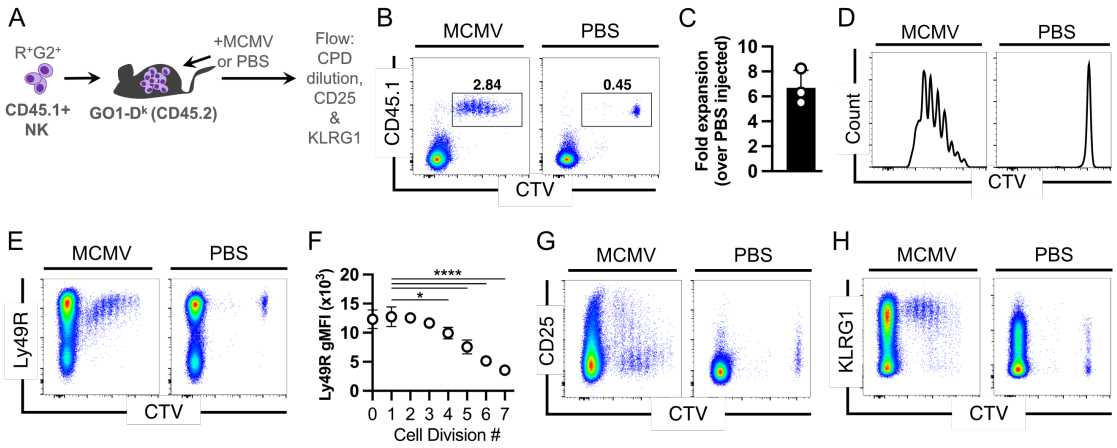


Figure 18. Ly49R downregulation during MCMV infection marks proliferating antiviral effectors.

Figure 18. Ly49R downregulation during MCMV infection marks proliferating antiviral effectors. (A and B) Schematic depicts adoptive transfer of congenically marked and CTV-labeled R^+ $G2^+$ NK cells (IL-15-expanded) from NKC^L-D^k donor mice into NKC^{GO1}-D^k recipients 24 h prior to MCMV infection (2.5×10^4 PFU) or PBS injection. (B) Flow plots show the percentage of enriched donor NK cells within the host spleen NK cell pool, 90 hpi. (C) Cumulative data for the fold expansion of donor NK cells. (D) Representative CTV dilution profiles for enriched donor NK cells. (E and F) Representative flow plots (E) and quantification (F) show Ly49R and CTV distribution profiles for enriched NK cells derived from recipient mice. (G and H) Representative flow plots show CD25 (G) or KLRG1 (H) and CTV distribution profiles for enriched NK cells derived from recipient mice. Data are representative of four experiments (3 mice per MCMV group). For statistical analysis, one-way ANOVA with post-hoc test was used in F. Error bars indicate mean ± SD. $*P < 0.05$, $****P < 0.0001$.

MCMV gp34 is required for infected cell recognition by Ly49R.

The MCMV immunoevasin gp34 has been shown to bind distinct polymorphic MHC I molecules (205) and can enhance cell surface D^k expression in MCMV-infected cells (129, 150). To test whether gp34-D^k complexes might represent an MCMV-modified D^k ligand of Ly49R, we generated a CRISPR mutant MCMV strain deficient for *m04*/gp34 **(Figures 19A-B)**. Notably, WT MCMV and Δ*m04* MCMV strains exhibited similar rates of *in vitro* replication and virion release by M2-10B4 cells **(Figure 19C)**. We further evaluated cell surface D^k and gp34 expression in MCMV-infected (MOI 0.5) WT or D^k-deficient M2-10B4 cells **(Figure 19D)**. As expected, Δ*m04* MCMV-infected M2-10B4 lacked cell surface gp34 expression. Likewise, we did not observe expression of gp34/D^k complexes in H-2^k SVEC4-10 cells infected with Δ*m04* MCMV (MOI 0.1) **(Figure 19E)**. Whereas we found a direct correspondence between MCMV IE1 and gp34-D^k expression in SVEC4-10 infected with WT virus, IE1 expression in Δ*m04*-infected cells coincided with loss of surface D^k expression **(Figure 19E)**.

To determine whether gp34 facilitated Ly49R stimulation, we engineered a Jurkat reporter cell line (J7) to express chimeric CD3ζ-R receptors **(Figure 20A)**. We then analyzed the ability of SVEC4-10 cells pre-treated with IFN-β or infected with WT or Δ*m04* MCMV to stimulate CD3ζ-R reporters. WT MCMV-infected targets consistently triggered CD3ζ-R reporters, whereas Δ*m04* MCMV-infected targets did not **(Figure 20B)**. A key difference, cell surface gp34-D^k complexes

were prevalent on the WT MCMV-infected targets **(Figure 20C)**. These data demonstrate that Ly49R recognition of MCMV-infected targets is facilitated by gp34. Taken together, our findings suggest that MCMV gp34 enables Ly49R to recognize virus-modified D^k molecules on MCMV-infected cells, which can lead to selective expansion of antiviral R^{Lo} $G2^+$ NK cells during MCMV infection.

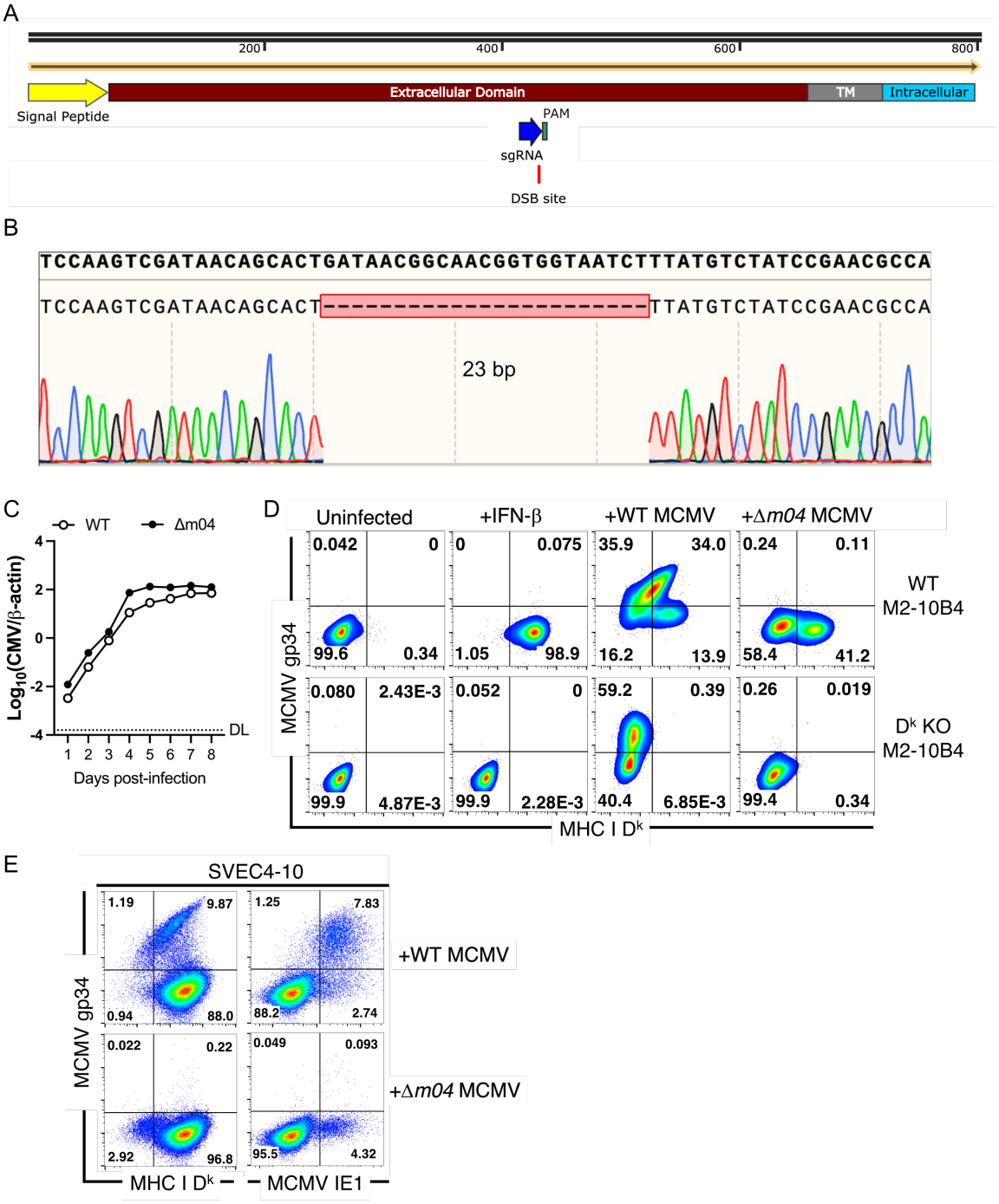


Figure 19. CRISPR/Cas9 editing and characterization of Δ*m04* MCMV.

Figure 19. CRISPR/Cas9 editing and characterization of Δ*m04* MCMV. (A) Diagram of CRISPR/Cas9-mediated editing of MCMV *m04*. Coding sequence (CDS) fragments predicted to encode the signal peptide, extracellular, transmembrane, and intracellular protein domains are indicated, along with the anticipated sgRNA double-stand break (DSB) site and the sgRNA protospacer adjacent motif (PAM). (B) Viral DNA was isolated from M2-10B4 cells infected with WT or Δ*m04* CRISPR-Cas9 modified MCMV used in these studies. The CRISPR target site in the m04 ORF was sequenced to assess on-target sequence variants. Primers used for PCR and subsequent sequence analysis are described in the Materials and Methods section. Variant nucleotides are identified in red boxes. (C) Multi-step *in vitro* growth kinetics of WT vs Δ*m04* MCMV. M2-10B4 cells and viral supernatants were harvested at indicated timepoints and quantified by PCR. (D) Representative surface MHC I D^k and MCMV gp34 distribution profiles on WT or D^k-deficient M2-10B4 cells treated with IFN-β for 24 h, left uninfected, or infected with 0.5 PFU/cell of WT or Δ*m04* TC-passaged MCMV for 24 h. (E) Representative surface MHC I D^k and MCMV gp34, or MCMV IE1 and MCMV gp34 distribution profiles on SVEC4-10 cells infected with 0.1 PFU/cell of WT or Δ*m04* TC-passaged MCMV for 24 h. Data are representative of three experiments.

