

**REGULATION AND HETEROGENEITY OF  
ANTIVIRAL NATURAL KILLER CELL RESPONSES**

by

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A Dissertation

Presented to the Faculty of the Louis V. Gerstner, Jr.

Graduate School of Biomedical Sciences,

Memorial Sloan Kettering Cancer Center

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

New York, NY

May, 2019

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Date

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## **Abstract**

In both mice and humans, natural killer (NK) cells are required for host defense against viral infection, most notably herpesvirus infection. NK cells contribute to viral clearance by killing infected cells, rapidly secreting cytokines, and robustly proliferating in an antigen-specific fashion. However, the molecular mechanisms governing these dynamic and varied NK cell responses to viral pathogens are incompletely defined. Here, we report on two different players that regulate antiviral NK cell responses: the transcription factor IRF8 and the expression level of the activating NK cell receptor Ly49H. We show that cell-intrinsic IRF8 was required for NK cell-mediated protection against mouse cytomegalovirus (MCMV) infection. During virus exposure, NK cells upregulated IRF8 through interleukin-12 (IL-12) signaling and the transcription factor STAT4. Moreover, IRF8 facilitated the proliferative burst of virus-specific NK cells by promoting expression of cell cycle genes, and directly controlling *Zbtb32*, a master regulator of virus-driven NK cell proliferation. Furthermore, we show that NK cells with a broad range of avidities for the MCMV glycoprotein m157 are initially recruited after virus infection, but those with highest avidity are selected to undergo the greatest clonal expansion and comprise the majority of the memory NK cell population. Our findings support a functional “division of labor” within Ly49H<sup>+</sup> NK cells determined by pre-established levels of Ly49H receptor expression, with lower avidity NK cells possessing greater capacity for interferon (IFN)- $\gamma$  production, and higher avidity NK cells possessing greater capacity for cytotoxicity and adaptive responses.

## **Acknowledgements**

I would first like to thank my thesis mentor, Joe Sun. You provided me the opportunity to fully immerse myself in the scientific process: not only performing experiments and writing up my stories but also sharing my work at meetings, participating in reviewing papers, helping with grant preparation, communicating with editors, and meeting with seminar speakers and the many PIs to whom you introduced me. With time I have begun to appreciate that the opportunities you afford your trainees is indeed quite rare, and I know that I have become a better scientist as a result. Furthermore, the avidity project would never have happened if you were not so supportive of my ideas (...well in this case, an idea based off of a finding you made years ago!) and so willing to entertain these ideas whenever I would bug you early in the morning in person or late at night by e-mail. This independence has undoubtedly given me the ability and confidence to ask and set out to answer scientific questions. Most importantly, you have helped me kindle my passion for scientific discovery, and you have been a true role model who has championed humility despite your scientific success. I absolutely made the right choice in joining your lab four years ago.

I now understand why lab cohesiveness is so important. It truly enriches the lab. It is because of the individuals that worked beside me that have made it enjoyable to come to work every day, especially those whom I have known for several years now. We certainly had some fun times! I want to thank Clair Geary in particular for always being willing to assist with experiments, reading manuscript drafts, and generally providing feedback on random ideas I have.

I also owe a great deal of gratitude to those outside the lab who have advised and supported me scientifically, including my thesis committee (Sasha Rudensky, Ming Li, David Artis) and NK club, especially Kathy Hsu and members of her lab. It was both a challenge and a privilege to learn from such giants in the field of immunology during graduate school. Your constructive feedback has challenged me to think more broadly and deeply about my work and has taken my projects in directions I would not have predicted.

Both GSK and the Tri-I MD-PhD program provided the framework within which all of this was made possible. Despite my limited undergraduate research experience, the MD-PhD program took a chance on me and opened the door for me to realize my scientific potential. The administrative staff and leadership at GSK and the MD-PhD programs have organized two of the most smoothly operating programs. Of course a little money never hurts, and thanks are due to the GSK Grayer Fellowship, the Medical Scientist Training Program Grant from the NIGMS of the NIH to the Tri-I MD-PhD Program (T32GM007739), and the F30 Predoctoral Fellowship from NIAID of the NIH (F30 AI136239).

Finally, I would like to thank all of my family and friends. I would not be where I am today if it were not for my mom. You taught me that education can never be taken away from you, and to that end, afforded me every opportunity you could and continue to push me to fulfill my potential. To Russell, you have been my best friend growing up, and your good humor always lightens the mood. To Diana, your unwavering belief in me has provided encouragement in times of doubt, and the happiness you bring me is always the perfect escape from the trials of a failed experiment. And to my grandpa, I know that

you are watching with immense pride, wishing that I could write up for you my latest news for inclusion in the Fairways Legend and so that you could properly describe it to your cardiologist at your next appointment. I so wish you could have been here.

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<sup>1</sup>Originally published in *The Journal of Immunology*. Adams, N.M., O’Sullivan, T.E., Geary, C.D., Karo, J.M., Amezquita, R.A., Joshi, N.S., Kaech, S.M., and Sun, J.C. 2016. NK Cell Responses Redefine Immunological Memory. *J. Immunol.* 197: 2963-2970. Copyright © [2016] The American Association of Immunologists, Inc. <https://doi.org/10.4049/jimmunol.1600973>

<sup>2</sup>Originally published in *Immunity*. Adams, N.M., Lau, C.M., Fan, X., Rapp, M., Geary, C.D., Weizman, O.E., Diaz-Salazar, C., and Sun, J.C. 2018. Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity*. 48: 1172-1182. Copyright © [2018] Elsevier, Inc. Reproduced with permission of Elsevier, Inc via Copyright Clearance Center. <https://doi.org/10.1016/j.immuni.2018.04.018>

<sup>3</sup>The material herein has been submitted for publication in a modified format. Adams, N.M., Geary, C.D., Santosa, E.K., Lumaquin, D., Le Luque, J.B., Sottile, R., van der Ploeg, K., Hsu, J., Whitlock, B.M., Jackson, B.T., Weizman, O.E., Huse, M., Hsu, K.C., and Sun, J.C. 2019. Cytomegalovirus Infection Drives Avidity Selection of Natural Killer Cells. *Immunity*. *in press*.

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<sup>1</sup>Originally published in *The Journal of Immunology*. Adams, N.M., O’Sullivan, T.E., Geary, C.D., Karo, J.M., Amezcua, R.A., Joshi, N.S., Kaech, S.M., and Sun, J.C. 2016. NK Cell Responses Redefine Immunological Memory. *J. Immunol.* 197: 2963-2970. Copyright © [2016] The American Association of Immunologists, Inc. <https://doi.org/10.4049/jimmunol.1600973>

<sup>2</sup>Originally published in *Immunity*. Adams, N.M., Lau, C.M., Fan, X., Rapp, M., Geary, C.D., Weizman, O.E., Diaz-Salazar, C., and Sun, J.C. 2018. Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity*. 48: 1172-1182. Copyright © [2018] Elsevier, Inc. Reproduced with permission of Elsevier, Inc via Copyright Clearance Center. <https://doi.org/10.1016/j.immuni.2018.04.018>

<sup>3</sup>The material herein has been submitted for publication in a modified format. Adams, N.M., Geary, C.D., Santosa, E.K., Lumaquin, D., Le Luduec, J.B., Sottile, R., van der Ploeg, K., Hsu, J., Whitlock, B.M., Jackson, B.T., Weizman, O.E., Huse, M., Hsu, K.C., and Sun, J.C. 2019. Cytomegalovirus Infection Drives Avidity Selection of Natural Killer Cells. *Immunity. in press.*

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## List of Abbreviations

**$\alpha$ LP:**  $\alpha$ -lymphoid progenitor

**ADCC:** antibody-dependent cellular cytotoxicity

**AID:** activation-induced cytidine deaminase

**AIM2:** absent in melanoma 2

**AMPK:** 5' adenosine monophosphate-activated protein kinase

**ANOVA:** analysis of variance

**AP-1:** activator protein 1

**ATAC:** assay for transposase-accessible chromatin

**Bcl-2:** B-cell lymphoma 2

**BCR:** B cell receptor

**Blimp-1:** B lymphocyte-induced maturation protein-1

**BNIP3:** BCL2/adenovirus E1B 19-kDa interacting protein 3

**BTB-ZF:** Broad complex, Tramtrack, Bric à Brac and Zinc Finger

**CD:** Cluster of Differentiation

**cDC:** conventional dendritic cell

**CFU:** colony-forming units

**cGAS:** cyclic GMP-AMP synthase

**CHILP:** common helper ILC progenitor

**ChIP:** chromatin immunoprecipitation

**CHS:** contact hypersensitivity

**CLP:** common lymphoid progenitor

**CNS:** conserved non-coding sequence

**CTV:** Cell Trace Violet

**CXCR:** C-X-C motif chemokine receptor

**D:** days post-infection

**DC:** dendritic cell

**DE:** differential expression/differentially expressed

**DNA:** deoxyribonucleic acid

**DNAM-1:** DNAX accessory molecule 1

**DNFB:** 1-fluoro-2,4-dinitrobenzene

**DTH:** delayed-type hypersensitivity

**EILP:** early innate lymphoid progenitor

**Eomes:** Eomesodermin

**ES:** enrichment score

**Ets:** E26 transformation-specific

**FC:** fold change

**FcR:** Fc receptor

**FDR:** false discovery rate

**FLICA:** fluorochrome-labeled inhibitors of caspases

**Foxp3:** forkhead box P3

**FSC:** forward scatter

**GEO:** Gene Expression Omnibus

**GREAT:** interferon-gamma reporter with endogenous polyA transcript

**GSEA:** gene set enrichment analysis

**H3K4me3:** trimethylation of histone H3 at lysine 4

**HBV:** hepatitis B virus

**HCMV:** human cytomegalovirus

**HCV:** hepatitis C virus

**HIV:** human immunodeficiency virus

**HLA:** human leukocyte antigen

**HSCT:** hematopoietic stem cell transplantation

**HSV:** herpes simplex virus

**ICSBP:** interferon consensus sequence binding protein (i.e. IRF8)

**IE-1:** immediate-early 1 (MCMV protein)

**IFN- $\gamma$ :** interferon gamma

**Ig:** immunoglobulin

**IL:** interleukin

**ILC:** innate lymphoid cell

**i.p.:** intraperitoneally

**IRES:** internal ribosome entry site

**IRF:** interferon regulatory factor

**ITAM:** immunoreceptor tyrosine-based activation motif

**i.v.:** intravenously

**JAK:** Janus kinase

**KEGG:** Kyoto Encyclopedia of Genes and Genomes

**KIR:** killer-cell immunoglobulin-like receptor

**KLRG1:** killer cell lectin-like receptor G1

**KO:** knockout

**LLO:** Listeriolysin O

***L.m.:*** *Listeria monocytogenes*

**LPS:** lipopolysaccharide

**MCMV:** mouse cytomegalovirus

**MFI:** median fluorescence intensity

**MHC:** major histocompatibility complex

**miR:** microRNA

**MPEC:** memory precursor effector cell

**mTOR:** mechanistic target of rapamycin

**NFIL3:** nuclear factor IL-3 regulated

**NK cell:** natural killer cell

**NKG2:** natural killer group 2

**NKR:** NK cell receptor

**PANTHER:** Protein ANalysis Through Evolutionary Relationships classification system

**PBMC:** peripheral blood mononuclear cells

**PC:** principal component

**pDC:** plasmacytoid dendritic cell

**PFU:** plaque-forming units

**PI:** post-infection

**PKC:** protein kinase C

**PLZF:** promyelocytic leukemia zinc finger protein

**PMA:** phorbol 12-myristate 13-acetate

**PRR:** pattern recognition receptor

**qPCR:** quantitative polymerase chain reaction

**RAG:** recombination-activating gene

**RNA:** ribonucleic acid

**ROS:** reactive oxygen species

**RPKM:** reads per kilobase million

**SCID:** severe combined immunodeficiency

**SEM:** standard error of the mean

**seq:** sequencing

**SHIV:** simian-human immunodeficiency virus

**SIV:** simian immunodeficiency virus

**SLEC:** short-lived effector cell

**SOCS:** suppressor of cytokine signaling

**SSC:** side scatter

**STAT:** signal transducer and activator of transcription protein family

**STING:** stimulator of interferon genes

**T-bet:** T-box-containing protein expressed in T cells

**TCR:** T cell receptor

**Tg:** transgenic

**Th:** T helper cell

**TLR:** Toll-like receptor

**TRAIL:** tumor necrosis factor-related apoptosis-inducing ligand

**TSS:** transcriptional start site

**UI:** uninfected



**UTR:** untranslated region

**VSV:** vesicular stomatitis virus

**WT:** wild-type

**YFP:** yellow fluorescent protein

**ZAP70:** zeta chain of T cell receptor associated protein kinase 70

**Zbtb32:** zinc finger and BTB domain containing 32

# Chapter 1: Introduction

## I. Introduction<sup>1</sup>

The immune system has classically been divided into two arms, innate and adaptive immunity. Innate immunity is poised for swift, short-lived effector responses mediated through recognition of evolutionarily conserved signals via germline-encoded receptors (Lanier, 2005). Although initially slow in onset, adaptive immunity is considered “specialized” based on the ability to somatically rearrange antigen receptor genes to generate a diverse repertoire of T and B cells that can amplify antigen-specific responses through prolific clonal expansion (Butz and Bevan, 1998; Murali-Krishna et al., 1998; Williams and Bevan, 2007). Because adaptive immune cells can persist long-term following recognition of cognate antigen and execute a quantitatively and qualitatively more robust response following re-challenge with the same antigen, T and B cells were thought to be the only immune population capable of generating “memory”.