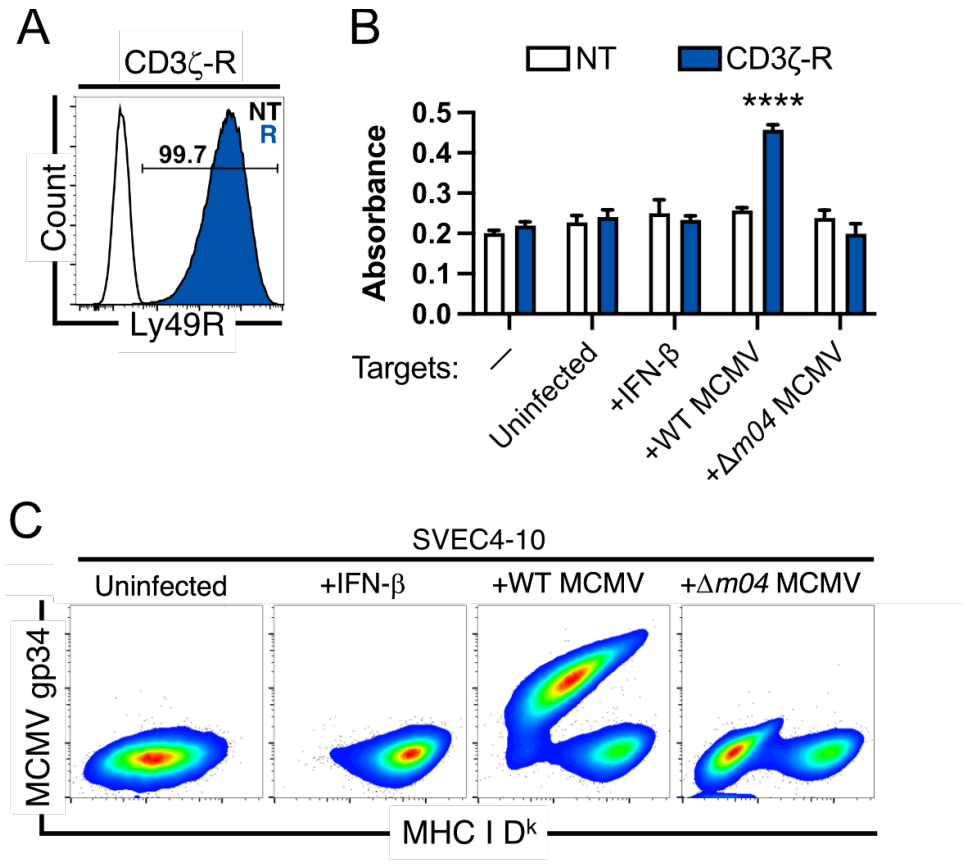


Figure 20. MCMV gp34 is required for infected cell recognition by Ly49R.

Figure 20. MCMV gp34 is required for infected cell recognition by Ly49R. (A) Representative histogram of CD3ζ-Ly49R expression on transduced J7 reporter cells. (B) SVEC4-10 cells were left uninfected, pre-treated with 1000 U/ml IFN-β, or infected with 25 PFU/cell of the indicated TC-passaged MCMV strains for 24 h. Targets and non-transduced (NT) or CD3ζ-Ly49R reporters were co-cultured for 8 h prior to analysis of LacZ activity. (C) Representative flow plots show MCMV gp34 and D^k expression on SVEC4-10 cells treated as indicated in (B). Data are representative of three experiments. For statistical analysis, unpaired two-tailed t test was used. Error bars indicate mean ± SD. ****$P < 0.0001$.

Ly49G2^{+} antiviral NK cells require both Ly49R and Ly49G2 for proliferation and expansion during MCMV infection.

We hypothesized that Ly49R is required for mature licensed NK cell virus sensing *in vivo*. To test this, we ablated Ly49R's expression in IL-15-expanded primary NK cells using *Klra18/Ly49r*-specific CRISPR/Cas9 ribonucleoprotein (cRNP) complexes and then transferred a 1:1 mix of sorted WT R^{+} G2^{+} (CFSE-labeled) and mutant R^{null} G2^{+} (CTV-labeled) NK cells into Ly49G2-deficient GO1-D^{k} mice prior to MCMV infection **(Figures 21A-B)**. Unexpectedly we found that donor R^{null} G2^{+} NK cell proliferation and CD25 upregulation was comparable to that of donor WT R^{+} G2^{+} NK cells during MCMV infection **(Figures 21C-E)**. However, R^{+} G2^{+} NK cells exhibited greater representation by percentage in the spleen following infection **(Figures 21C and 21F)**. Thus, although Ly49R is not explicitly required to expand IL-15-primed antiviral Ly49G2^{+} NK cells during MCMV infection, Ly49R signaling is nonetheless essential for optimal accumulation of Ly49G2^{+} antiviral effectors.

Ly49R^{+} NK cells lacking endogenous Ly49G2, or those from Ly49G2-deficient GO1-D^{k} mice, exhibit severe defects in NK cell proliferation during MCMV infection (11). However, it is unknown whether Ly49G2's essential role is limited to NK cell development, or if it dynamically affects Ly49R^{+} NK cell effector functions. Thus, we ablated Ly49G2 from primary Ly49R^{+} NK cells using *Klra7/Ly49g2*-specific cRNP complexes to assess its role in antiviral NK cells

during MCMV infection **(Figures 22A-B)**. Whereas both sorted subsets of Ly49R^{+} NK cells exhibited progressive downregulation of Ly49R coinciding with NK cell division during infection, mutant G2^{null} NK cells displayed significantly less accumulation in the spleen and impaired proliferation in comparison to WT G2^{+} NK cells **(Figures 22C-G)**, possibly stemming from less effective CD25 upregulation during cell division **(Figure 22H)**. Antiviral NK cells thus require sustained Ly49G2 expression to efficiently accumulate during MCMV infection. We infer that direct recognition of MCMV-modified D^{k} ligands by Ly49R facilitates the expansion of R^{Lo} G2^{+} effectors with enhanced antiviral activity.

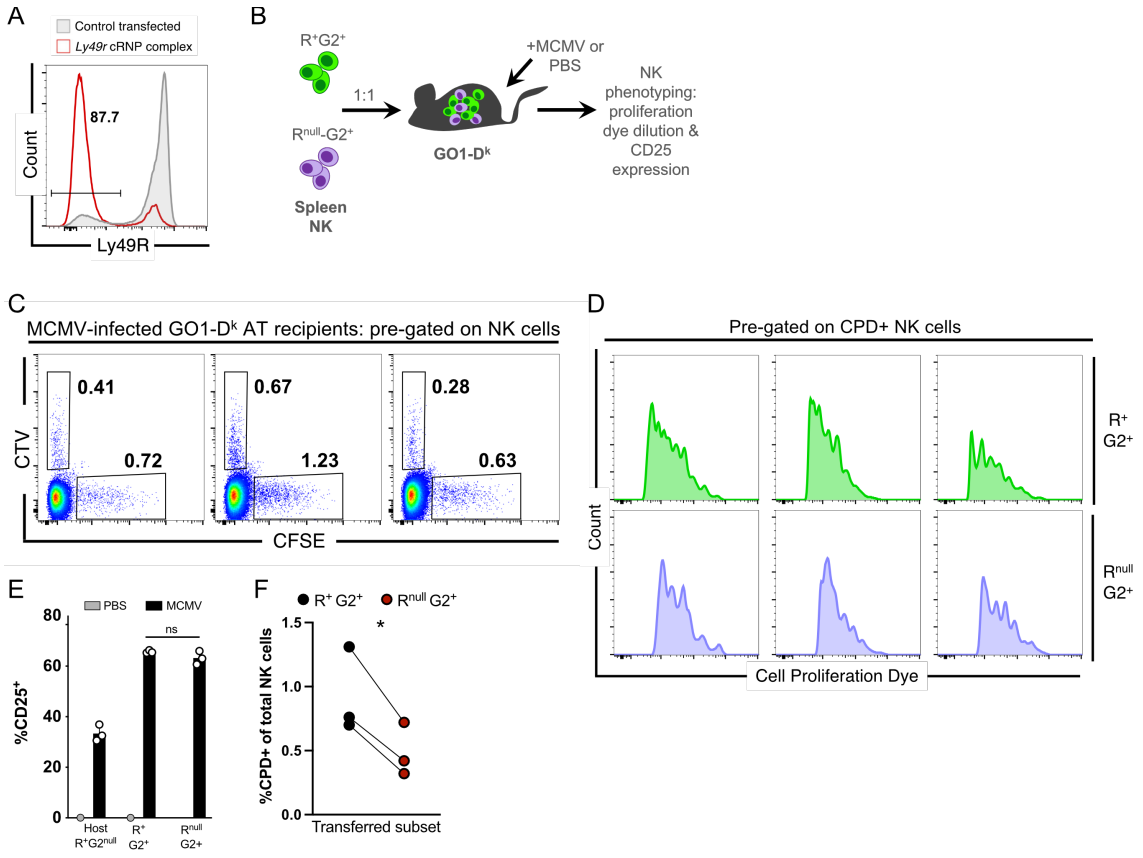


Figure 21. Ly49R signaling enhances the accumulation of IL-15-stimulated antiviral Ly49G2+ NK cells.

Figure 21. Ly49R signaling enhances the accumulation of IL-15-stimulated antiviral Ly49G2^{+} NK cells. (A) Representative histograms and frequency of Ly49R expression 5 d after electroporation of 2.5 x 10^5 IL-15-preactivated purified splenic NK cells with *Klra18* cRNP complexes compared to controls electroporated in the presence of Cas9 protein alone. (B) Schematic illustrating that splenic NK cells derived from NKC^{L}-D^{k} donor mice were negatively enriched, preactivated and transfected as described in (A), and subsequently labelled with CFSE or CTV prior to adoptive transfer at a 1:1 ratio into NKC^{GO1}-D^{k} recipients 24 h prior to MCMV infection (2.5 × 10^4 PFU) or injection with PBS. (C) Flow plots show the percentage of CFSE^{+} or CTV^{+} enriched donor NK cells within the splenic NK cell pool. (D) CFSE and CTV dilution profiles of enriched donor NK cells from the spleens of 90 h MCMV infected NKC^{GO1}-D^{k} mice. (E) Quantification of CD25^{+} enriched donor NK cells from the spleens of 90 h MCMV infected or PBS injected NKC^{GO1}-D^{k} mice. (F) Quantification of the percentage of cell proliferation dye (CPD)$^{+}$ enriched donor NK cells from the spleens of 90 h MCMV infected NKC^{GO1}-D^{k} mice. Each pair of symbols represents CPD^{+} NK cells derived from the same recipient mouse. For statistical analysis, one-way ANOVA with post-hoc test was used in E and paired two-tailed t test was used in F. Data are representative of two experiments with three mice per group. Error bars indicate mean ± SD. $*P < 0.05$.