The emergence of immunological memory in the adaptive immune system can be traced to lower vertebrates, including the jawless fish such as lamprey and hagfish. Early studies demonstrated that lampreys immunized with the killed bacterium *Brucella abortus* produce long-lived immunoglobulin capable of agglutinating *Brucella* cells upon re-challenge but not capable of agglutinating typhoid-paratyphoid cells, underscoring the antigen-specific nature of these antibody titers (Finstad and Good, 1964). The lamprey can also mediate delayed-type hypersensitivity (DTH) reactions following stimulation with *Mycobacterium tuberculosis*-fortified complete Freund’s adjuvant (Finstad and Good, 1964). The basis for these phenomena may be attributable to the recently

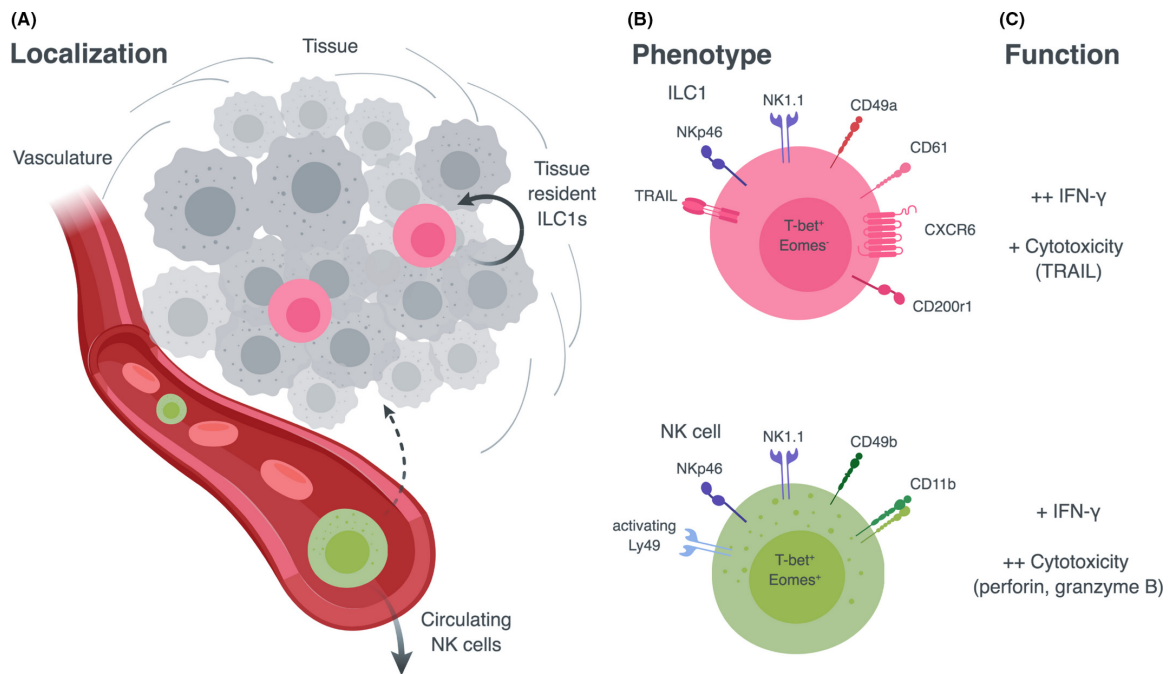
discovered lymphocyte-like populations and variable lymphocyte receptors, akin to primordial T and B lymphocytes and their antigen receptors, respectively (Boehm et al., 2012).

However, evidence also exists for immunological memory in invertebrates devoid of an adaptive immune system. Protection of the American cockroach *Periplaneta americana* against the bacterium *Pseudomonas aeruginosa* was enhanced by prior immunization with killed *P. aeruginosa* but not with saline or immunization with an array of other gram-negative organisms (Faulhaber and Karp, 1992). Interestingly, this protection against *P. aeruginosa* re-challenge persisted for 14 days post-immunization (Faulhaber and Karp, 1992), demonstrating both specificity and memory. Similar findings were demonstrated in the copepod *Macrocyclus albidus*, in which exposure to larvae of their natural parasite, the tapeworm *Schistocephalus solidus*, resulted in fewer infections from sibling but not unrelated parasites, the first evidence of innate immune memory in crustaceans (Kurtz and Franz, 2003). Similar protective memory responses against bacteria, parasites and fungi in other invertebrates add to the mounting evidence for innate immunity's capacity for memory responses in lower organisms (Bergin et al., 2006; Moret and Siva-Jothy, 2003; Pham et al., 2007; Rodrigues et al., 2010; Roth and Kurtz, 2009; Roth et al., 2009; Sadd and Schmid-Hempel, 2006), suggesting that the ability to "remember" may be evolutionarily conserved across both innate and adaptive immunity.

## **II. NK Cells: Founding Member of the Heterogeneous Innate Lymphoid Cell Family**

We now appreciate that the NK cell is the founding member of the innate lymphoid cell (ILC) family. A defining feature of ILCs is the absence of somatically rearranged antigen receptors, which gave rise to the prevailing notion that ILC responses lack antigen specificity (Artis and Spits, 2015; Spits and Di Santo, 2011). Furthermore, all ILCs share a common developmental origin, the common lymphoid progenitor (CLP), which subsequently loses its potential for differentiation into adaptive lymphocytes at the level of downstream multipotent progenitors, the  $\alpha$ -lymphoid progenitor ( $\alpha$ LP) and the early innate lymphoid progenitor (EILP) (Zook and Kee, 2016). The first developmental step that generates heterogeneity in the ILC compartment is the transition from these multipotent progenitors to the common helper ILC progenitor (CHILP), which begets all the “helper” ILC subsets but not “cytotoxic” NK cells (Klose et al., 2014). An extensive body of literature characterizing the transcriptional requirements and functional properties of ILC subsets has demonstrated striking similarities between ILCs and helper T cells (Spits et al., 2013). ILCs have been named to reflect these similarities, and divided into group 1, group 2, and group 3 ILCs to indicate that they are the innate counterparts to adaptive Th1, Th2, and Th17 cells respectively (Spits et al., 2013). Although there is an ongoing debate about the role for ILCs in the context of a healthy adaptive immune system (Vely et al., 2016), “helper” ILCs are nonetheless uniquely located and maintain long-term tissue residency at barrier surfaces, which enables them to act as sentinels by sensing and responding to signals of perturbed homeostasis (Artis and Spits, 2015; Spits and Di Santo, 2011).

NK cells have been classified within group 1 ILCs given their dependence on IL-15 and the transcription factor T-box-containing protein expressed in T cells (T-bet) for their development, and their production of IFN- $\gamma$ , features they share with another group 1 ILC family member, the ILC1 (Artis and Spits, 2015; Serafini et al., 2015). It is worth noting some of the unique features of NK cells that set them apart from ILC1s (Figure 1), and the rest of the ILC family. Transcriptionally, in addition to T-bet, both ILC1s and NK cells require nuclear factor IL-3 regulated (NFIL3) for their development (Gascoyne et al., 2009; Geiger et al., 2014; Kamizono et al., 2009; Seillet et al., 2014; Xu et al., 2015b; Yu et al., 2014). However, whereas ILC1s develop independently of Eomesodermin (Eomes), NK cells strictly require it (Daussy et al., 2014; Gordon et al., 2012; Robinette et al., 2015). Both T-bet and Eomes are T-box family members and are thought to share many binding sites, suggesting that it is the balance between the activity of these two transcription factors that may regulate group 1 ILC development. Furthermore, NK cells and ILC1s are phenotypically distinct (Figure 1). NK cells are best distinguished by their expression of integrins  $\alpha_2$  (CD49b) and Ly49 receptors (both inhibitory and activating) whereas ILC1s express integrins  $\alpha_1$  and  $\beta_3$  (CD49a and CD61 respectively), the chemokine receptor CXCR6, the cytotoxic molecule tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and the inhibitory receptor CD200r1 (Adams and Sun, 2018). The stability of the NK cell and ILC1 lineages and the relationship between them has often been questioned, but carefully performed transfer experiments have conclusively proven that NK cells and ILC1s maintain their phenotype during homeostasis and inflammation (Daussy et al., 2014; O'Sullivan et al., 2016b; Peng et al., 2013; Weizman et al., 2017). Functionally, both NK cells and ILC1s are potent sources of



**Figure 1. Heterogeneity of Group 1 ILCs<sup>4</sup>**

(A) Localization. During homeostasis, NK cells are primarily circulating cells transiting through the vasculature, whereas ILC1s are resident within tissue. During inflammation, ILC1 residency is maintained, and NK cells may also be recruited into the tissue.

(B) Phenotype. Mouse NK cells and ILC1s both express the activating NK cell receptors NKp46 and NK1.1 as well as the transcription factor T-bet. Only NK cells express and require Eomes for their development, and bear activating Ly49 receptors, CD11b, and CD49b on their surface. In contrast, ILC1s develop independently of Eomes, and can be characterized by expression of CD200r1 and a distinct integrin profile, including CD49a and CD61. CXCR6 expression is unique to hepatic ILC1s. NK cells and ILC1s are stable cell lineages during homeostasis and MCMV infection, although NK cells have been observed to adopt an ILC1-like phenotype in various cancer models.

(C) Function. ILC1s are thought to be less cytotoxic than NK cells, and may mediate cytotoxicity through a distinct set of effector molecules (eg, TRAIL). However, ILC1s are potent and early IFN- $\gamma$ -producing cells in response to cytokine stimulation.

Figure created with BioRender.

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IFN- $\gamma$ ; however, NK cells possess a greater knack for perforin- and granzyme-mediated cytotoxicity, although ILC1s can mediate some cytotoxicity through TRAIL (Cortez et al., 2014; Daussy et al., 2014; Robinette et al., 2015). Finally, ILC1s and NK cells have distinct localizations and establish different relationships with the tissues they occupy (Figure 1). Whereas NK cells are circulating cells that traffic through the vascular compartment of both lymphoid and non-lymphoid tissues, ILC1s, as well as other ILC subsets, are embedded within the tissues in which they reside and maintain long-term residency within these tissues, even during states of inflammation (Gasteiger et al., 2015; O'Sullivan et al., 2016b; Peng et al., 2013; Sojka et al., 2014; Weizman et al., 2017).

Thus, even among the diverse array of innate and innate-like lymphocytes, NK cells occupy a distinct niche and provide critical innate functions. In the following sections, we will review the ways in which NK cells possess “adaptive” features, which has led the field to reconsider the way in which it classifies immune cells and their functions: on an innate-adaptive continuum rather than a binary choice of innate or adaptive.