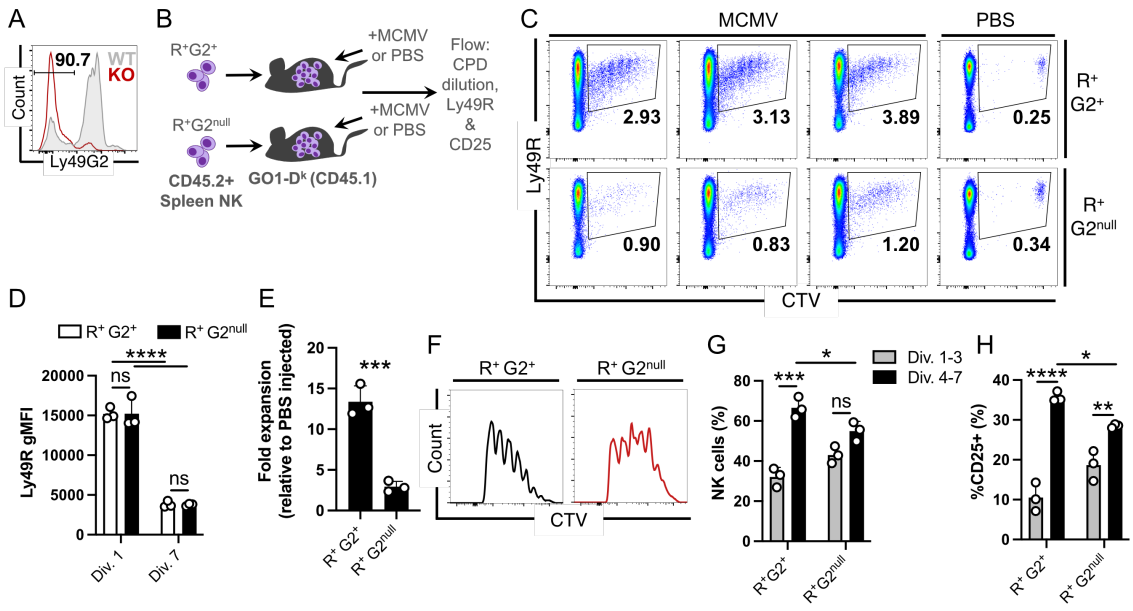


Figure 22. Ly49G2 promotes proliferation and accumulation of CD25+ Ly49R^{dim} NK cells during MCMV infection.

Figure 22. Ly49G2 promotes proliferation and accumulation of CD25^{+} Ly49R^{dim} NK cells during MCMV infection. (A) Shown are representative histograms of Ly49G2 expression (5 d post-electroporation) in IL-15-activated splenic NK cells transfected with *Ly49g2/Klra7* cRNP complexes (KO) or Cas9 protein only (WT). (B) A schematic diagram for adoptive transfer of congenically marked donor NKC^{L}-D^{k} Ly49R^{+} G2-disparate NK cells (transfected as described in (A)) into NKC^{GO1}-D^{k} recipients prior to PBS injection or MCMV infection (2.5 × 10^{4} PFU). CPD, cell proliferation dye. (C) Flow plots show the percentage of Ly49R^{+} CTV^{+} enriched donor NK cells within the splenic NK cell pool. (D) Ly49R gMFI values are shown at division 1 vs division 7 for donor Ly49G2-disparate R^{+} NK cells identified in (C). (E-H) The fold expansion (E), CTV dilution profiles (F), cumulative of NK cell frequency by divisions (1-3 v 4-7) (G), and cumulative CD25^{+} NK cell frequency by divisions (1-3 v 4-7) (H) is shown for adoptively transferred Ly49R^{+} G2-disparate NK cells during MCMV infection. Data are representative of two experiments (3 mice per MCMV group). For statistical analysis, unpaired two-tailed t test was used in D and E, and two-way ANOVA with post-hoc test in G and H. Error bars indicate mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Ly49G2 is required to counter MCMV gp34-mediated immune evasion.

Previous studies have shown that MHC I-gp34 complexes can engage Ly49 IRs to facilitate MCMV evasion of licensed NK cell antiviral immunity (129, 150). Nonetheless, Ly49G2 is critical for cytotoxic control of MCMV in NKC^{L}-D^{k} mice **(Figure 16A)**. We thus questioned whether the Ly49G2^{L} receptor also binds gp34-D^{k} complexes. To test this, we generated J7 reporter cells expressing chimeric CD3z-G2 receptors **(Figure 23A)**, and evaluated whether they can be stimulated by SVEC4-10 cells pre-treated with IFN-β or infected with WT or Δ*m04* MCMV. Whereas IFN-β treated or WT MCMV-infected targets readily stimulated CD3ζ-G2 reporters, Δ*m04* MCMV-infected targets were much less effective **(Figure 23B)**. Remarkably, although WT MCMV-infected cells exhibited significantly lower cell surface D^{k} expression relative to IFN-β treated cells **(Figure 23C)**, CD3ζ-G2 reporters were equivalently stimulated by both targets. MCMV gp34-D^{k} complexes thus represent a bona fide ligand of the Ly49G2^{L} IR.

Ligand binding can promote IR clustering and ITIM phosphorylation, driving recruitment and activation of SH2 domain-containing phosphatases (8). We thus tested whether Ly49G2 inhibits Ly49R signaling in CD3ζ-R reporter cells engineered to express doxycycline-inducible G2^{WT} or ITIM-mutant G2^{Y8F} receptors **(Figure 24A)**. Whereas Ly49G2 crosslinking impeded Ly49R stimulation in dox-induced CD3ζ-R.G2^{WT} reporter cells **(Figure 24B)**, it had no effect on CD3ζ-R.G2^{Y8F} reporters **(Figure 24C)**. Likewise, induction of G2^{WT} receptors inhibited

Ly49R engagement of gp34-D^k complexes on MCMV-infected SVEC4-10 cells, in contrast to $G2^{Y8F}$ receptors **(Figures 24D-F)**. These results demonstrate that Ly49G2 is functionally competent to inhibit Ly49R signaling.

We then evaluated gp34's role in D^k- and Ly49G2-dependent NK cell antiviral immunity. Whereas MCMV gp34 did not significantly alter viral burden in NKC^L-D^k, *Prf*$^{-/-}$-D^k, or NKC^L mice **(Figure 23D)**, GO1-D^k mice were selectively susceptible to gp34-expressing MCMV **(Figure 23D)**. These data thus demonstrate that MCMV gp34 facilitates immune evasion in the absence of Ly49G2-mediated D^k sensing. Nonetheless, higher Δ*m04* virus levels in GO1-D^k than NKC^L-D^k spleen tissues further establish a gp34-indpendent role of Ly49G2 in MCMV control **(Figure 23D)**. Indeed, we observed striking Ly49G2- and D^k-dependent accumulation of NK cells in response to WT or gp34-deficient MCMV strains **(Figure 23E-F)**. Ly49G2-mediated self MHC I sensing is thus a focal point of antiviral NK cell immunity in this model system.

We reasoned that Ly49G2 inhibitory signals might protect $Ly49R^+$ NK cells from disarming in response to gp34-D^k molecules displayed by MCMV-infected targets. Indeed, mature NK cells can decrease their responsiveness to activating receptor stimulation within days of transfer into MHC I-deficient hosts (86), presumably due to lack of engagement of self-specific IRs. We thus sought to elucidate whether MCMV gp34 disarms $Ly49R^+$ NK cells in the absence of Ly49G2. To test this, we blocked Ly49R in NKC^L-D^k or Ly49G2-deficient GO1-D^k mice by administering the Ly49R-specific 12A8 mAb. Whereas this treatment fully

abrogated MCMV resistance in NKC^{L}-D^{k} mice, it did not result in higher GO1-D^{k} spleen virus titers **(Figure 23F)**. Thus, despite its essential role in D^{k}-dependent antiviral immunity (11), Ly49R signaling is ineffectual in mice lacking the Ly49G2 inhibitory receptor.

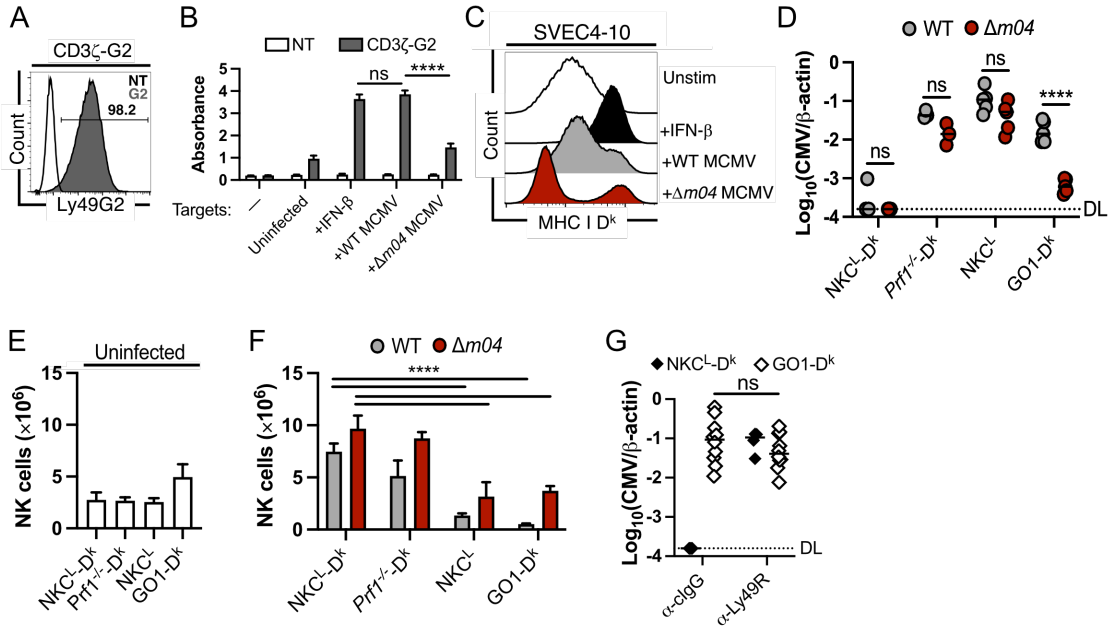


Figure 23. Ly49G2 is required to counter MCMV gp34-mediated immune evasion.

Figure 23. Ly49G2 is required to counter MCMV gp34-mediated immune evasion. (A) Representative histogram of CD3ζ-Ly49G2 expression on transduced J7 reporter cells. (B) SVEC4-10 cells were left uninfected, pre-treated with 1000 U/ml IFN-β, or infected with 25 PFU/cell of the indicated MCMV strains for 24 h prior to coculture for 8 h with reporter cells. (C) Representative histograms plots show MHC I D^k expression on SVEC4-10 cells treated as described in (B). (D) Spleen virus levels (90 hpi) for the indicated mice infected with TC-passaged WT or Δ*m04* MCMV (1×10^7 PFU). DL, detection limit. (E and F) Total spleen NK cell numbers from uninfected mice of the indicated genotype, or mice infected as in (D). (G) Spleen virus levels (90 hpi) from infected (2×10^5 PFU WT MCMV) NKC^L-D^k or GO1-D^k mice pre-treated with control IgG (cIgG) or an anti-Ly49R blocking mAb (12A8) prior to infection. DL, detection limit. Data (A-C) are representative of two experiments. Data in D-F are representative of two experiments with 3-5 mice per group. Data in G are combined results of two experiments with 2-5 mice per group. For statistical analysis, one-way ANOVA with post-hoc test was used in B, and unpaired two-tailed t test was used in D, F, and G. Error bars indicate mean ± SD. $^*P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

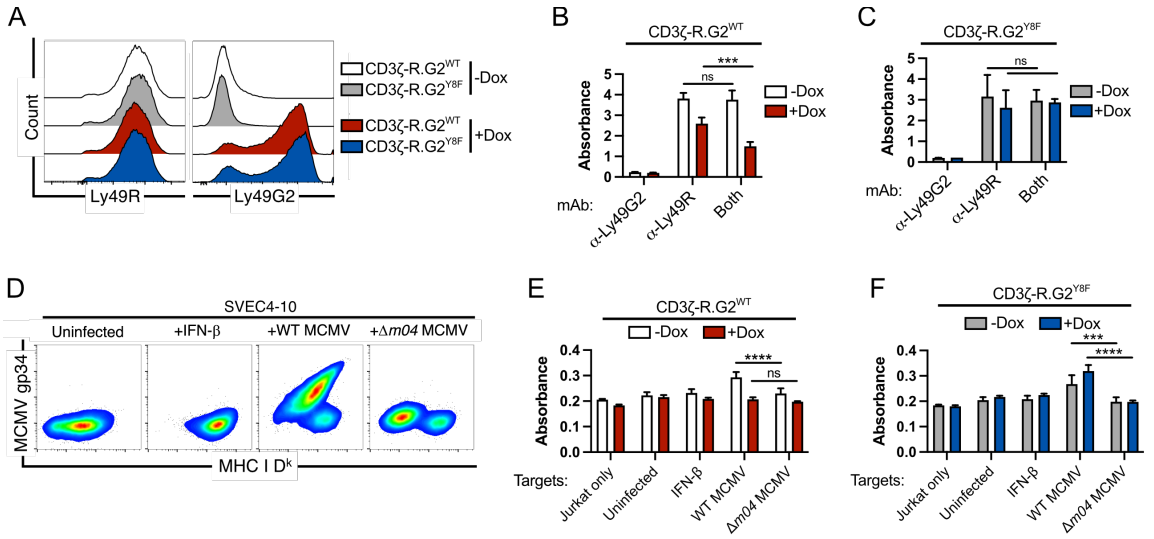


Figure 24. Ly49G2 is functionally competent to inhibit Ly49R.

Figure 24. Ly49G2 is functionally competent to inhibit Ly49R. (A) Representative histograms show CD3ζ-Ly49R, Ly49G2^{WT}, or Ly49G2^{Y8F} expression on transduced CD3ζ-Ly49R J7 reporter cells cultured for 24 h +/- doxycycline. (B, C) Reporter cell lines were stimulated with respective platebound mAbs for 8 h prior to analysis of LacZ activity. (D) Representative flow plots show MCMV gp34 and D^k expression on SVEC4-10 cells left uninfected, treated with 1000 U/ml IFN-β, or infected with 25 PFU/cell WT or Δ*m04* TC-passaged MCMV for 20 h as indicated. (E, F) SVEC4-10 cells were left uninfected, pre-treated with 1000 U/ml IFN-β, or infected with 25 PFU/cell of the indicated TC-passaged MCMV strains for 24 h. Targets and reporters were co-cultured for 8 h prior to analysis. Data are representative of two experiments. For statistical analysis, unpaired two-tailed t test was used. Error bars indicate mean ± SD. ***$P < 0.001$, ****$P < 0.0001$.

Ly49G2 licensing of cytokine responsiveness and Ly49R together enhance CD25 upregulation in antiviral NK cells during MCMV infection.

Primary NK cells cultured in IL-12 and antiviral NK cells responding to MCMV infection require IL-12R/STAT4 signaling to efficiently upregulate CD25 (69). We reasoned therefore that a selective increase in CD25 expression in R^+ $G2^+$ antiviral NK cells during MCMV infection might be related to enhanced sensitivity to IL-12R signaling. To test this, we enriched splenic NK cells from D^k- or Ly49G2-disparate mice and stimulated them with different cytokine conditions. IL-12 stimulation resulted in selective increases in CD25 expression and phosphorylated STAT4 (p-STAT4) in R^+ $G2^+$ antiviral NK cells from mice with D^k and Ly49G2 **(Figures 25A-B)**. Conversely, CD25 expression and p-STAT4 were substantially lower in NK cells from mice lacking either D^k or Ly49G2 **(Figures 25A-B)**. These data suggest D^k-licensing of Ly49G2$^+$ NK cells can enhance activation downstream of the IL-12R, thereby promoting CD25 upregulation of R^+ $G2^+$ antiviral NK cells during infection. To this end, we co-cultured NKC^L-D^k splenocytes with control IFN-β-treated or MCMV-infected M2-10B4 cells and evaluated CD25 expression on R^+ $G2^+$ vs R^+ $G2^-$ NK cells. Although both subsets of NK cells specifically increased CD25 expression in response to MCMV-infected targets, R^+ $G2^+$ NK cells had significantly higher CD25 expression in comparison to their R^+ $G2^-$ counterparts **(Figure 25C)**. Thus Ly49G2's licensing effect on IL-

12R signaling and subsequent CD25 upregulation may confer a proliferative advantage to R^+ $G2^+$ NK cells responding to MCMV infection.

Our findings in *Prf1*$^{-/-}$-D^k mice raised the possibility that Ly49R engagement of MCMV target ligands may promote CD25 upregulation in R^{Lo} $G2^+$ NK cells. To pursue this, we blocked Ly49R in NKC^L-D^k mice using the Ly49R-specific 12A8 mAb. Ly49R-blocked $G2^+$ NK cells from MCMV-infected mice were detected using the anti-Ly49ROV mAb (4E5) as described **(Figure 25D)** (11, 155). ROV^{Lo} $G2^+$ NK cells from Ly49R-blocked mice failed to differentially acquire increased CD25 or KLRG1^{Hi} expression in comparison to cIgG-treated mice **(Figures 25E-F)**. Furthermore, when gating on $CD25^+$ subsets, Ly49R-blocked ROV^+ $G2^+$ NK cells exhibited significantly lower CD25 MFI **(Figures 25G-H)**. Thus, endogenous antiviral R^+ $G2^+$ NK cells rely on Ly49R activating receptor signaling to increase CD25 upregulation, maturation, and survival during MCMV infection.

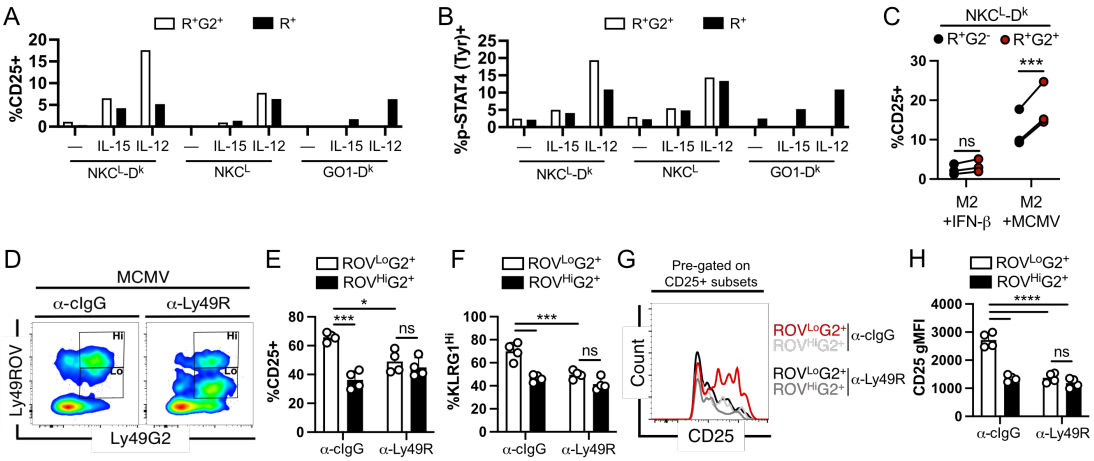


Figure 25. Ly49G2 licensing of cytokine responsiveness and Ly49R together enhance CD25 upregulation in antiviral NK cells during MCMV infection.

Figure 25. Ly49G2 licensing of cytokine responsiveness and Ly49R together enhance CD25 upregulation in antiviral NK cells during MCMV infection. (A and B) Purified splenic NK cells from the indicated mouse strains were cultured in media alone or respective cytokines for 24 h. The frequencies of $CD25^+$ (A) or p-STAT4 $(Tyr)^+$ (B) R^+ $G2^+$ or $Ly49R^+$ NK cells are shown. (C) IFN-β pre-activated (60 min) NKC^L-D^k splenocytes were co-cultured with IFN-β-treated or infected (TC-passaged MCMV, MOI 1.5) M2-10B4 cells for 24 h. Paired symbols represent flow-gated NK cells from a single mouse. (D-H) Representative flow plots (D) show gated Ly49ROV $Ly49G2^+$ expression on NKC^L-D^k splenic NK cells from infected (2×10^5 PFU) mice pre-treated with control IgG (cIgG) or an anti-Ly49R blocking mAb (12A8) prior to infection. Cumulative data for the frequency of $CD25^+$ (E) or $KLRG1^{Hi}$ (F) Ly49ROV expression-disparate $Ly49G2^+$ NK cells from infected mice are shown. Representative histograms (G) and cumulative CD25 expression data (H) for $CD25^+$ NK cells identified in (D) are shown. Each bar in A and B represents pooled enriched NK cells from three mice and data are representative of four experiments. Data in C-H are representative of two experiments (4 mice per group). For statistical analysis, paired two-tailed t test was used in C, and two-way ANOVA with post-hoc test in E, F and H. Error bars indicate mean ± SD. $*P<0.05$, $***P<0.001$, $****P<0.0001$.

Discussion

Herein we provide mechanistic insight into how recognition of self-MHC I by functionally disparate NK cell receptors regulates the responsiveness of antiviral NK cells responding to MCMV infection. Prior work has shown that select Ly49 activating and inhibitory receptors bind MCMV gp34-associated MHC I complexes *in vitro* (129, 150, 154, 167), suggesting that altered-self ligands might contribute to NK cell detection of MCMV *in vivo*. Indeed, we found that the Ly49R activating receptor selectively binds to altered-self gp34-D^k complexes at the surface of MCMV-infected target cells, but not IFN-β-induced D^k molecules. Ly49R thus may be sensitive to conformational changes in D^k molecules induced by gp34, or another MCMV immunoevasin such as MATp1 (150), or possibly an MCMV peptide. By contrast, we found that the C57L allele of Ly49G2 binds both self and altered-self D^k molecules. Inasmuch as neither $Ly49R^L$ nor $Ly49G2^L$ possess putative amino acid residues (R/M^{223}, L^{234}, and N^{244}) thought required for binding gp34-MHC I complexes (167), these receptors may contact gp34 differently than other Ly49 receptors (e.g. Ly49P, Ly49D2, or Ly49L). These findings further suggest that coincident recognition of altered-self by co-expressed, "paired" activating and inhibitory receptors synergistically enhances NK cell-mediated virus sensing.

MCMV gp34-MHC I engagement of self-MHC I licensing IRs is posited to interfere with NK cell immunity to MCMV by facilitating evasion of the NK cell

missing-self response (129, 150). Remarkably, we show that gp34 does not impede R^+ $G2^+$ antiviral NK cells from controlling MCMV infection. Rather, our findings suggest that altered-self gp34-D^k sensing by Ly49G2 is necessary for Ly49R^+ NK cell immunity to MCMV. Ly49G2 is required to prevent gp34-mediated immune evasion, as evidenced by selective gp34-dependent MCMV spread in the Ly49G2-deficient GO1-D^k mice. Preferential control of Δ*m04* MCMV in GO1-D^k animals hints that another missing-self IR may have a role in D^k-dependent antiviral immunity. Alternatively, gp34 may be able to disarm NK cells by tolerizing signaling through gp34-D^k specific activating receptors like Ly49R or Ly49P. Nonetheless, our data demonstrate that altered-self complexes facilitate evasion of these recognition systems. Paired altered-self detection via signaling-disparate MHC I recognition receptors thus may underpin MCMV sensing via R^+ $G2^+$ antiviral NK cells and increased effector functionality, including proliferation, expansion, and activation during infection.