### **III. Natural Killer Cells: Innate Lymphocytes with Adaptive Features<sup>1</sup>**

NK cells were first described in 1975, when several groups identified a lymphocyte population in athymic (nude) mice capable of mediating “natural” (i.e. without the requirement of prior target exposure) cytotoxicity against both syngeneic and allogeneic tumor cell lines (Herberman et al., 1975a; Herberman et al., 1975b; Kiessling et al., 1975a; Kiessling et al., 1975b). Cytotoxic activity was maintained despite filtration of splenocytes through an anti-immunoglobulin column or treatment with carbonyl iron/magnet, excluding a contribution from T and B lymphocytes or macrophages (Herberman et al., 1975a; Kiessling et al., 1975a). Because of their capacity to rapidly secrete lytic molecules and the proinflammatory cytokine IFN- $\gamma$  upon sensing pathogen-derived or host stress ligands through a repertoire of germline-encoded receptors, and because they lack RAG-mediated rearrangement of receptor genes, NK cells were characterized as a component of innate immunity (Anegón et al., 1988; Arase et al., 1996; Ho et al., 2002; Mason et al., 1996; Smith et al., 2000).

However, current evidence has revealed striking similarities between NK cells and adaptive immune cells. NK cells develop from the common lymphoid progenitor (CLP), from which T and B cells and the newly described lineage of innate lymphoid cells (ILCs) are also derived (Kondo et al., 1997). Similar to T and B cells, NK cell development, homeostasis, survival, and function require common- $\gamma$ -chain-dependent cytokine signaling, particularly IL-15 (Cooper et al., 2002; Kennedy et al., 2000; Lodolce et al., 1998). Although NK cells do not require expression of the RAG recombinase for development or generation of their receptor repertoire, nearly a third of peripheral NK cells have a history of RAG expression during ontogeny (Ichii et al., 2010; Karo et al.,



2014). Furthermore, analogous to thymic T cell education, NK cells acquire functional competence through a “licensing” or “arming” process; during development, NK cells become self-tolerant (and thus gain effector function) due to engagement of self-MHC by inhibitory receptors (Orr and Lanier, 2010).

Lastly, NK cells can initiate antigen-specific recall responses. Perhaps the earliest evidence suggesting the possibility of NK cell memory was reported in 1964 in a model of F1 hybrid resistance. Adult F1 hybrid mice (B10 x B10.D2) primed with a bone marrow graft from one parent (B10) rapidly rejected a second bone marrow graft from that same parent (B10) but not the other parent (B10.D2) (Cudkowicz and Stimpfling, 1964). Conversely, priming the F1 hybrids with a B10.D2 or allogeneic graft did not precipitate B10 graft rejection (Cudkowicz and Stimpfling, 1964). Together, these findings suggested that a non-T or -B cell responded in a qualitatively different manner upon re-exposure to previously encountered antigens. The later discovery of the NK cell and the “missing-self” hypothesis (that NK cells selectively kill cells lacking self-MHC class I) implicated NK cells as the cell type mediating F1 hybrid resistance (Kärre et al., 1986). Since then, NK cells have been found to possess a number of adaptive features that have redefined their role in immunity.

## IV. Antigen-Dependent NK Cell Memory<sup>1</sup>

The first evidence of anamnestic NK cell responses was in the setting of contact hypersensitivity responses to chemical haptens. *Rag2*<sup>-/-</sup> mice lacking both T and B lymphocytes exhibited a severe inflammatory reaction when sensitized and re-challenged with the same hapten (either DNFB or oxazolone) (O'Leary et al., 2006). Depletion of NK cells abrogated the contact hypersensitivity, suggesting that NK cells either directly or indirectly were responsible for the observed recall response (O'Leary et al., 2006). Adoptive transfer of DNFB-sensitized NK cells into *Rag2*<sup>-/-</sup> *Il2rg*<sup>-/-</sup> mice was also sufficient to drive contact hypersensitivity upon recipient challenge with DNFB, although transferable hapten-specific recall was retained only in hepatic, but not splenic NK cells (O'Leary et al., 2006; Paust et al., 2010). Specifically, contact hypersensitivity depended on a subset of hepatic NK cells expressing the chemokine receptor CXCR6, which was required for the homeostasis but not antigen-recognition of these cells (Paust et al., 2010). Thus, hepatic NK cells can generate antigen-specific recall responses to haptens, although whether these cells are truly mature NK cells or a distinct subpopulation of the type 1 ILC family is unresolved (O'Sullivan et al., 2016c).

NK cells were also discovered to undergo recall responses against viral pathogens (Sun et al., 2009). During the “expansion” phase of the NK cell response to MCMV infection that peaks at day 7 post-infection, the antigen-specific NK cell compartment has been measured to undergo ~100-1000-fold growth in size (Sun et al., 2009). This proliferative burst is driven by antigen-specific interactions between the activating receptor Ly49H on NK cells and the MHC-I-like viral glycoprotein m157 on the surface of infected cells (Arase et al., 2002; Dokun et al., 2001; Smith et al., 2002). Following

robust expansion of Ly49H<sup>+</sup> NK cells following MCMV infection, these effector cells contract and form a long-lived pool of memory NK cells in both lymphoid and non-lymphoid tissues that is detectable at least 70 days after MCMV infection (Sun et al., 2009). These memory NK cells exhibit enhanced IFN- $\gamma$  production and degranulation compared to naïve NK cells (Sun et al., 2009). The response of memory NK cells re-challenged with MCMV was found to be comparable in both kinetics and magnitude to that of naïve NK cells, yet memory NK cells conferred greater protection to susceptible neonate mice against MCMV challenge (Sun et al., 2009). Thus, MCMV-experienced NK cells are capable of recall responses, enhanced functionality, and protection against repeated MCMV exposure.

Evidence for secondary NK cell responses against different viral pathogens continues to build. Analogous to hapten-specific memory NK cell-mediated contact hypersensitivity responses, adoptively transferred hepatic, but not splenic NK cells, from *Rag1*<sup>-/-</sup> mice immunized with virus-like particles expressing influenza A virus, vesicular stomatitis virus (VSV) or HIV-1 antigens afforded protection to *Rag2*<sup>-/-</sup> *Il2rg*<sup>-/-</sup> hosts challenged with the immunizing, but not unrelated, virus (Paust et al., 2010). Similar immunization-dependent and virus-specific NK cell protective responses in the absence of adaptive immunity have been described for HSV-2, vaccinia virus and influenza virus (Abdul-Careem et al., 2012; Gillard et al., 2011; Helden et al., 2012).

Similar to the expansion of NK cells in MCMV-infected mice, human NK cells expressing the activating heterodimeric CD94/NKG2C receptor are preferentially found in the peripheral blood of healthy individuals seropositive for HCMV, compared to donors who were HCMV-seronegative or seropositive for other herpesvirus infections

(Gumá et al., 2004; Gumá et al., 2006a; Lopez-Vergès et al., 2011). This CD94/NKG2C<sup>+</sup> subset commonly co-expresses the maturation marker CD57 and lacks expression of the inhibitory NKG2A receptor (Foley et al., 2012a; Hendricks et al., 2014; Lopez-Vergès et al., 2010; Lopez-Vergès et al., 2011). Although the ligand driving expansion of CD94/NKG2C<sup>+</sup> NK cells *in vivo* has yet to be elucidated, *in vitro* studies demonstrated that shRNA-mediated knockdown of HLA-E on HCMV-infected fibroblasts abrogated this expansion (Rölle et al., 2014). HCMV reactivation in patients receiving allogeneic hematopoietic stem cell grafts, umbilical cord blood grafts or solid-organ transplants also precipitates expansion of CD94/NKG2C<sup>+</sup> NK cells, followed by persistence of these cells for months to years (Chiesa et al., 2012; Foley et al., 2012b; Horowitz et al., 2015; Lopez-Vergès et al., 2011; Muccio et al., 2016). Furthermore, the transfer of NKG2C<sup>+</sup> NK cells in grafts from HCMV-seropositive donors resulted in enhanced target cell-induced IFN- $\gamma$  production upon secondary CMV exposure in the recipient compared to grafts from HCMV-seronegative donors (Foley et al., 2012a). In combination, these studies highlight the antigen-specificity, longevity and transplantability of human memory NK cell responses to HCMV. A recent study also identified a subset of CD16<sup>+</sup>CD56<sup>+</sup> Fc $\epsilon$ RI $\gamma$ <sup>-</sup> NK cells associated with prior HCMV and HSV-1 infection that persisted upwards of 9 months and was capable of mediating enhanced antibody-dependent cellular cytotoxicity (ADCC) in the presence of HCMV and HSV-1-infected cells coated by virus-specific antibodies (Hwang et al., 2012; Zhang et al., 2013). Thus, human NK cells appear to have evolved redundant mechanisms to generate immunological memory.

Several studies have also demonstrated expansion of long-lived CD94/NKG2C<sup>+</sup> human NK cells in response to HIV-1, hantavirus, chikungunya virus, hepatitis B virus (HBV) or hepatitis C virus (HCV), although it should be noted that this expansion occurred only in individuals previously infected with HCMV (Béziat et al., 2012; Björkström et al., 2011; Brunetta et al., 2010; Gumá et al., 2006b; Petitdemange et al., 2011), suggesting that superinfection with these viruses may trigger HCMV reactivity. Interestingly, a recent study in rhesus macaques established that primate NK cells can achieve pathogen-specific memory independent of HCMV exposure (Reeves et al., 2015). Infection of rhesus macaques with simian-human immunodeficiency virus (SHIV) or SIV elicited splenic and hepatic memory NK cells capable of lysing Gag- and Env-pulsed dendritic cells in an NKG2A- and NKG2C-dependent fashion for as long as 5 years post-infection (Reeves et al., 2015). Vaccinating the macaques with recombinant adenovirus expressing HIV-1 *Env* or SIV *Gag* likewise produced robust, stable, and antigen-specific NK cell memory (Reeves et al., 2015).

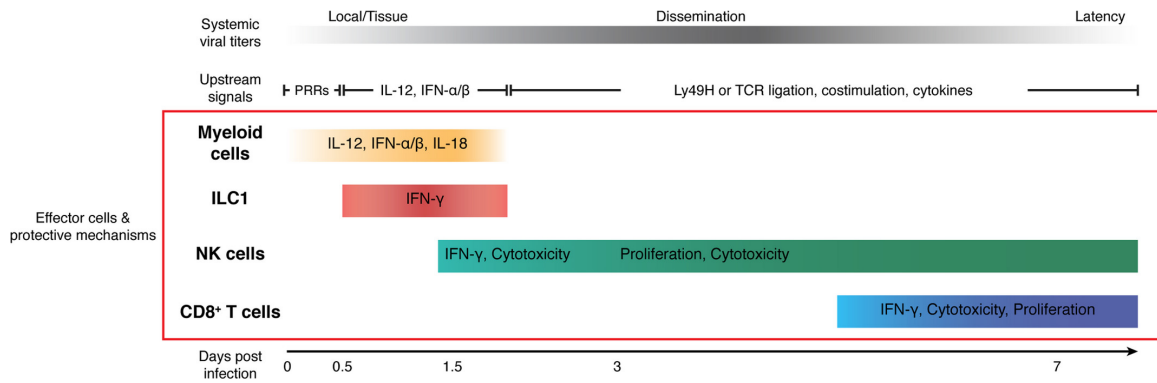
The benefit of pathogen-specific NK cell memory is clear in the case of persistent or repeated encounter with the same virus. However, there is some evidence that suggests there may be a costly trade-off associated with NK cell memory. A recent study highlighted that human NK cell receptor diversity increases with age (Strauss-Albee et al., 2015). CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells that expand during HCMV infection are far from clonal, displaying substantial heterogeneity for other receptors (Horowitz et al., 2013). However, high NK cell receptor diversity was associated with greater risk of HIV-1 acquisition in Kenyan women (Strauss-Albee et al., 2015). Given that viral challenge may enhance NK cell receptor diversity, human NK cells appear to risk unresponsiveness

to novel antigens in order to better protect against previously encountered pathogens (Strauss-Albee et al., 2015). The consequence of NK cell receptor diversity for human health and disease thus requires further exploration.

## **V. NK Cells: One Critical Wave of the Host Antiviral Response to MCMV**

Given that the majority of the evidence for “adaptive” NK cell responses in both mice and humans is in the context of CMV infection, an exploration into the role that NK cells play in combatting CMV infection is warranted. The earliest studies in mice, using adoptive transfer of NK cells into susceptible hosts and depletion of NK cells in immunocompetent hosts, demonstrated that NK cells are required to control MCMV infection (Bukowski et al., 1985; Bukowski et al., 1983; Welsh et al., 1990). Follow-up genetic studies led to the understanding that NK cell-mediated control was dependent on the activating NK cell receptor Ly49H (Brown et al., 2001; Daniels et al., 2001; Lee et al., 2001; Scalzo et al., 1990; Scalzo et al., 1995). NK cells are similarly necessary for the control of HCMV in humans as evidenced by patients with rare NK cell deficiencies who present with complications related to herpesvirus infections (Biron et al., 1989; Etzioni et al., 2005; Orange, 2013). Although NK cells are essential for herpesvirus control, their responses do not occur in isolation. Here, we will briefly describe the waves of antiviral responses to CMV that precede and succeed those of NK cells (Figure 2).

The first antiviral wave comes in the form of proinflammatory cytokine release by diverse populations of myeloid cells upon recognition of viral products by pattern recognition receptors (PRRs), reviewed in (Adams and Sun, 2018). In particular, a spike in the serum concentration of type 1 IFNs, IL-12, and IL-18 are detected 36 hours post-infection (Pien et al., 2000; Ruzek et al., 1997). The necessity of these proinflammatory cytokines is illustrated by impaired viral control and death in mice in which the



**Figure 2. Regulation, Localization, and Function of the Cellular Components Mediating Immunity to MCMV<sup>4</sup>**

PRR activation and signaling (including TLR7, TLR9, AIM2, and cGAS-STING) induces proinflammatory cytokine production by myeloid cells (antiviral wave 1). IL-12 production by cDC1s in turn stimulates secretion of protective ILC1-derived IFN- $\gamma$  (antiviral wave 2). These first two waves are initiated in tissue at the site of viral entry. Myeloid cell-derived IL-12 and IL-18 also trigger IFN- $\gamma$  production by NK cells, and type 1 IFNs enhance NK cell cytotoxicity. Along with Ly49H engaging MCMV-encoded m157 on infected cells, IL-12, type 1 IFN, and costimulatory signals synergize to drive prolific clonal expansion of Ly49H<sup>+</sup> NK cells (antiviral wave 3) as MCMV disseminates. During this time, an antigen-specific T-cell response, dependent on the canonical three signals (TCR, costimulation, and proinflammatory cytokines), begins to develop against MCMV to keep the virus in latency (antiviral wave 4). Colored bars span the duration of a given cellular response, with the color saturation representing the magnitude of the response (or viral burden in peripheral blood) at that time.

<sup>4</sup>Copyright © 2018 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd in *Immunological Reviews*. Adams, N.M., and Sun, J.C. Spatial and temporal coordination of antiviral responses by group 1 ILCs. *Immunol. Rev.* 286: 23-36. Reproduced with permission of John Wiley & Sons A/S via Copyright Clearance Center. <https://doi.org/10.1111/imr.12710>



production or sensing of IL-12 or type 1 IFNs is abolished (Gil et al., 2001; Orange and Biron, 1996; Pien et al., 2000). We now appreciate that myeloid-derived proinflammatory cytokine production is critical for initiating and regulating the subsequent responses of group 1 ILCs.

The second wave of antiviral host protection against MCMV is mediated by ILC1-derived IFN- $\gamma$  at the initial sites of viral infection, particularly in the peritoneal cavity and the liver following i.p. administration of MCMV (Weizman et al., 2017). ILC1 production of IFN- $\gamma$  is critically dependent on IL-12 production by tissue-resident cDC1s, the major source of this cytokine during early infection (Weizman et al., 2017). This wave of ILC1-derived IFN- $\gamma$  precedes IFN- $\gamma$  production by a number of other innate and adaptive lymphocytes. The status of ILC1s as “poised” effectors is facilitated by a number of factors, including 1) the positioning of ILC1s within the infected tissue, where they are likely to experience a spike in IL-12 prior to appreciable IL-12 appearance in blood, 2) their IFN- $\gamma$  production being independent of IL-18, and 3) high basal expression of IL-12 receptor components and chromatin accessibility of the *Ifng* promoter (Weizman et al., 2017). Together, these enable ILC1s to restrict MCMV replication prior to the initiation of NK cell and CD8<sup>+</sup> T cell responses.

As proinflammatory cytokines and virus disseminate from tissue into the bloodstream, the response of circulating NK cells is recruited. NK cells provide varied responses, including production of IFN- $\gamma$ , cytotoxicity against infected or stressed cells, and a robust proliferative burst mediated by signaling through the activating receptor Ly49H (the “adaptive” response). The molecular mechanisms regulating the “adaptive”

NK cell response, which lead to the generation of the memory NK cell pool, is the topic of discussion in the next section of the introduction.

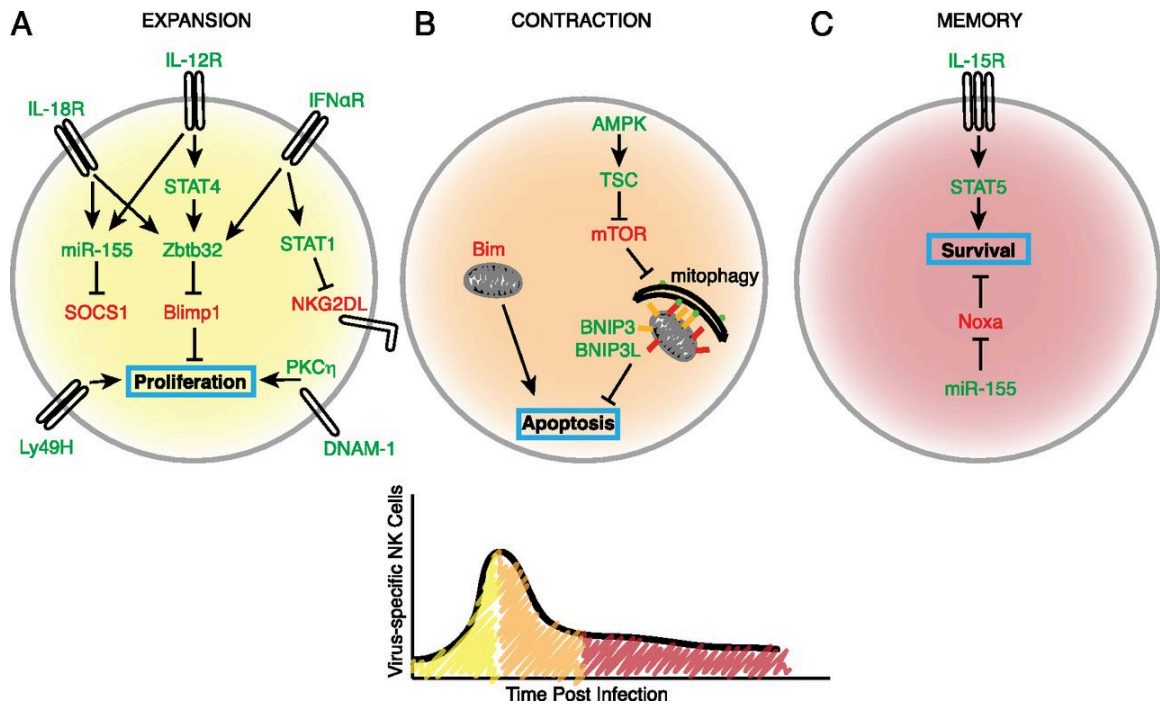
Although NK cells are necessary for control of CMV, they are not sufficient by themselves. This is evidenced by the fact that adaptive lymphocyte-deficient mice (C57BL/6 SCID and RAG1-deficient) ultimately succumb to MCMV infection (French et al., 2004). Indeed CD8<sup>+</sup> T cells have been shown to mount multiple epitope-specific responses to MCMV that span four different kinetic patterns and lead to control of MCMV through both perforin and IFN- $\gamma$  (Klenerman and Oxenius, 2016; Munks et al., 2006). Furthermore, CD4<sup>+</sup> T cells can restrict the viral replication and shedding of MCMV in the salivary gland through production of IFN- $\gamma$  (Jonjic et al., 1989; Walton et al., 2011). Importantly, NK cells can regulate T cell responses through a number of mechanisms, including at the level of T cell priming by DCs, Th1 polarization, and their prolonged activation (Vivier et al., 2011; von Burg et al., 2015).

Thus, the waves of antiviral responses mediated by myeloid cells, innate lymphocytes, and adaptive lymphocytes are highly integrated and coordinated to achieve optimal control of MCMV. In the next section, we will review the molecular mechanisms that drive “adaptive” NK cell responses during MCMV infection.

## **VI. Mechanisms of NK Cell Memory Generation during Viral Infection<sup>1</sup>**

Analogous to the generation of T cell memory against pathogens (Williams and Bevan, 2007), NK cells progress through three phases during their response to MCMV: expansion, contraction, and memory maintenance (Sun and Lanier, 2011). During each stage, both intracellular and extracellular cues are necessary for establishing a long-lived memory NK cell pool (Figure 3). In addition to antigen engagement by activating receptors (analogous to TCR engagement for T cell activation), NK cells require proinflammatory cytokine signaling for robust expansion (Williams and Bevan, 2007). The proinflammatory cytokine IL-12, through a STAT4-dependent, but IFN- $\gamma$ -independent mechanism, is indispensable for optimal MCMV-specific NK cell clonal expansion as well as memory NK cell formation (Sun et al., 2012). IL-12 and STAT4 may be responsible for programming activated NK cells early during MCMV infection for memory formation (Sun et al., 2012). Members of the IL-1 family of cytokines, particularly IL-33 and IL-18, are similarly necessary for amplifying NK cell proliferation in response to MCMV, but are dispensable for recall responses (Madera and Sun, 2015; Nabekura et al., 2015). A recent study also identified a role for type I interferon and downstream STAT1 signaling in shielding proliferating NK cells from fratricide (killing by other NK cells) by modulating their cell surface expression of NK group 2 member D (NKG2D) ligands (Madera et al., 2016).

Interestingly, IL-12, IL-18, and type I interferon signaling act synergistically to drive maximal expression of the BTB-ZF family transcription factor Zbtb32, which controls the proliferative burst of virus-specific NK cells by antagonizing the anti-proliferative factor Blimp-1 (Beaulieu et al., 2014). In parallel, IL-12- and IL-18-



**Figure 3. Regulation of NK Cells during Each Phase of the Response to Viral Infection<sup>1</sup>**

(A) Expansion. IL-12, IL-18, and type I IFN converge to drive Ly49H<sup>+</sup> NK cell proliferation by inducing Zbtb32 and subsequently suppressing Blimp-1. IL-12 and IL-18 also cooperate to regulate SOCS1 in an miR-155-dependent mechanism. Signaling through IFN- $\alpha$ R also independently contributes to memory formation by protecting against NKG2D-mediated fratricide. The costimulatory molecule DNAX accessory molecule 1 (DNAM-1) facilitates a protein kinase C isoform  $\eta$  (PKC $\eta$ )-dependent proliferative burst.

(B) Contraction. Memory formation hinges on averting Bim-mediated mitochondrial apoptosis. Recycling dysfunctional mitochondria via mitophagy mediated by BNIP3 and BNIP3-like (BNIP3L) promotes the contraction-to-memory phase transition.

(C) Memory maintenance. IL-15 signaling is required as NK cells contract and for the maintenance of memory cells in peripheral tissues. miR-155-mediated suppression of Noxa promotes memory NK survival. Green font represents positive regulators of memory. Red font represents negative regulators of memory. Cell colors correspond to the infection time course. AMPK, AMP-activated protein kinase; mTOR, mechanistic target of rapamycin; NKG2DL, NKG2D ligand.

<sup>1</sup>Originally published in *The Journal of Immunology*. Adams, N.M., O’Sullivan, T.E., Geary, C.D., Karo, J.M., Amezquita, R.A., Joshi, N.S., Kaech, S.M., and Sun, J.C. 2016. NK Cell Responses Redefine Immunological Memory. *J. Immunol.* 197: 2963-2970. Copyright © [2016] The American Association of Immunologists, Inc. <https://doi.org/10.4049/jimmunol.1600973>

mediated induction of miR-155 regulates effector and memory NK cell numbers during MCMV infection by regulating targets such as Noxa and SOCS1 (Zawislak et al., 2013). Although the mechanism by which SOCS1 impairs the development of effector NK cells is unclear, the potent restraint that constitutive SOCS1 activity places on STAT signaling may have some influence. Lastly, akin to the necessity of costimulation for T cell activation (“signal 2”), NK cells require the costimulatory molecule DNAX accessory molecule 1 (DNAM-1) and downstream signaling through the Src-family tyrosine kinase Fyn and the serine-threonine protein kinase C isoform  $\eta$  (PKC $\eta$ ) for optimal expansion of effector NK cells and their differentiation into memory NK cells (Nabekura et al., 2014).