Inasmuch as prior studies indicate that Ly49 IRs facilitate missing-self recognition of MCMV (129, 150, 202), our findings suggest that R^+ $G2^+$ NK cell MCMV sensing is mechanistically distinct. Neutralization of Ly49R via blocking antibodies revealed its role in driving Ly49$G2^+$ NK cell antiviral immunity (11), yet Ly49R is ineffectual in this context without co-expression of the Ly49G2 IR. In contrast, transgenic expression of Ly49H is sufficient for MHC I-independent MCMV resistance (152). These distinct requirements for antiviral immunity might suggest a difference in the magnitude or quality of activating signals propagated

by the DAP12 signaling adaptor. Whereas Ly49H has a relatively high affinity for m157 (206), Ly49R reporter cells are modestly stimulated by gp34-D^k complexes on MCMV-infected cells. Because lower, albeit not significantly so, spleen viral titers were observed in Ly49R-blocked GO1-D^k mice in comparison to control IgG-treated controls, our data might suggest that ongoing interaction with altered-self ligands resulting in sustained Ly49R – DAP12 signaling in the absence of Ly49G2 may serve to disarm NK cells during MCMV infection. This interpretation is in line with previous studies showing that active engagement of MHC I molecules by self-specific IRs is essential to maintain NK cell reactivity towards target cells with altered MHC I expression levels (86, 185). Moreover, sustained signaling through select DAP10/12-dependent receptors has been shown to render NK cells broadly hyporesponsive to activating receptor stimulation (207–209). Thus, Ly49G2 inhibitory signals may engender NK cell reactivity during MCMV infection by preventing tolerance induction via virus-specific activating receptors. Alternatively, active *cis*-engaged Ly49G2 receptors in licensed NK cells may sequester SH2-domain containing phosphatases away from Ly49R activating receptor signaling substrates (210), thus further amplifying Ly49R signaling output. Regardless of the mechanism, we infer that Ly49G2 augments $Ly49R^+$ NK cell recognition of viral infection and antiviral immunity via altered-self D^k sensing.

Given its importance in driving D^k-dependent antiviral immunity, it was surprising that Ly49R ablation had little, if any, effect on MCMV-induced proliferation of IL-15-activated primary $Ly49G2^+$ NK cells. Notably, IL-15 primes

the PI3K/mTOR pathway in NK cells, resulting in enhanced signaling downstream of cytokine receptors and enhanced cytokine production following activating receptor stimulation (211). IL-15 treatment might render Ly49G2^{+} NK cells more sensitive to stimulatory signals via other ITAM-linked activating receptors or cytokine receptors such that Ly49R is not explicitly required to induce expansion of cytokine-primed Ly49G2^{+} NK cells. Indeed, high-dose IL-15 priming can rewire NK cell metabolism such that NK cell activation is no longer dependent on glucose-driven oxidative phosphorylation (212). Nonetheless, we consistently observed that R^{null} G2^{+} NK cells were underrepresented in comparison to their WT counterparts after several days of infection which suggests Ly49R signaling may be needed to support NK cell survival during MCMV infection. Conversely, ablation of Ly49G2 in IL-15-activated Ly49R^{+} NK cells revealed its intrinsic role in enhancing proliferation. These data further demonstrate that cytokine priming is insufficient to compensate for Ly49G2-deficiency. We conclude that IR-mediated licensing of NK cell functionality is essential in MHC I-dependent antiviral immunity mediated by NK cells.

We provide evidence that Ly49G2 and Ly49R signaling contribute to selective upregulation of CD25 on licensed NK cells responding to MCMV infection. Our data further suggest that Ly49G2 drives CD25 upregulation in response to IL-12 by promoting STAT4 phosphorylation. Although self-specific IR ITIM signals have previously been shown to enhance NK cell stimulation downstream of activating receptor ligation (89), our study implies an additional role

for licensing receptors in amplification of cytokine receptor signaling. The PI3K-AKT-mTOR pathway is critical for priming of NK cells with cytokine (211), and SHP-1 enhances the basal mTOR reactivity of licensed NK cells (75). Ly49G2 may amplify licensed NK cell responses to cytokine receptor signaling by modulating the abundance, localization, or phosphorylation status of downstream phosphatases (92, 142, 210). The resulting differential sensitivity to IL-2, IL-12, or IL-15 cytokines may enable rapid accumulation of $R^+ G2^+$ antiviral effectors during infection, which is supported by recent studies showing that IL-2 and IL-15 non-redundantly drive proliferation or survival of MCMV-specific $Ly49H^+$ cells (70).

A hallmark of this MCMV detection mechanism is scrutinization of a shared self MHC I molecule by functionally disparate paired MHC I recognition receptors. We predict that such paired recognition systems may be a conserved feature of NK cell immunity to viral infection. Indeed, CMV employs several MHC I mimics or MHC I-binding immunoevasins to evade cytolysis by NK cells, including MCMV m157, m04, m12, and HCMV UL40 (141). Notably, each of these proteins has been shown to exhibit high mutability (213–218), and both activating and ITIM-bearing NK cell receptors have been implicated in direct recognition of these molecules displayed by virally infected cells (10, 129, 157, 167, 217, 219, 220). It follows that a combined effect of polymorphism in the viral genome, in the altered-self ligands, and in the signaling disparate self-receptors themselves likely dictates the extent to which a given receptor pair is beneficial or suppressive to the antiviral NK cell response (195). We propose that efficient altered-self recognition via

activating receptors and licensing IRs is critical for paired-self receptor-mediated NK cell sensing of viral infection. Further study thus is warranted to understand the contexts in which paired-self receptor recognition can be harnessed to improve disease outcomes.

Chapter IV

Conclusions and Future Directions

Introduction

Nearly two decades ago, researchers described IR/MHC I-dependent licensing of NK cell reactivity against missing-self tumor targets (72–74). Although numerous studies in human (12–19, 107, 108, 110–115, 122) and mouse (11, 20–23, 44, 125–128, 138, 139) have implicated licensed NK cells in viral resistance, others have shown that licensing receptors instead impede antiviral immunity in certain contexts (130, 133). Thus, our understanding of the role of self-MHC I-specific IRs in regulating licensed NK cell reactivity during viral infection is incomplete.

In prior work, we linked an MHC I-binding activating NKR with licensed NK cell-mediated virus control (11). However, a basis for viral detection by the activating NKR was undefined. Furthermore, while a role of self-IRs in detection of missing-self MHC I targets is well established (8), whether this recognition mechanism contributes to licensed NK cell-mediated virus control *in vivo* has remained unclear. Indeed, many viruses have evolved intricate strategies to evade missing-self detection by NK cell IRs (129, 141, 150).

This study addressed the functional significance of self-IRs in virus control and evaluated the contribution of a well-known viral immunoevasin to recognition of infected targets by functionally disparate NKRs sharing an MHC I ligand. We discovered that self-IRs are vital for host defense, and that paired sensing of altered-self MHC I is an important feature of licensed NK cell-mediated antiviral

immunity. Further study thus is warranted to understand the basis for synergistic enhancement of viral detection by paired activating and inhibitory NKRs.

Ly49G2 is vital for MHC I D^k-dependent MCMV resistance

In this work, we have uncovered the functional significance of the $Ly49G2^{C57L}$ IR in D^k-dependent MCMV control. Using CRISPR/Cas9, we were the first to demonstrate specific disruption of a single polymorphic *Ly49* receptor gene. Considering that Ly49G2 is >95% identical to other $Ly49^{C57L}$ genes with respect to nucleic acid content, and there >10 *Ly49* genes within the C57L-derived NKC **(Table 1)**, our study has highlighted the remarkable utility and specificity of CRISPR/Cas9 for gene-editing. Using the resultant Ly49G2-deficient mice, we have discovered a protective role for the IR in NK cell-mediated MCMV resistance. Inasmuch as 1) licensing receptors were previously shown to impede MCMV control in B6 mice (130), and 2) $Ly49G2^+$ NK cells are hypothesized to exhibit MHC I-independent expansion during MCMV infection, and in IL-2- or HSCT-treated recipient B6 mice (221), our findings challenged prior notions regarding the importance of Ly49G2 and licensed NK cells in antiviral immunity.

Mechanistically, we have shown that interactions between Ly49G2 and D^k license NK cells, which endows them with enhanced sensitivity to activating receptor and IL-12R stimulation. Furthermore, we have demonstrated enhanced

D^k-dependent activation, maturation, proliferation, and accumulation of $Ly49R^+$ $Ly49G2^+$ NK cells (relative to $Ly49R^+$ Ly49G2- NK cells) during MCMV infection. Whether the enhanced antiviral effector activities of R^+ $G2^+$ NK cells can be solely attributed to their lower threshold for stimulation via activating or IL-12 receptors remains unclear. In this regard, it is intriguing that other D^k-binding licensing IRs in NKC^L mice (e.g., Ly49I, Ly49O, and Ly49V; **Table 1**; (155)) have negligible effects on licensed NK cell sensitivity to activating or IL-12 receptor stimulation **(Figure 11B and Figure 25A-B)**.

Our results imply that a hierarchy of licensing receptors exists such that select IRs (i.e., Ly49G2) engender greater responsiveness than others. The heightened responsiveness of $Ly49G2^+$ NK cells during MCMV infection may be related to the magnitude of inhibitory signals propagated by Ly49G2 during IR/self-MHC I licensing interactions (85). A prediction of the tuning model (85) is that greater Ly49G2 IR signaling following engagement of self-MHC I during licensing would engender heightened reactivity to licensed NK cells against missing-self targets. Greater missing-self reactivity of licensed $Ly49G2^+$ NK cells may be related to Ly49G2/*cis*-D^k modulation of the activation status or localization of downstream phosphatases, as was recently shown for Ly49A/D^d and SHP-1 (210), enabling greater signaling through downstream activating receptor adaptors. Alternatively, considering that phosphorylated Crk affects NK cell inhibition by preventing F-actin network formation and limiting activating receptor translocation (58, 59), Ly49G2-driven SHP-1 activities may influence the phosphorylation status

of Crk during activating receptor crosslinking **(Figure 1).** Moreover, since ligation of the IL-12R leads to JAK2-STAT4 signal transduction (222), and that SHP-1 has been shown to directly associate with and dephosphorylate JAK2 (223), sequestration of SHP-1 by Ly49G2/*cis*-D^k may facilitate IL-12R signaling and subsequent upregulation of CD25. To address these hypotheses, it will be necessary to evaluate the phosphorylation status and localization of the aforementioned phosphatases and downstream kinases **(Figure 1)** in D^k-licensed NK cells following activating or cytokine receptor ligation. One could specifically use a combinatorial approach (e.g., phosphoflow and confocal/stimulated emission depletion (STED) microscopy) to evaluate subsets of licensed NK cells singly positive for Ly49G2, Ly49I, Ly49O, or Ly49V. Ultimately, these experiments will provide a molecular basis for Ly49G2-mediated enhancement of licensed NK cell activation and proliferation during MCMV infection.