Following viral infection, contraction of effector lymphocytes serves to eliminate activated CD8<sup>+</sup> T and NK cells to stave off immunopathology (Hildeman et al., 2007; Hildeman et al., 2002). Mitochondrial apoptosis mediated by the proapoptotic factor Bim shapes the size, maturity, and functionality of the memory NK cell pool in response to MCMV (Min-Oo et al., 2014), similar to CD8<sup>+</sup> T cells (Prlic and Bevan, 2008). The accumulation of depolarized mitochondria and mitochondrial-released reactive oxygen species (ROS) in effector NK cells after virus-driven expansion results in either cell death, or clearance of damaged mitochondria, resulting in NK cell survival (O'Sullivan et al., 2015). Analogous to the autophagy-dependent survival and memory formation of virus-specific effector CD8<sup>+</sup> T cells (Puleston et al., 2014; Schlie et al., 2015; Xu et al., 2014), surviving NK cells undergo the self-catabolic process of mitophagy during the contraction-to-memory phase transition, requiring the autophagosome machinery component *Atg3* and the mitophagy-specific receptors BCL2/adenovirus E1B 19-kDa

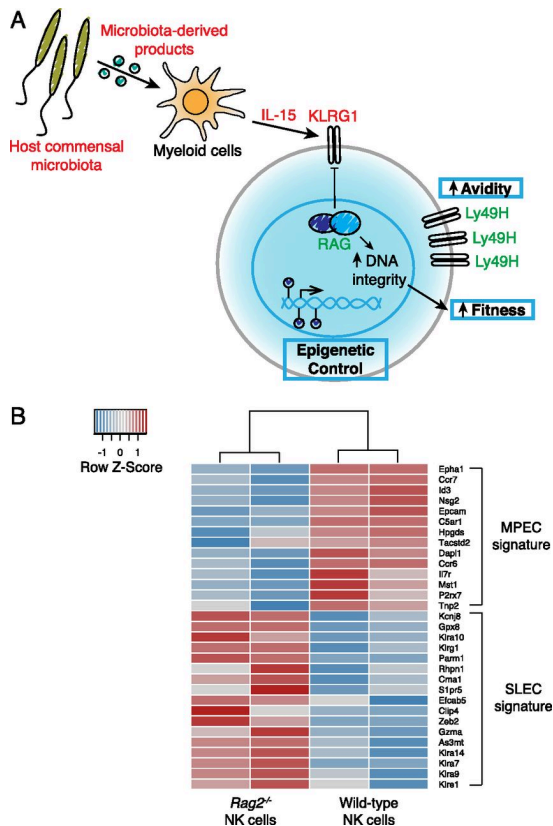
interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L or Nix) to promote their survival (O'Sullivan et al., 2015). Mitophagy in contracting NK cells is induced by mechanistic target of rapamycin (mTOR) inhibition or AMP-activated protein kinase (AMPK) activation (O'Sullivan et al., 2015), suggesting that mitophagy may also link other cellular metabolic processes, similar to the catabolic processes memory T cells employ to fuel oxidative phosphorylation during non-proliferative states (Buck et al., 2015).

In addition to intracellular mechanisms, extracellular cues can also promote the maintenance of NK cell memory. Adoptive transfer of Ly49H<sup>+</sup> NK cells isolated from MCMV-infected hosts at day 7 or day 21 post-infection into IL-15-deficient recipients led to decreased persistence of the transferred cells, supporting a role for IL-15-dependent maintenance of NK cells during contraction (Firth et al., 2013). IL-15 was previously shown to promote NK cell survival via Mcl-1 (Huntington et al., 2007). miR-155-mediated suppression of Noxa may also aid long-term survival of memory NK cells (Zawislak et al., 2013). Thus, generating long-lived, MCMV-specific memory NK cells requires a complex combination of intracellular and extracellular signals during both the early and late phases of the antiviral response.

## VII. Identifying Memory NK Cell Precursors<sup>1</sup>

During viral infection, two subsets of effector CD8<sup>+</sup> T cells have been described to develop: terminally differentiated KLRG1<sup>hi</sup> short-lived effector cells (SLECs) that die after infection, and KLRG1<sup>lo</sup> memory precursor effector cells (MPECs) that are long-lived and participate in secondary responses (Kaech and Wherry, 2007). Analogous to CD8<sup>+</sup> T cells, recent evidence supports the idea of heterogeneity within antiviral NK cell populations that can dictate memory potential. KLRG1<sup>lo</sup> Ly49H<sup>+</sup> NK cells preferentially generate memory NK cells compared to KLRG1<sup>hi</sup> cells, which have a limited capacity for MCMV-driven expansion (Kamimura and Lanier, 2015). KLRG1 is associated with NK cell maturation, as indicated by the greater percentage of KLRG1<sup>hi</sup> NK cells that are also of the most mature CD11b<sup>+</sup> CD27<sup>-</sup> phenotype, suggesting that NK cell maturation is antagonistic to their memory potential (Kamimura and Lanier, 2015). However, because memory NK cells are themselves KLRG1<sup>hi</sup> CD11b<sup>+</sup> CD27<sup>-</sup>, yet still competent to undergo robust secondary expansion following MCMV challenge, KLRG1 alone does not dictate proliferative potential (Kamimura and Lanier, 2015; Sun et al., 2009).

Interestingly, KLRG1 expression on naïve NK cells may be modulated by prior RAG activity. NK cells with a history of RAG activity during ontogeny preferentially expand and persist as memory cells following MCMV infection (Figure 4A), and thus preferential memory formation correlates with lower KLRG1 expression in the NK cells that have expressed RAG (Karo et al., 2014). The absence of RAG expression in developing NK cells also results in diminished expression of the DNA damage repair machinery and a subsequent impairment in DNA double-strand break (DSB) resolution following DNA damage (Karo et al., 2014). Thus, RAG activity confers NK cells with



**Figure 4. Functional Heterogeneity within the Effector NK Cell Pool<sup>1</sup>**

(A) Multiple mechanisms regulate memory precursor identity in NK cells. KLRG1 expression inversely correlates with NK cell memory potential. RAG expression during ontogeny not only negatively regulates KLRG1 expression but also promotes enhanced NK cell fitness by supporting optimal expression of DNA damage repair enzymes to maintain DNA integrity. Host commensal microbiota-derived products and IL-15 signaling, the availability of which is determined by competition with conventional T cells, drive NK cell expression of KLRG1. Greater avidity for ligand via higher cell surface concentration of Ly49H and epigenetic programs that drive a particular suite of memory genes may also converge to dictate NK cell memory precursor identity. Green font represents positive regulators of memory potential. Red font represents negative regulators of memory potential.

(B) Comparison of the gene expression profile of *Rag2*<sup>-/-</sup> and wild-type NK cells with that of MPECs and SLECs. *Rag2*<sup>-/-</sup> and wild-type NK cells were purified from mixed bone marrow chimeric mice and RNA sequencing was performed. Heat map shows the relative mRNA expression in *Rag2*<sup>-/-</sup> and wild-type NK cells of the top differentially expressed genes between MPEC and SLEC populations, as previously described (Dominguez et al., 2015). The transcriptional signature of *Rag2*<sup>-/-</sup> NK cells resembles that of SLECs whereas wild-type NK cells exhibit an MPEC-like signature.

<sup>1</sup>Originally published in *The Journal of Immunology*. Adams, N.M., O’Sullivan, T.E., Geary, C.D., Karo, J.M., Amezcua, R.A., Joshi, N.S., Kaech, S.M., and Sun, J.C. 2016. NK Cell Responses Redefine Immunological Memory. *J. Immunol.* 197: 2963-2970. Copyright © [2016] The American Association of Immunologists, Inc. <https://doi.org/10.4049/jimmunol.1600973>



enhanced cellular fitness by maintaining global genomic stability and survival despite the DNA damage that occurs during rapid proliferation. Furthermore, *Rag2*<sup>-/-</sup> NK cells display a transcriptional signature markedly similar to that observed in SLECs rather than MPECs (SLEC and MPEC signatures published in (Dominguez et al., 2015)) (Figure 4B), supporting the idea that RAG dictates functional heterogeneity within the NK cell compartment. It will be of interest to determine whether the degree of genomic integrity similarly specifies SLEC versus MPEC fate.

KLRG1 expression is also influenced in a cell-extrinsic manner. T cells can restrain NK cell maturation by limiting the availability of IL-15, driving the preferential generation of KLRG1<sup>lo</sup> memory progenitors at the expense of KLRG1<sup>hi</sup> NK cells (Figure 4A) (Kamimura and Lanier, 2015). Signals derived from the host commensal microbiota also appear to control expression of KLRG1 in NK cells (Figure 4A) (Kamimura and Lanier, 2015). Treatment with broad-spectrum antibiotics in the drinking water diminished KLRG1 expression and boosted the frequency of memory NK cells compared with untreated wild-type mice, suggesting that the host microbiota regulates the NK cell pool containing memory potential (Kamimura and Lanier, 2015). Thus, the foundation for NK cell memory formation may be laid even prior to encountering virus.

MCMV-specific memory NK cells display greater cell surface expression of not only KLRG1 but also Ly49H compared with naïve NK cells, and expression of these receptors is further enhanced in secondary memory NK cells (Sun et al., 2010). In contrast, naïve, memory and secondary memory NK cells express comparable levels of the activating receptors NK1.1 and Ly49D (Sun et al., 2010). These data together imply that there may be selection for NK cells with the greatest avidity for ligand during

successive rounds of MCMV infection (Figure 4A). T cells undergo a process of affinity maturation, whereby T cells bearing TCRs with highest affinity for peptide rise to clonal dominance during the response to a pathogen (Bachmann et al., 1997; Busch and Pamer, 1999; Busch et al., 1998; McHeyzer-Williams and Davis, 1995). A similar process may occur with NK cells responding against infection. There is currently no evidence to support the idea that NK cells undergo somatic mutation of their antigen receptor genes (i.e. the affinity of each Ly49H receptor for m157 is equivalent), indicating that NK cell avidity for viral antigen is determined solely by the number of Ly49H receptors on the surface of a given NK cell. However, it remains to be determined at what stage of the antiviral response activating signals through Ly49H select for memory precursors to constitute the long-lived pool.

Emerging evidence also illustrates the contribution of the activating receptor Ly49D and the inhibitory receptor Ly49A, both of which recognize the MHC class I molecule H-2D<sup>d</sup>, during the NK cell memory response to MCMV (Nabekura and Lanier, 2016). Adoptive transfer of B10.D2 (H-2D<sup>d</sup>-sufficient background) NK cells into MCMV-infected syngeneic Ly49H-deficient recipients demonstrated that Ly49D<sup>+</sup>Ly49A<sup>-</sup>Ly49H<sup>+</sup> NK cells preferentially differentiated into memory NK cells compared with Ly49D<sup>-</sup>Ly49A<sup>+</sup>Ly49H<sup>+</sup> NK cells (Nabekura and Lanier, 2016). Similar to how MCMV infection can break the anergic state of unlicensed NK cells in B6 mice, it appears that acute viral infection can also breach tolerance of activating receptors for self-MHC class I, a phenomenon that has functional consequences for host protection and NK cell memory (Nabekura and Lanier, 2016; Orr et al., 2010; Sun and Lanier, 2008; Tay et al., 1995).

Recent human studies have suggested that epigenetic heterogeneity is also associated with differential capacity for NK cell longevity (Figure 4A). The aforementioned CD16<sup>+</sup> CD56<sup>+</sup> FcεRIγ<sup>-</sup> NK cells are a subset of memory-like NK cells that can be isolated from HCMV-seropositive individuals, although they can be characterized by either the absence or presence of CD94/NKG2C (Hwang et al., 2012; Lee et al., 2015; Schlums et al., 2015; Zhang et al., 2013). Interestingly, this population lacks expression of the tyrosine kinase SYK, the signaling adaptors DAB2 and EAT-2, and the transcription factors promyelocytic leukemia zinc finger protein (PLZF) and Helios due to promoter hypermethylation at several of these loci (Lee et al., 2015; Schlums et al., 2015). Another study has identified epigenetic imprinting at the *Ifng* conserved non-coding sequence (CNS) 1 in NKG2C<sup>+</sup> NK cells from HCMV-seropositive individuals that is critical for *Ifng* transcriptional activity in response to stimulation through NKG2C (Luetke-Eversloh et al., 2014). It is unclear whether this epigenetic heterogeneity is a cause or consequence of NK cell memory. Nevertheless, receptor heterogeneity, the microbiota, and history of RAG expression during ontogeny precipitate differential fitness of cells within the naïve NK pool. During viral infection, selective pressure may then be acting on the effector pool to preferentially select memory precursors to establish the memory pool.

## VIII. Antigen-Independent NK Cell Memory<sup>1</sup>

Although proinflammatory cytokine signaling is critical in driving the clonal expansion and maintenance of MCMV-specific memory NK cells, proinflammatory cytokines alone were found to be capable of supporting NK cell memory properties in the absence of antigen. Splenic NK cells pre-activated with a cocktail of IL-12, IL-15, and IL-18, and adoptively transferred into *Rag1*<sup>-/-</sup> mice become long-lived and can be identified in these recipients up to 3 weeks following transfer (Cooper et al., 2009). These cytokine-induced memory NK cells retained a cell-intrinsic capacity for enhanced IFN- $\gamma$  production, but not cytotoxicity, when re-stimulated with cytokine, plate-bound antibody or target cells (Cooper et al., 2009; Keppel et al., 2013). Human NK cells pre-activated with cytokines also displayed the same properties (Romee et al., 2012). The progeny of cytokine-activated NK cells similarly exhibited enhanced effector functions (Cooper et al., 2009; Romee et al., 2012), suggesting that NK cell memory properties may be epigenetically inherited. These data may have implications for the antigen-independent self-renewal of memory NK cells following clearance of pathogens, although future studies are necessary to dissect the contribution of cytokine-induced memory NK cells during a recall response to a pathogen against which pathogen-specific memory NK cells already exist. Given that antigen-specific NK cells demonstrate diminished bystander activation to heterologous infection (Min-Oo and Lanier, 2014), cytokine-induced memory NK cells may represent a strategy for the host to nonspecifically respond to a new proinflammatory stimulus. Recent studies have shown that NK cells pre-activated with IL-12, IL-15, and IL-18 demonstrate enhanced persistence and antitumor activity against established, irradiated mouse tumors compared with naïve NK cells, suggesting that harnessing the sustained

effector functions of cytokine-activated NK cells may represent a potential enhancement to adoptive NK cell immunotherapy (Ni et al., 2012). ILCs are thought to respond exclusively to cytokine cues (Sonnenberg and Artis, 2015), and it will be of interest to determine whether cytokines can similarly support longevity in other innate lymphocytes.

Lastly, in an NK cell-deficient lymphopenic host, adoptively transferred NK cells undergo a rapid, antigen-independent homeostatic proliferation to fill the empty niche, a process that requires common- $\gamma$ -chain-dependent cytokine signaling (Jamieson et al., 2004; Prlic et al., 2003; Ranson et al., 2003). Following homeostatic proliferation in *Rag2<sup>-/-</sup> Il2rg<sup>-/-</sup>* or sublethally irradiated recipients, adoptively transferred NK cells contract to form a long-lived population that persists in both lymphoid and nonlymphoid organs for at least 6 months (Sun and Lanier, 2011). These NK cells display an enhanced capacity for IFN- $\gamma$  production and degranulation when stimulated *ex vivo* 10 days after transfer, but the functionality of these homeostatically-driven NK cells following contraction is unclear (Sun and Lanier, 2011). Nevertheless, homeostatically-expanded NK cells self-renew and are capable of mounting a robust proliferative response when challenged with MCMV 6 months after transfer, thus sharing some properties with MCMV-specific memory NK cells (Sun and Lanier, 2011). Emerging evidence demonstrates that *Atg5*-mediated autophagy is critical for the survival of NK cells during homeostatic proliferation, suggesting that this is one mechanism by which NK cells acquire memory properties following lymphopenia-driven proliferation (O'Sullivan et al., 2016a).

## **IX. Conclusions**

NK cells are versatile cells that contribute to CMV control through both classical innate and newly appreciated “adaptive” responses. However, we are only beginning to understand the molecular mechanisms that regulate the “adaptive” responses they exhibit during their dynamic lifespan. Here, we identify the transcription factor IRF8 as a key driver of NK cell proliferation and expansion during MCMV infection that promotes optimal viral control. We also describe how the avidity of the interaction between NK cells and infected cells, imparted by the expression level of the activating receptor Ly49H, dictates the effector function of a given NK cell during MCMV infection. With a growing body of knowledge and a good model system, the field is now ripe for further exploration into whether other ILC subsets can similarly generate “adaptive” responses, whether other activating NK cell receptors can support “adaptive” responses, whether human NK cells share the same mechanistic requirements for their adaptive responses, and the physiological role that NK cell memory plays in host defense.

# **Chapter 2: Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response<sup>2</sup>**

## **I. Introduction<sup>2</sup>**

### **1. “Adaptive Responses of NK Cells”**

Natural killer (NK) cells are innate lymphocytes capable of killing stressed, transformed, or infected cells without prior sensitization (Lanier, 2005). Their germline-encoded receptor repertoire and status as poised effectors classically position NK cells as cells of the innate immune system. However, more recent evidence suggests that NK cells possess features of adaptive immunity, including their derivation, requirements for homeostatic maintenance, and acquisition of functional competence (Sun and Lanier, 2011). Recent studies demonstrate that NK cells undergo a robust burst of clonal proliferation during mouse cytomegalovirus (MCMV) infection to promote viral clearance (Daniels et al., 2001; Dokun et al., 2001; Sun et al., 2009), and establish a long-lived memory population with enhanced protective function against MCMV reinfection (Sun et al., 2009), functions thought to be limited solely to T and B cells of the adaptive immune system.

During MCMV infection, NK cells mediate this “adaptive” antiviral response by binding the viral glycoprotein m157 on infected cells with the DAP12-dependent activating receptor Ly49H (Arase et al., 2002; Daniels et al., 2001; Dokun et al., 2001; Sun et al., 2009). In addition to detection of viral ligands, these adaptive NK cell responses critically require proinflammatory cytokines, particularly interleukin-12 (IL-

12), IL-18, and type I interferons, which play differential roles in supporting NK cell proliferation and survival during expansion, and imprinting the effector to memory NK cell transition (Madera et al., 2016; Madera and Sun, 2015; Sun et al., 2012). Nevertheless, the transcriptional regulators NK cells employ to integrate these signals, and the transcriptional programs they drive to generate antiviral responses, are only beginning to be elucidated.

## **2. IRF8 in the Immune System**

The interferon regulatory factor (IRF) family of transcription factors consists of nine members in mammals with differential dependencies on type I and type II interferon signaling and pleiotropic functions both within and outside of the immune system (Tamura et al., 2008). Our current understanding of the requirement for IRF family members in mouse NK cell development and function is limited to IRF1 and IRF2. NK cell development is impaired in germline *Irf1*<sup>-/-</sup> mice (Duncan et al., 1996; Ogasawara et al., 1998; Taki et al., 1997); however, this was demonstrated to be secondary to IRF1-dependent IL-15 production by radiation-resistant bone marrow stromal cells that support NK cell development (Ogasawara et al., 1998). In contrast, IRF2 is thought to be required in a cell-intrinsic manner to support the survival of mature peripheral NK cells (Lohoff et al., 2000; Taki et al., 2005). More recently, a clinical study identified compound heterozygous or homozygous *IRF8* mutations that segregated with severe, and in some cases fatal, viral susceptibility in 3 unrelated families (Mace et al., 2017). These patients possessed a greatly diminished number of mature NK cells and reduced NK cell cytolytic



function, suggesting a role for IRF8 in NK cell development and function. However, the direct *in vivo* function of IRF8 in NK cells has not been established.

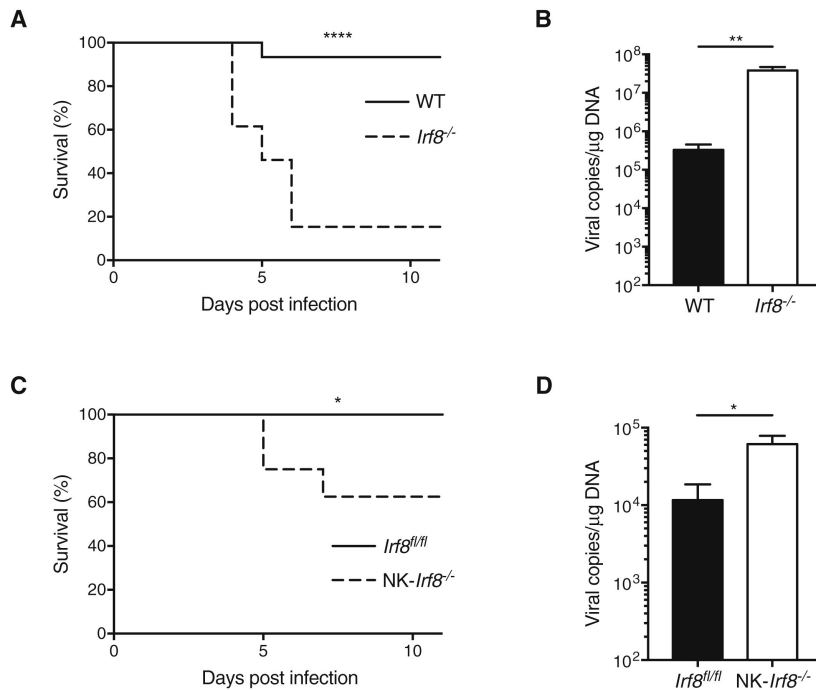
Expression of IRF8 (also known as ICSBP) is restricted to the immune system, and a growing number of studies has revealed the critical and divergent roles that IRF8 plays in the transcriptional regulation of hematopoiesis and peripheral immune responses, including monocyte and dendritic cell (DC) lineage commitment (Holtschke et al., 1996; Schiavoni et al., 2002; Tamura et al., 2000), B cell development (Lu et al., 2003) and germinal center reactions (Lee et al., 2006; Xu et al., 2015a), T helper-1 (Th1) cell differentiation (Giese et al., 1997; Scharon-Kersten et al., 1997), and thymic selection (Herzig et al., 2017). Given its prominent role in lymphocyte biology, and its frequent mutation in familial cases of NK cell deficiency and viral susceptibility, we hypothesized that IRF8 may act as an essential regulator of NK cell antiviral responses.

In this study, we show that the transcription factor IRF8 played a critical and non-redundant role in facilitating the proliferative burst of virus-specific NK cells during acute viral infection, but was dispensable for their development. We used conditional *Irf8* ablation to demonstrate that IRF8 was required in a cell-intrinsic manner for host protection against lethal viral challenge, consistent with the induction of IRF8 during early NK cell activation. We found that the IL-12 and STAT4 signaling axis promoted IRF8 upregulation, which drove the proliferation of antigen-specific NK cells by activating a transcriptional program that promoted cell cycle progression and expression of the pro-proliferative transcription factor *Zbtb32*. These findings attribute a function and regulation for IRF8 in NK cell-mediated host immunity.