Ly49G2 is required to counter MCMV gp34-mediated immune evasion

We demonstrated that WT MCMV selectively spreads in mice lacking Ly49G2 **(Figure 23D)**. By contrast, gp34-deficient MCMV is selectively attenuated in D^k-bearing Ly49G2-deficient mice **(Figure 23D)**. Whereas gp34 is known to facilitate cell surface expression of altered-self MHC I molecules in MCMV-infected cells **(Figure 7 and Figure 20C)**, gp40 and gp48 promote downregulation of MHC I molecules and give rise to missing-self targets. Our findings suggest that, in

contrast to Ly49I, Ly49O, or Ly49V, Ly49G2 is unimpeded by altered-self gp34/D^k complexes. Although these D^k-binding IRs may facilitate missing-self sensing, Ly49G2 is nonetheless the dominant altered-/missing-self receptor in this mouse model.

Our findings indicate that Ly49G2^{C57L}-bearing NK cells are uniquely poised to recognize and eliminate viral targets bearing virus-altered-D^k molecules. Relatedly, the selectivity of a given IR for the MHC I molecules expressed in NKC^L-D^k mice (D^b, K^b, D^k) may influence the capacity of an individual NK cell to respond to stimulation during MCMV infection. For example, Ly49G2 and Ly49O selectively bind D^k but not D^b or K^b; by contrast, Ly49I binds D^k and K^b, and Ly49V binds D^b, K^b, and D^k (155). Gp34 can engage each of these MHC I molecules (149) and may therefore influence IR-mediated MHC I sensing.

To cleanly test whether MHC I selectivity is related to the ability of a given IR$^+$ subset to control gp34-expressing MCMV, one could generate reporter cell lines expressing chimeric Ly49G2, Ly49I, Ly49O, or Ly49V receptors and evaluate the amount of reporter cell stimulation by uninfected or MCMV-infected target cells singly expressing D^b, K^b, or D^k. One could also evaluate *in vitro* activating receptor stimulation of primary NK cells singly expressing each IR using NK cells from NKC^L-D^k (D^b, K^b, D^k), NKC^L (D^b, K^b) KODO.NKC^L-D^k (D^k only), or KODO.NKC^L (no D^b, K^b or D^k) mice. Such experiments would enable testing of the contribution of individual MHC I alleles or self-IRs to enhancement of NK cell responsiveness. Finally, one could evaluate selective activation and expansion of each IR$^+$ NK cell

subset during MCMV infection. Collectively, these experiments are expected to provide insight into Ly49G2's role in countering MCMV gp34-mediated immune evasion.

Ly49R recognition of MCMV-infected targets is gp34-dependent

Others have found that select Ly49 activating NKRs recognize MCMV-infected targets *in vitro* in a manner dependent on MHC I and MCMV gp34 expression (150, 167). In this work, we have shown that MCMV-specific and D^k-dependent triggering of Ly49R corresponds with NK cell activation and proliferation during MCMV infection **(Figures 16-18)**. We have also demonstrated that Ly49R activating NKR recognition of MCMV is likewise dependent on MHC I/gp34 **(Figure 20).** Gp34-dependent stimulation of Ly49R was somewhat surprising given that Ly49R lacks putative gp34-binding amino acids (167). Our findings thus beg the question whether distinct Ly49 activating NKRs differently contact gp34-MHC I complexes. Moreover, although we have shown MCMV gp34 is required for efficient cell surface D^k expression in infected targets, we cannot exclude the possibility that Ly49R recognition of MCMV is modified by an additional MCMV-encoded protein or peptide. Taken together, these studies warrant structural elucidation of Ly49 activating NKRs bound to gp34-MHC I complexes. The results of such investigations could provide insight into how innate immune recognition receptors distinguish self- from altered-self MHC I molecules.

Both Ly49P and Ly49R activating NKRs have been implicated in MCMV resistance in D^k-bearing mice with either MA/My- or C57L-derived Ly49 haplotypes (11, 153). Inasmuch as NKC^L-D^k mice also express Ly49P **(Table 1)**, and Ly49R blockade abrogates D^k-dependent MCMV control (11), our findings suggest that Ly49P is ineffectual when Ly49R is neutralized. However, we cannot exclude a role for Ly49P in D^k-dependent MCMV resistance. It remains possible that the combined sensing of altered-self gp34-D^k complexes by Ly49R and Ly49P synergistically enhances NK cell recognition of MCMV-infected cells. This hypothesis is further supported by amino acid alignments showing that Ly49R and Ly49P differ at critical gp34/MHC I contact residues (167), suggesting the receptors may differently bind gp34/MHC I complexes. To test whether Ly49R and Ly49P work together to enhance D^k-dependent antiviral immunity, we could generate Ly49R and Ly49P knockout mice (as described for Ly49G2) and test each mouse for MCMV resistance. We would further test whether sorted 1) $Ly49R^+$ $Ly49P^+$ $Ly49G2^+$, 2) $Ly49R^-$ $Ly49P^+$ $Ly49G2^+$, 3) $Ly49R^+$ $Ly49P^-$ $Ly49G2^+$, or 4) $Ly49R^-$ $Ly49P^-$ $Ly49G2^+$ NK cell subsets transferred protection to MCMV-susceptible Ly49G2-deficient D^k-bearing recipient mice. Additionally, we would generate reporter cell lines singly expressing or coexpressing Ly49R and Ly49P receptors, and test R^+, P^+, or R^+ P^+ reporter cell line stimulation with gp34-D^k bearing target cells. These studies may inform our understanding of how licensed NK cells break tolerance during viral infection.

Intriguingly, the Yokoyama group has shown that licensed NK cells do not require DAP10 or DAP12 adaptor signaling to control MCMV in D^d-transgenic B6 mice (202). Considering that the Ly49 activation receptors mentioned above (Ly49R and Ly49P in D^k-bearing mice) propagate signals primarily via DAP12, these findings (202) suggest distinct activating receptors and adaptors are required in D^d-dependent MCMV control. Nonetheless, the basis for activation of licensed NK cells in the different mouse models may be partially conserved, despite their mechanistic differences. For instance, NK cells have been shown to require combined signaling via multiple activating receptors to induce target cell cytotoxicity (8). Thus, reliance on multiple redundant activating receptors may enable virus-responsive licensed NK cells to control MCMV infection in DAP12-deficient D^d animals.

Recognition of altered-self by functionally disparate paired NK cell receptors

Others have shown that activating and inhibitory Ly49 NKRs derived from MA/My, BALB, or B6 mice (e.g., $Ly49P^{MA/My}$, $Ly49L^{BALB}$, $Ly49A^{BALB}$, and $Ly49A^{B6}$; **Table 1**) bind altered-self gp34/MHC I complexes. Whereas activating NKR engagement of gp34/MHC I is thought to facilitate NK cell-mediated lysis of MCMV-infected targets (150, 167), gp34/MHC I complexes are predicted to counter IR-mediated missing-self recognition by binding to IRs and inhibiting NK cell stimulation (129). However, despite compelling evidence for gp34/MHC I

engagement by Ly49G2^{C57L} **(Figure 23A-C)** and Ly49A^{B6} (129), both of these licensing IRs have been shown essential in MCMV resistance **(Figure 16** and (11, 202)**)**. Thus, the assumption that gp34 binding to licensing IRs dominantly impedes activating receptor stimulation is inconsistent with recent studies.

Our findings have demonstrated that both Ly49G2^{C57L} and Ly49R^{C57L} engage altered-self gp34/D^{k} complexes on MCMV-infected cells and that each receptor is essential to D^{k}-dependent NK cell-mediated MCMV resistance **(Figure 26)**. Furthermore, we have observed selective expansion of NK cells coexpressing both receptors during MCMV infection. We predict that Ly49R^{+} Ly49G2^{+} NK cell MCMV sensing represents an example of paired recognition, whereby the paired receptors 1) are coexpressed, 2) share a ligand, and 3) exhibit synergy.

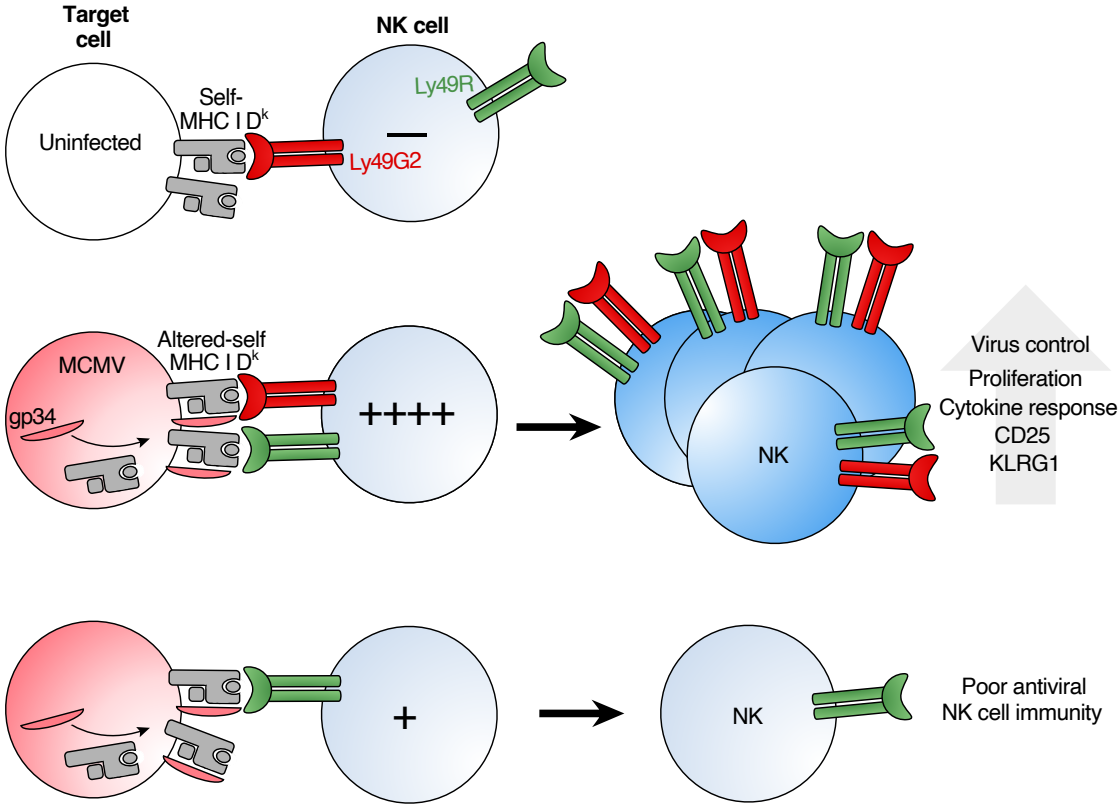


Figure 26. Altered-self recognition by functionally disparate paired NK cell receptors unleashes expansion of antiviral NK cells during MCMV infection.