## II. Results<sup>2</sup>

### 1. NK Cells Require IRF8 for Antiviral Immunity<sup>2</sup>

NK cells play an essential, dominant role in control of MCMV and human cytomegalovirus (HCMV) infection in mice and humans, respectively (Biron et al., 1989; Bukowski et al., 1985; Etzioni et al., 2005). To determine whether IRF8 was required for host antiviral immunity, we first challenged wild-type (WT) and *Irf8*<sup>-/-</sup> mice with a lethal dose of MCMV. Compared to WT mice, *Irf8*<sup>-/-</sup> mice rapidly succumbed to MCMV infection due to elevated viral burden (Figures 5A and 5B). Loss of IRF8-dependent CD8α<sup>+</sup> DCs in *Irf8*<sup>-/-</sup> mice impairs IL-12 production (Giese et al., 1997; Schariton-Kersten et al., 1997), which is required to prime NK cells (Sun et al., 2012). Therefore, to investigate whether NK cells require IRF8 in a cell-intrinsic fashion for MCMV control, we began by generating a genetic mouse model with conditional deletion of *Irf8* exon 2 specifically in NK cells (*Nkp46*<sup>iCre/+</sup> *Irf8*<sup>fl/fl</sup>, herein designated NK-*Irf8*<sup>-/-</sup>) (Figure 6A). NK cell numbers, frequency, maturation, and receptor repertoire were unaffected by loss of IRF8 (Figures 6B-6E), suggesting this transcription factor was dispensable during NK cell development and homeostasis. To test the protective capacity of IRF8-deficient NK cells, we challenged NK-*Irf8*<sup>-/-</sup> and Cre-negative littermate control mice (*Irf8*<sup>fl/fl</sup>) with a lethal dose of MCMV. NK-*Irf8*<sup>-/-</sup> mice exhibited poorly controlled viral replication, and many failed to survive beyond 4 days post infection (PI) (Figures 5C and 5D). Thus, IRF8 is required in a cell-intrinsic manner for protective antiviral responses by NK cells.



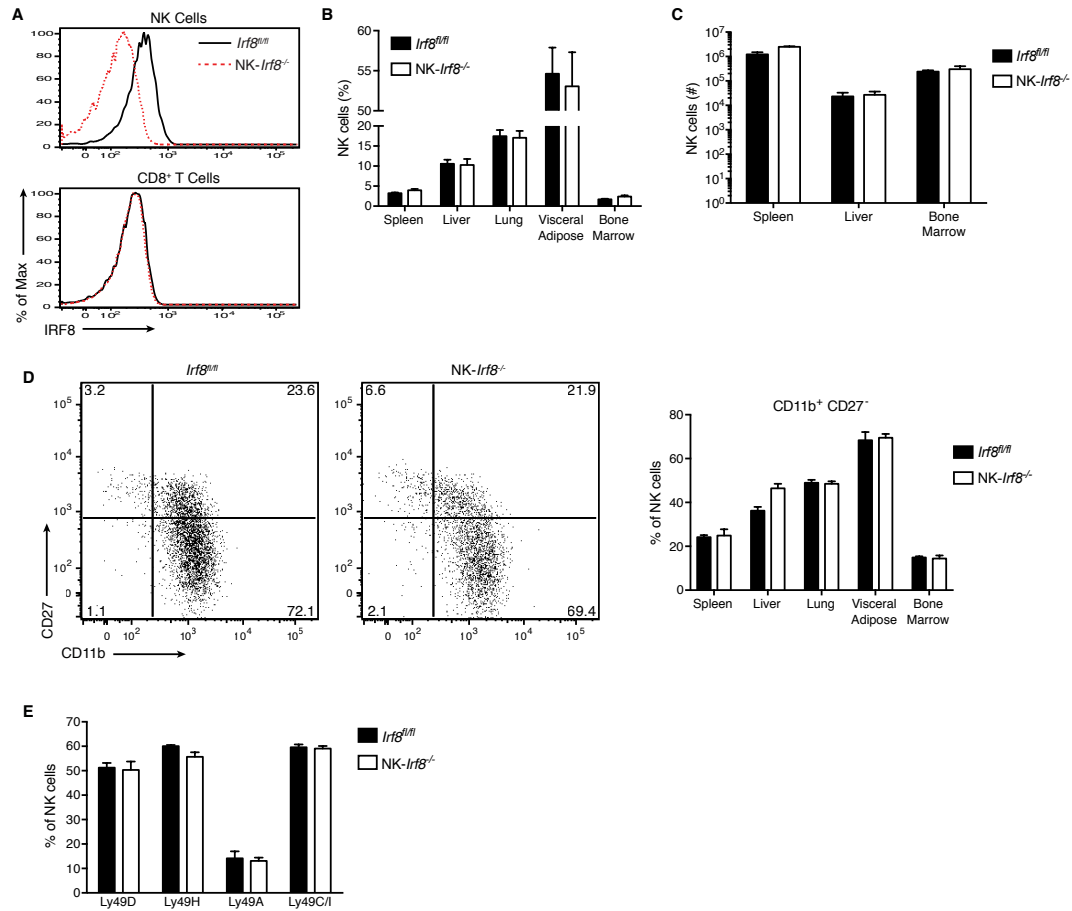
**Figure 5. IRF8 Is Required for NK-Cell-Mediated Host Protection against MCMV Infection<sup>2</sup>**

(A and B) Kaplan-Meier survival curves (A) and peripheral-blood viral titers at PI day 4 (B) for WT mice (solid line) and *Irf8*<sup>-/-</sup> mice (dashed line) challenged intraperitoneally (i.p.) with a lethal dose of MCMV.

(C and D) Kaplan-Meier survival curves (C) and peripheral-blood viral titers at PI day 4 (D) for littermate control *Irf8*<sup>fl/fl</sup> mice (solid line) and NK-*Irf8*<sup>-/-</sup> mice (dashed line) challenged i.p. with a lethal dose of MCMV.

Data are representative of three independent experiments each with three to six mice per group. Groups were compared with the Gehan-Breslow-Wilcoxon test (A and C) or an unpaired, two-tailed Student's t test (B and D). Data are presented as the mean ± SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.0001.

<sup>2</sup>Originally published in *Immunity*. Adams, N.M., Lau, C.M., Fan, X., Rapp, M., Geary, C.D., Weizman, O.E., Diaz-Salazar, C., and Sun, J.C. 2018. Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity*. 48: 1172-1182. Copyright © [2018] Elsevier, Inc. Reproduced with permission of Elsevier, Inc via Copyright Clearance Center. <https://doi.org/10.1016/j.immuni.2018.04.018>



## Figure 6. IRF8 Is Dispensable for NK Cell Development and Terminal Maturation<sup>2</sup>

(A) Representative histograms of IRF8 expression in NK cells (TCR $\beta$ <sup>-</sup>CD3<sup>-</sup>CD19<sup>-</sup>F4/80<sup>-</sup>CD45<sup>+</sup>NK1.1<sup>+</sup>) and CD8<sup>+</sup> T cells (NK1.1<sup>-</sup>TCR $\beta$ <sup>+</sup>CD3<sup>+</sup>CD45<sup>+</sup>CD8 $\alpha$ <sup>+</sup>) from *NK-Irf8<sup>-/-</sup>* and littermate control *Irf8<sup>fl/fl</sup>* mice.

(B and C) Percent NK cells of total CD45<sup>+</sup> lymphocytes (B) and NK cell numbers (C) in various organs of 7 week old *NK-Irf8<sup>-/-</sup>* and *Irf8<sup>fl/fl</sup>* mice.

(D) Representative flow plots showing CD11b and CD27 co-expression on blood NK cells from *NK-Irf8<sup>-/-</sup>* and *Irf8<sup>fl/fl</sup>* mice (left two panels). Percent NK cells of the most mature (CD11b<sup>+</sup>CD27<sup>-</sup>) phenotype in various organs (right panel).

(E) Percentage of blood NK cells expressing various activating (Ly49D, Ly49H) and inhibitory (Ly49A, Ly49C/I) NK cell receptors.

Data are representative of two independent experiments with 3-5 mice per group per experiment. Data are presented as the mean  $\pm$  SEM.

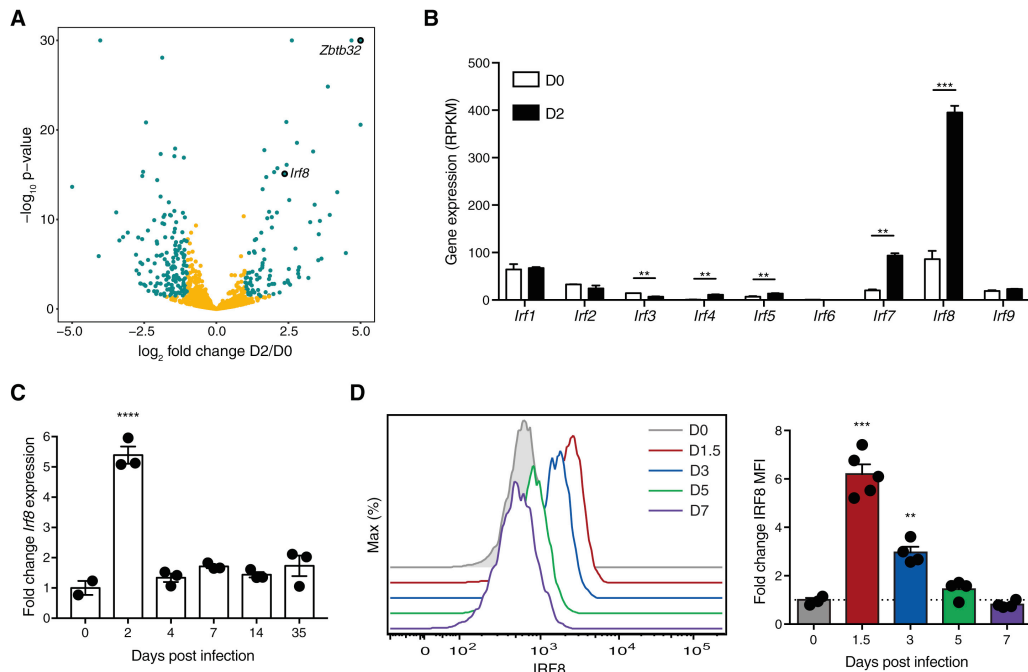
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<https://doi.org/10.1016/j.immuni.2018.04.018>

## **2. NK Cells Rapidly Upregulate IRF8 during MCMV Infection in Response to IL-12 and STAT4<sup>2</sup>**

To assess IRF8 regulation in antigen-specific NK cells during MCMV infection, we performed comparative transcriptome analysis by RNA-seq during their adaptive responses. Consistent with a critical role for IRF8 in early viral control by NK cells, *Irf8* was one of the most highly induced transcription factors in activated Ly49H<sup>+</sup> NK cells (isolated at day 2 PI) compared to naïve NK cells (Figure 7A), and was highly expressed and upregulated compared with other IRF family members (Figure 7B). During MCMV infection, IRF8 was upregulated during early NK cell activation at both the transcript and protein levels (Figures 7C and 7D). However, maintenance of maximal IRF8 expression was transient, with both transcript and protein returning nearly to baseline within 1 week and at memory time points (Figures 7C and 7D), suggesting that IRF8 functions in the early programming of antiviral NK cell responses.

IRF8 was first described to be induced in lymphocytes and macrophages by IFN- $\gamma$  (Driggers et al., 1990); however, IRF8 upregulation in NK cells during MCMV infection was not dependent upon IFN- $\gamma$  (Figure 8A). Because peak IRF8 expression was observed at early time points *in vivo*, when inflammation is maximal (Biron and Tarrio, 2015), we hypothesized that proinflammatory cytokines such as IL-12, IL-18, or type I interferons may instead be regulating IRF8 induction in activated NK cells. We generated mixed bone marrow chimeric mice, harboring both WT cells and cells deficient in receptors for various proinflammatory cytokines (IL-12, IL-18, or type I interferons) and downstream