In order for the Ly49G2 IR to synergistically enhance Ly49R signaling, the inhibitory impact of Ly49G2 must be overcome. We envision several non-mutually exclusive possibilities may allow for this outcome. 1) Ly49G2-mediated licensing may lower the threshold for activation of the Ly49R/DAP12 signaling cascade such that Ly49R stimulation overrides Ly49G2-mediated inhibition. 2) Stimulation via Ly49R and Ly49P together may overcome inhibitory signals propagated by Ly49G2. 3) The density of Ly49R activating NKRs may be greater than Ly49G2 IRs in licensed NK cells, such that low-avidity activating NKR ligation dominates high-affinity inhibitory receptor ligation. 4) Ly49G2/SHP-1-mediated dephosphorylation of p-Crk may enhance actin-dependent Ly49R activating NKR clustering **(Figure 1)**. 5) High-affinity Ly49G2-mediated ligand engagement could facilitate synapse formation and conjugation with infected target cells. 6) We have not observed significant reduction of cell surface D^k expression ~20 hrs post WT MCMV infection of M2-10B4 or SVEC4-10 cell lines compared to uninfected cells (unpublished timecourse data). However, it remains possible that MCMV gp40 and gp48 may act to downregulate D^k expression at earlier timepoints post-infection **(Figure 7)**, resulting in infected target cells with lower D^k expression. Ly49G2-mediated detection of such missing-self targets may alter the activation status of licensed $Ly49R^+$ $Ly49G2^+$ NK cells. An increase in the number of *cis*-engaged relative to *trans*-engaged Ly49G2 IRs **(Figure 6)** could further enable free Ly49R receptors to better detect an increase in gp34-D^k complexes on MCMV-infected targets. Addressing these possibilities experimentally should provide

insight into how paired activating and inhibitory NKRs synergize to promote recognition and clearance of viral targets.

We predict that paired detection of altered-self is a conserved mechanism of NK cell-mediated host defense against herpesviruses. In particular, CMV encodes multiple MHC I-like and MHC I-binding immunoevasins which can engage NK cell IRs and evade lysis, including MCMV m04/gp34, m12, m157, and HCMV UL40 (141). Remarkably, both activating and inhibitory NKRs have been shown to directly recognize these molecules on the surface of viral targets (10, 129, 157, 167, 217, 219, 220). Furthermore, each of these viral immunoevasins (MCMV gp34, m12, m157, and HCMV UL40) is highly mutable (213–218), which may significantly impact recognition by a given activating or inhibitory NKR. Polymorphism in NKRs, MHC I ligands, or viral immunoevasins may therefore combine to give rise to select pairs of activating and inhibitory NKRs which together facilitate viral detection (195). Further study is thus warranted to understand which other pairs of activating and inhibitory NKRs can be harnessed to promote NK cell-mediated virus control.

Closing remarks

The results presented herein demonstrate that licensed NK cells equipped with self-MHC I recognition receptors impart essential MCMV protection. Our discovery of Ly49G2's vital role in MHC I-dependent MCMV resistance has

fundamentally advanced the field's understanding of the importance of self-IRs in NK cell-mediated antiviral immunity. Nonetheless, certain self-IRs instead impede NK cell resistance to viral infection. This distinction is likely influenced by licensing IR polymorphism, which has been shown to affect IR interactions with MHC I molecules and MHC I-dependent rewiring of NK cell responsiveness. Yet, it remains unclear why only select self-IRs (e.g., Ly49G2^{C57L}) effectively sense virus-altered MHC I molecules during MCMV infection and impart viral resistance. A specific requirement for certain IRs in viral detection may be linked to 1) the affinity of IR/self-MHC I interactions, 2) IR/self-MHC I-dependent tuning of licensed NK cell responsiveness, and/or 3) IR-mediated discrimination of self- vs altered- vs missing-self MHC I molecules on infected targets. Notwithstanding, our findings further show that signals emanating from paired virus-specific activating NKRs or licensing IRs are insufficient in isolation. Rather, coordinate recognition of infection by paired, coexpressed licensing and activating NKRs is required for efficient virus control. Activating and licensing NKR/MHC I associations in certain human chronic viral infections may likewise represent paired recognition systems. Considering the importance of licensing IRs in self-tolerance, we predict that such paired recognition systems might enhance adaptive immune outcomes by protecting bystander APCs and secondary lymphoid structures, ultimately promoting antigen-specific T and B cell responses. Resolving these questions may facilitate personalized therapeutic strategies to improve NK cell cytotoxicity against select viruses and tumors which downregulate MHC I molecules to evade T cell immunity.

Materials and Methods

Ethics Statement. All mouse experiments were performed in accordance with the Animal Welfare Act and approved by the UVA ACUC.

Mice. All mice used in this study were bred and maintained at UVA under specific pathogen free conditions. B6.Cg-NKC^{C57L}-D^{k} (NKC^{L}-D^{k}) and B6.Cg-GO1-D^{k} (GO1-D^{k}) mice were previously generated (11, 170). B6.SJL-*Ptprc^{a} Pepc^{b}*/BoyJ mice were purchased (Jackson Laboratory) and crossed with NKC^{L}-D^{k} or GO1-D^{k} mice to generate congenically marked CD45.1.NKC^{L}-D^{k} and CD45.1.GO1-D^{k} mice. B6-*Prf1^{tm1Sdz}*/J mice were purchased (Jackson Laboratory) and crossed with NKC^{L}-D^{k} mice to generate perforin-deficient NKC^{L}-D^{k} mice (*Prf1$^{-/-}$*-D^{k}).

Cell lines. WT and D^{k}-deficient M2-10B4 (H-2$^{k/b}$; ATCC) cells or YB2/0 and YB2/0-D^{k} cells (a kind gift of Kevin Kane) were cultured in complete RPMI (Roswell Park Memorial Institute) 1640 media supplemented with 10% FBS and 100 U/ml Penicillin/Streptomycin). SVEC4-10 (H-2^{k}; ATCC) cells were cultured in complete DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS and 100 U/ml Penicillin/Streptomycin). Ly49-CD3ζ reporter cell lines were generated by lentiviral transduction as described (11). Briefly, C57L-derived CD3ζ-Ly49G2, CD3ζ-Ly49R, Ly49G2^{WT}, or ITIM-mutant Ly49G2^{Y8F} coding sequences were

cloned into pCpp2E (kindly provided by Sam Kung; (224)) or pCW57-MCS1-P2A-MCS2 (Blast) (a kind gift of Adam Karpf; Addgene plasmid #80921), respectively. Transfers plasmids were then transfected into 293T cells together with psPAX2 and pMD2.G (both kindly provided by Didier Trono (Addgene plasmid #12260 and 12259, respectively) using Lipofectamine 3000. Titered lentiviral supernatants (MOI 4) were used to transduce J7 Jurkat T cells (kindly provided by Koho Iizuka; (171)). High expressors were sorted or selected with blasticidin (10 mg/ml), respectively, and cultured in RPMI 1640 media supplemented with 10% FBS, 10 mM HEPES, 1 mM Sodium Pyruvate, 1 mM b-2-mercaptoethanol, and 100 U/ml Penicillin/Streptomycin.

MCMV Infections. Salivary gland-passaged (SGV) or tissue culture (TC)-propagated MCMV (Smith Strain; ATCC) were titered on M2-10B4 monolayers and i.p. injected at indicated doses, as described (22, 225). SGV was used for *in vivo* virus infections unless indicated otherwise (e.g., for Δ*m04* MCMV infections, TC-passaged WT and Δ*m04* MCMV were compared). Ly49R was neutralized using 200 μg mAb 12A8 (a gift from John Ortaldo, National Cancer Institute, National Institutes of Health, Frederick, MD) given i.p. 72 and 24 h before infection (11). For $CD4^+$ and $CD8^+$ T cell depletion studies, 200 μg mAbs 2.43 and and GK1.5 (kindly provided by Timothy Bullock, University of Virginia) were administered 48 h before and 24 h after infection. Infected spleen genomic DNA was measured for MCMV genomes via quantitative PCR as described (226). For *in vitro* MCMV

infections, M2-10B4 cells were infected with MOI 0.5, whereas SVEC4-10 cells were infected with MOI 0.1 or MOI 25 as indicated. Adherent monolayers were incubated at 37 °C, 5% CO_2 in a small volume of diluted virus for 30 min, followed by centrifugation at 800 *x g* for 30 min at 25 °C, as described (150).

Flow Cytometry and Cell Sorting. Splenocytes (single-cell suspension) from uninfected or infected mice were pre-blocked with Fc receptor blocking mAb 2.4G2 (hybridoma maintained by UVA Lymphocyte Culture Center). Cell surface staining was performed using fluorophore-conjugated antibodies (BD Biosciences, eBioscience, BioLegend, or University of Rijeka Center for Proteomics). Flow cytometry and cell sorting were performed on the Attune NxT (ThermoFisher Scientific) and Influx (BD) cytometers, respectively. Data were analyzed with FlowJo software (version 10.8). The following fluorophore-conjugated or biotin-conjugated antibodies were used: CD3ε (145-2C11), CD19 (6D5), CD49b (DX5), NKp46 (29A1.4), Ly49G2 (4D11 and Cwy-3), Ly49R (12A8; kindly provided by John Ortaldo), Ly49ROV (4E5), CD27 (LG.7F9), CD11b (M1/70), KLRG1 (2F1), CD25 (PC61), BrdU (BU20a), IFN-γ (XMG1.2), H-2D^k (15-5-5), H-2K^k (36-7-5), H-2K^b (AF7-88.5), H-2D^b (28-14-8), NKG2D (CX5), p-STAT4 (Y693; Clone 38), MCMV m04/gp34 (m04.16), and MCMV m123/IE1 (IE1.01). LIVE/DEAD™ fixable dyes (ThermoFisher Scientific) were used to assess cell viability. p-STAT4 was detected in cytokine-stimulated NK cells after cell surface staining. NK cells were fixed with 4% PFA for 10 min at 37 °C, washed with PBS, permeabilized with ice-

cold 100% methanol for 10 min and washed again with PBS + 2% FBS, as described (227). Cells were stained with the p-STAT4 mAb (Y693; Clone 38). M2-10B4 or SVEC4-10 cells were stained for cell surface MCMV gp34 (anti-m04.16 (150); mouse IgG2b isotype) and D^k (mAb 15-5-5) followed by staining with an anti-mouse IgG2b-PE secondary antibody and fluorophore-conjugated streptavidin.

BrdU Incorporation Assay. Mice were i.p. injected with BrdU (1 mg/ 200 μL PBS) 3 h prior to sacrifice. BrdU staining was performed using a kit (BD Biosciences) per the manufacturer's instructions.

Generation and Validation of Ly49G2-deficient GO Mice.

Design and In Vitro Transcription of Single-Guide RNA. An allele-specific single-guide RNA (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 https://zlab.bio/guide-design-resources, as previously described (228). 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,

Broad Institute of MIT, Cambridge, MA; 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, New England Biolabs), pX330-*Ly49g2-exon4* vector, and a primer designed to append the T7 promoter to the *Ly49g2*-sgRNA encoding oligonuceltide (229). The template was purified using a kit (Qiagen QIAQuick PCR purification) followed by dialysis against 1× TE. The template was then transcribed and its product purified using Ambion, MEGAshortscript, and MEGAclear T7 kits.

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 previously (230). Primers (Integrated DNA Technologies) were optimized and used in HRM PCR as described previously (230–232).

Generation and Validation of Ly49g2 Deficient GO Mice. B6 (NKC^{B6}) (Jackson Labs) males were bred to superovulated B6.NKC^{C57L} (NKC^{L}) females to generate B6.NKC$^{B6/L}$ embryos that were microinjected with Cas9 protein (Integrated DNA Technologies) 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 previously (233). Briefly, GO1 and GO2 FastQ files were separately aligned to the *Ly49g2*^{L} reference sequence using BWA-MEM in Sequencher (Gene Codes Corporation). A Burrows–Wheeler Aligner (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 **Tables 3-5.**

Primary NK cell gene-editing. CRISPR RNAs (crRNAs) were selected using the CRISPOR (www.crispor.tefor.net) online platform (234). The first ~35% of the coding sequence for each gene was prioritized for targeting. sgRNAs (*Klra18* 5-GCA GAA CGA GAU GAG GCU CA-3; *Klra7* 5-GCG UGG UGC UGC AGU UAU CG-3 (11)) were selected for their maximal likelihood of specific targeting with minimal potential to edit off-target genes. Synthetic crRNAs were purchased from IDT (www.idtdna.com/CRISPR-Cas9) in Alt-R format. To prepare duplexes,

custom Alt-R crRNA and synthetic Alt-R trans-activating crRNA (IDT) were reconstituted to 100 mM (100 pMol/ml) with TE buffer (Life Technologies). Oligos were mixed at equimolar concentrations in a sterile PCR tube and annealed by heating to 95 °C for 5 min in a PCR thermocycler. Annealed duplexes were then removed from the thermocycler and allowed to slowly cool to room temperature. cRNP complexes were prepared essentially as described (235). Briefly, 1.2 ml (120 pMol) of annealed oligo duplexes, 0.9 ml of 100 mM Alt-R Cas9 Electroporation Enhancer (IDT) and 3.9 ml water were added to a sterile strip tube per sample (total volume 6 ml). 40 pMol of recombinant Alt-R S.p. Cas9 nuclease V3 (IDT) was diluted with water to a final volume of 6 ml in a separate sterile strip tube. 6 ml of diluted Cas9 was gently mixed with 6 ml of duplex-enhancer mixture for a total of 12 ml cRNP complex at a 1:3 molar ratio. The cRNP complex was allowed to incubate for at least 10 minutes at room temperature. Resting enriched (Miltenyi mouse NK Isolation Kit II) spleen NK cells (~2 x10^5 cells/well; 96-well round-bottom plate) were preactivated in complete NK cell media (IMDM containing 10% FBS, 2 mM L-Glutamine, 10 mM HEPES, 1X Glutamax™, 100 U/ml Penicillin/Streptomycin, 50 mM b-2-mercaptoethanol plus recombinant mouse IL-15 (20 ng/ml, Preprotech) for 18 h before transfection. IL-15-preactivated NK cells were nucleofected using a method similar to that described for activated primary mouse T cells (236). 200 μl complete NK cell media was prewarmed per well of a 96-well round-bottom plate. Approximately 5 x10^5 NK cells were resuspended in 20 μl P4 Primary Cell 4D-Nucleofection Solution

(Lonza), mixed with 12 μl cRNP complex and incubated for 2 min at room temperature. The NK cell–cRNP mix was transferred to Nucleofection Cuvette Strips (Lonza) for electroporation using a 4D-Nucleofector X Unit (Lonza). Different electroporation pulses were tested (CM137, CM138, DS137, DS138, DS150, DN100, and EH100). Pulse code CM138 was found optimal for gene-editing with minimal loss in activated mouse NK cell viability. After nucleofection, 200 μl prewarmed NK cell media was added to each cuvette well, and transfected cells were transferred to 96-well round-bottom plates. NK cells were then incubated at 37 °C (90 min) before centrifugation and resuspension in complete NK cell media plus IL-15 (20 ng/ml). NK cells were cultured in vitro for 5 d prior to analysis of gene-editing efficiency by flow cytometry, or sorting for use in adoptive transfer experiments.

Adoptive Transfers. IL-15-expanded spleen NK cells were flow-sorted into $Ly49^+$ NK subsets and then labeled with respective cell proliferation dyes (CellTrace™ CFSE or CellTrace™ Violet (CTV); ThermoFisher Scientific). For analysis of cell proliferation, sorted NK cells resuspended in PBS plus FBS (1X) were labeled with an equal volume of freshly prepared proliferation dye (10 μM in PBS), immediately inverted 3 times, and gently vortexed (10 s). Labeled NK cells were incubated at room temperature for 5 min and then quenched in 10 ml of FBS. For each NK cell subset, 5×10^5 labeled NK cells were i.v. injected per recipient mouse 24 h prior to MCMV infection.

In vitro lymphocyte stimulation and co-culture. For analysis of primary NK cell IFN-γ production, mouse splenocytes cultured in complete RPMI plus IL-2 (200 U/mL; Peprotech). IL-2-treated splenocytes (1 to 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 h prior to brefeldin A (BFA) addition, and an additional 4 h with BFA. Stimulated cells were fixed and permeabilized using a kit (Cytofix/Cytoperm; BD Biosciences) followed by staining for intracellular cytokines at 4 °C. For analysis of CD25 expression and STAT4 phosphorylation, enriched spleen NK cells were cultured for 24 h in complete NK media +/- 10 ng/ml IL-15 or IL-12 (Preprotech). For analysis of CD25 induction, IFN-β-pre-treated (R&D Systems, 500 U/ml, 60 min) splenocytes resuspended in complete NK media plus IL-2 (500 U/ml) were co-cultured with M2-10B4 cells pre-treated with IFN-β (1000 U/ml) or infected with TC-passaged MCMV (MOI 1.5) for 24 h prior to flow cytometry analysis. For reporter cell assays, SVEC4-10 cells were pre-treated with IFN-β (1000 U/ml) or infected with TC-passaged MCMV (WT or Δ*m04*; MOI 25). For mAb stimulations, 96-well plates were coated with 10 μg/well α-Ly49G2 (4D11), α-Ly49R (12A8), or both mAbs overnight at 4 °C. For doxycycline-inducible reporter cell lines, reporters were grown in media +/- 2 μg/mL doxycycline hyclate for 24 h prior to coculture. Ly49-bearing J7 reporter cells (2×10^5) were stimulated for 8-12 h with target cells (±IFN-β or MCMV infection; 2×10^4 for SVEC4-10 or M2-10B4, 2×10^5 for YB20 and

YB20-D^k), respective platebound mAbs, or PMA + ionomycin. LacZ activity was determined using the substrate chlorophenol red-D-galactoside (CPRG), as described (11, 171).

Generation of D^k-Deficient M2-10B4 cells. The second exon of the *D^k* gene was targeted using CRISPR-Cas9 editing. A *D^k*-specific sgRNA (5-GCGA GAG AUG AGC CGC GGG UG-3) was selected using CRISPOR (234) based on published *H-2D^k* coding sequence (GenBank accession no. M18524.1). The allele-specific sgRNA was selected for maximal likelihood to specifically target *D^k* with minimal potential to edit related *K^k, K^b, and D^b* genes also expressed by H-$2^{k/b}$ M2-10B4 cells. A 5' G was appended to the sgRNA to ensure efficient *in-vitro* transcription with the U6 promoter. *D^k*-specific oligonucleotides (Integrated DNA Technologies) were cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 (kindly provided by Feng Zhang; Addgene plasmid #42230) and then transfected into M2-10B4 cells using Lipofectamine 3000. Single cell clones (n=100) obtained by limiting dilution were stimulated with IFN-β (200 U/ml, 18 h) and screened for cell surface D^k, K^k, K^b, and D^b expression using flow cytometry. Five D^k-deficient single cell clones were identified. A single D^k-deficient clonal cell line was analyzed in *in vitro* assays.

Generation of Δm04 MCMV. The MCMV *m04* ORF was targeted using CRISPR-Cas9 editing. A *m04*-specific sgRNA (5-GAG CAC UGA UAA CGG CAA CGG-3) was selected using CRISPOR (234) based on published Smith strain *m04* coding

sequence (GenBank accession no. GU305914.1). The sgRNA was designed to introduce a frameshift mutation upstream of m04's transmembrane domain, which is required for MHC I association (205). Notably, a 5' G was appended to the sgRNA to ensure efficient transcription with the U6 promoter. *m04*-specific oligonucleotides (Integrated DNA Technologies) were cloned into plentiCRISPR v2 (kindly provided by Feng Zhang; Addgene plasmid #52961) and then transfected into 293T cells together with psPAX2 and pMD2.G (both kindly provided by Didier Trono (Addgene plasmid #12260 and 12259, respectively) using Lipofectamine 3000. Titered lentiviral supernatants were used to transduce M2-10B4 cells (MOI 1) followed by selection in media supplemented with 2 μg/ml puromycin. SGV was propagated in sgRNA/Cas9-coexpressing M2-10B4 cells (MOI 0.01) as described (237). Plaque-purified Δ*m04* MCMV was isolated from heterogenous CRISPR-modified viral supernatants by two rounds of limiting dilution on WT M2-10B4 cells. *m04*-specific PCR amplicons spanning the anticipated CRISPR/Cas9 cleavage site were generated from clonal virions using *m04*-specific primers 5-TCA CTC CCA TGC ACG GAT TA-3 and 5-CCT CAT CCG GAG CTG TCA TT-3. Sequence variants were confirmed and clonal virions were further propagated in WT M2-10B4 cells for use in *in vivo* studies. TC-passaged MCMV lacking m04 was compared to WT MCMV in a multi-step growth curve on M2-10B4 cells (MOI = 0.1). Viral genome copies were quantified from cell lysates and culture supernatants as described (22).

Statistical Analyses. Statistical analyses were performed using Graphpad Prism (version 9.2.0). 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 U rank test was used assess the significance for two independent measurements.

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VITA

John (Jack) Cronk was raised in Crozet, Virginia. Growing up in the foothills of the Blue Ridge Mountains fostered his curiosity in the natural world, and eventually led him to pursue scientific research in Dr. Michael Brown's laboratory at the University of Virginia. In 2016, he graduated with his Bachelor of Science in Biochemistry and his undergraduate thesis, which employed CRISPR/Cas9 gene-editing to create a novel knockout mouse, was awarded honors of highest distinction. Through his undergraduate research, he discovered his passion for scientific discovery and understanding how cytotoxic lymphocytes limit disease spread. This led him to initially pursue his Ph.D. in Immunology at the University of Pennsylvania, before transferring back to the University of Virginia, where his graduate studies have focused on natural killer cell recognition of viral infection. He has published his work in the *Journal of Leukocyte Biology*, the *Journal of Immunology*, *Pathogens,* and the *Proceedings of the National Academy of Sciences*, and presented his work at the Annual Meeting of the American Association of Immunologists and the Meeting of the Society for Natural Immunity. In addition, he was nominated for the Robert R. Wagner Prize for Outstanding Research in Microbiology.

PUBLICATIONS

1. Cronk, J. M., K. H. Dziewulska, P. Puchalski, R. B. Crittenden, M.-L. Hammarskjöld, and M. G. Brown. 2022. Altered-self MHC class I sensing via functionally disparate paired NK cell receptors counters murine CMV gp34-mediated immune evasion. *J Immunol* (In Press).

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