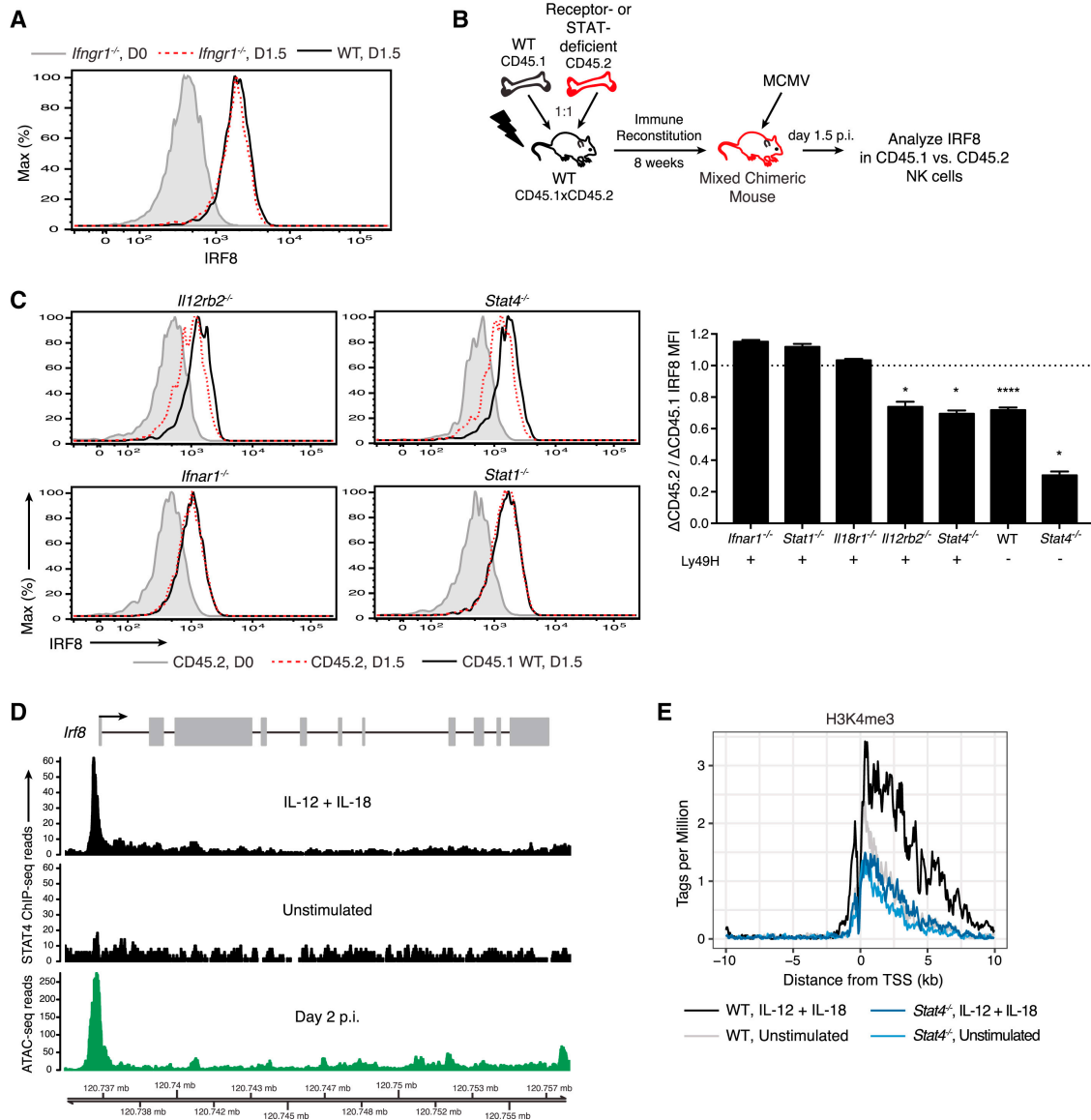
### Figure 7. NK Cells Upregulate IRF8 Early during MCMV Infection<sup>2</sup>

(A–C) Splenocytes were adoptively transferred into Ly49H-deficient mice 1 day before MCMV infection, and Ly49H<sup>+</sup> NK cells (TCR $\beta$ <sup>-</sup>CD3<sup>-</sup>CD19<sup>-</sup>F4/80<sup>-</sup>CD45<sup>+</sup>NK1.1<sup>+</sup>Ly49H<sup>+</sup>) were sorted from the spleen at various time points after infection and then RNA sequenced (two to three replicates per time point). (A) Volcano plot comparing expression of loci encoding transcription factors in Ly49H<sup>+</sup> NK cells isolated before (PI day 0) and after (PI day 2) MCMV infection. Cyan points indicate genes with  $|\log_2(D2/D0)| > 1$  and  $p_{adj} < 0.1$ . The p values were calculated in DESeq2. (B) RNA-seq reads for IRF family members at PI days 0 and 2. Reads are normalized per exon length and total mapped reads (RPKM). For each gene, time points were compared with an unpaired, two-tailed Student's t test. (C) *Irf8* expression is displayed as the fold change in normalized read number compared with the mean value in NK cells isolated from uninfected mice.

(D) Representative flow-cytometric histograms of IRF8 expression in splenic Ly49H<sup>+</sup> NK cells in MCMV-infected WT mice (left). Data are represented as the fold change in IRF8 median fluorescence intensity (MFI) in splenic Ly49H<sup>+</sup> NK cells relative to the mean value in uninfected mice (right) at the indicated time points after MCMV infection. Data are representative of three independent experiments each with four to five mice per time point. Each time point was compared against a hypothetical value of 1 with a one-sample t test.

Data are presented as the mean  $\pm$  SEM. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

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**Figure 8. NK Cell Induction of IRF8 Is Dependent on IL-12 and STAT4<sup>2</sup>**

(A) Representative histograms of IRF8 expression in *Ifngr1*<sup>-/-</sup> NK cells from uninfected (gray line) and infected (red dashed line) chimeric mice and in WT NK cells from infected chimeric mice (black line).

(B) Experimental schematic. Mixed-bone-marrow chimeric mice harboring both WT NK cells (CD45.1) and NK cells deficient in various proinflammatory cytokine receptors and STAT molecules (CD45.2) were infected with MCMV. IRF8 expression in splenic Ly49H<sup>+</sup> and Ly49H<sup>-</sup> NK cells was assessed before infection and at PI day 1.5.

(C) Representative histograms of IRF8 expression in various CD45.2 genetically deficient Ly49H<sup>+</sup> NK cells from uninfected (gray line) and infected (red dashed line) chimeric mice and in CD45.1 WT Ly49H<sup>+</sup> NK cells from infected chimeric mice (black line, left). Data are presented as  $\Delta$ CD45.2 MFI /  $\Delta$ CD45.1 WT MFI ( $\Delta$  = difference in IRF8 MFI between PI days 0 and 1.5; right). Data are representative of two independent

experiments each with 2–13 mice per group per time point. Groups with a ratio < 1 were compared against a hypothetical value of 1 with a one-sample t test. Data are presented as the mean ± SEM. \*p < 0.05, \*\*\*p < 0.0001.

(D) Sorted splenic NK cells were stimulated with IL-12 and IL-18 for 16 hr or unstimulated and then subjected to STAT4 ChIP-seq. STAT4 ChIP-seq reads mapping to the *Irf8* locus are shown in black (top and middle). Data are representative of three independent experiments. Ly49H<sup>+</sup> NK cells were sorted from the spleen at PI day 2 and then subjected to ATAC-seq. ATAC-seq reads mapping to the *Irf8* locus are shown in green (bottom). Data are representative of three independent experiments each with two to three replicates. *Irf8* exons are shown as gray boxes, and the black arrow denotes the origin and directionality of transcription.

(E) NK cells sorted from the spleens of WT or *Stat4*<sup>-/-</sup> mice were stimulated with IL-12 and IL-18 for 30 min or unstimulated and then subjected to H3K4me3 ChIP-seq. H3K4me3 signal from the *Irf8* locus is plotted as normalized fragment counts binned at 50 bp across a 20-kb window centered on the transcriptional start site (TSS). Data are representative of two independent experiments.

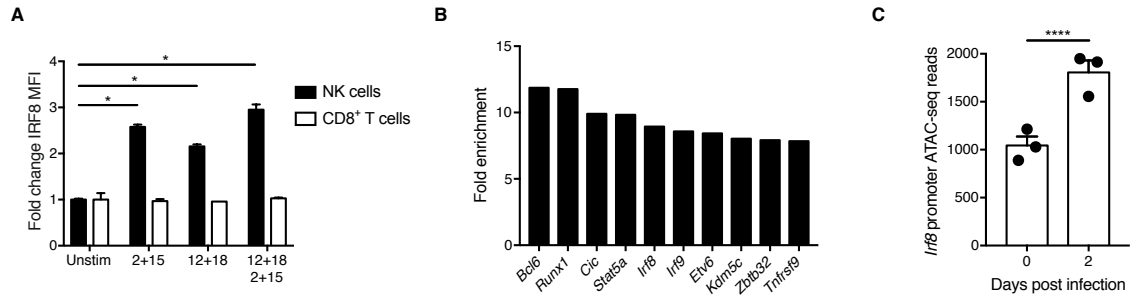
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STAT signaling molecules (STAT1 or STAT4), and challenged them with MCMV (Figure 8B). Compared to WT NK cells, NK cells that were unresponsive to IL-12 signaling (either through absence of the IL-12 receptor or its downstream effector STAT4) were impaired in their ability to maximally upregulate IRF8 after infection (Figure 8C). In contrast, IRF8 upregulation in *Ifnar1*<sup>-/-</sup>, *Il18r1*<sup>-/-</sup>, and *Stat1*<sup>-/-</sup> NK cells remained unaffected (Figure 8C). Collectively, these data suggest an NK cell-specific mechanism of IRF8 regulation by IL-12 and STAT4.

However, IRF8 induction *in vivo* was not fully abrogated in the absence of IL-12 and STAT4 signaling, suggesting a role for other factors. Indeed, Ly49H signaling in NK cells synergized with IL-12 to drive maximal IRF8 induction (Figure 8C), consistent with an analogous report of T cell receptor (TCR) signaling contributing to IRF8 expression in antigen-specific CD8<sup>+</sup> T cells (Miyagawa et al., 2012). Furthermore, *ex vivo* stimulation with IL-12 or common  $\gamma$ -chain-dependent cytokines (IL-2, IL-15) was also sufficient to promote IRF8 upregulation in NK cells (Figure 9A). Given the dependence of NK cells on common  $\gamma$ -chain-dependent cytokines for their survival in the periphery (Jamieson et al., 2004; Prlic et al., 2003; Ranson et al., 2003), the contribution of common  $\gamma$ -chain-dependent cytokines in IRF8 induction during MCMV infection *in vivo* cannot be assessed in the same manner as for IL-12. Nevertheless, signaling through these two pathways (Ly49H and common  $\gamma$ -chain-dependent receptors) likely synergized with IL-12 signaling to regulate IRF8 expression in NK cells during MCMV infection.

In agreement with the necessary role for IL-12 and STAT4 signaling in IRF8 induction, STAT4 occupied the *Irf8* promoter by genome-wide chromatin



### Figure 9. IRF8 Is a Top STAT4-Bound Target in NK Cells<sup>2</sup>

(A) IRF8 MFI in splenic NK cells or CD8<sup>+</sup> T cells following 16 hour *ex vivo* stimulation with indicated interleukin cytokines. IRF8 MFI is displayed as fold change relative to the mean in unstimulated cells. Data are representative of three independent experiments with 2-3 replicates per stimulation condition per experiment. Conditions were compared against a hypothetical value of 1 using a one sample t test.

(B) Experimental design as in Figure 8D. The 10 genes with greatest fold-enrichment of STAT4 binding over input calculated by MACS2 among transcription factor promoter peaks (+2kb to -0.5kb from annotated transcriptional start site), filtered by peaks with > 1.5-fold reads compared to *Stat4*<sup>-/-</sup> STAT4 ChIP samples.

(C) Experimental design as in Figure 8D. Normalized ATAC-seq reads mapping to the *Irf8* promoter peak (chr8:120,735,838-120,736,837) in Ly49H<sup>+</sup> NK cells isolated prior to and following (day 2 PI) MCMV infection. P value was calculated in DESeq2 and adjusted for testing multiple hypotheses.

Data are presented as the mean ± SEM. \* p < 0.05, \*\*\*\* p < 0.0001.

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immunoprecipitation coupled to DNA sequencing (ChIP-seq) (Figure 8D), and *Irf8* was among the top ten STAT4-bound transcription factor loci in activated NK cells (Figure 9B). STAT4 binding co-localized with a region of increased accessibility in the *Irf8* promoter in Ly49H<sup>+</sup> NK cells at day 2 PI, revealed by an assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq) (Figures 8D and 9C). Furthermore, we detected STAT4-dependent trimethylation of histone H3 at lysine 4 (H3K4me3, which marks transcriptionally active promoters) at the *Irf8* locus in NK cells stimulated with proinflammatory cytokines (Figure 8E). Together, these data suggest that STAT4 may be actively remodeling the epigenetic landscape to promote IRF8 induction in activated NK cells. Given the requirement for IL-12 and STAT4 for the clonal expansion of effector NK cells and the generation of memory NK cells (Sun et al., 2012), IRF8 appears to represent a key effector molecule downstream of the IL-12 and STAT4 axis driving a transcriptional program that supports antiviral NK cell responses.

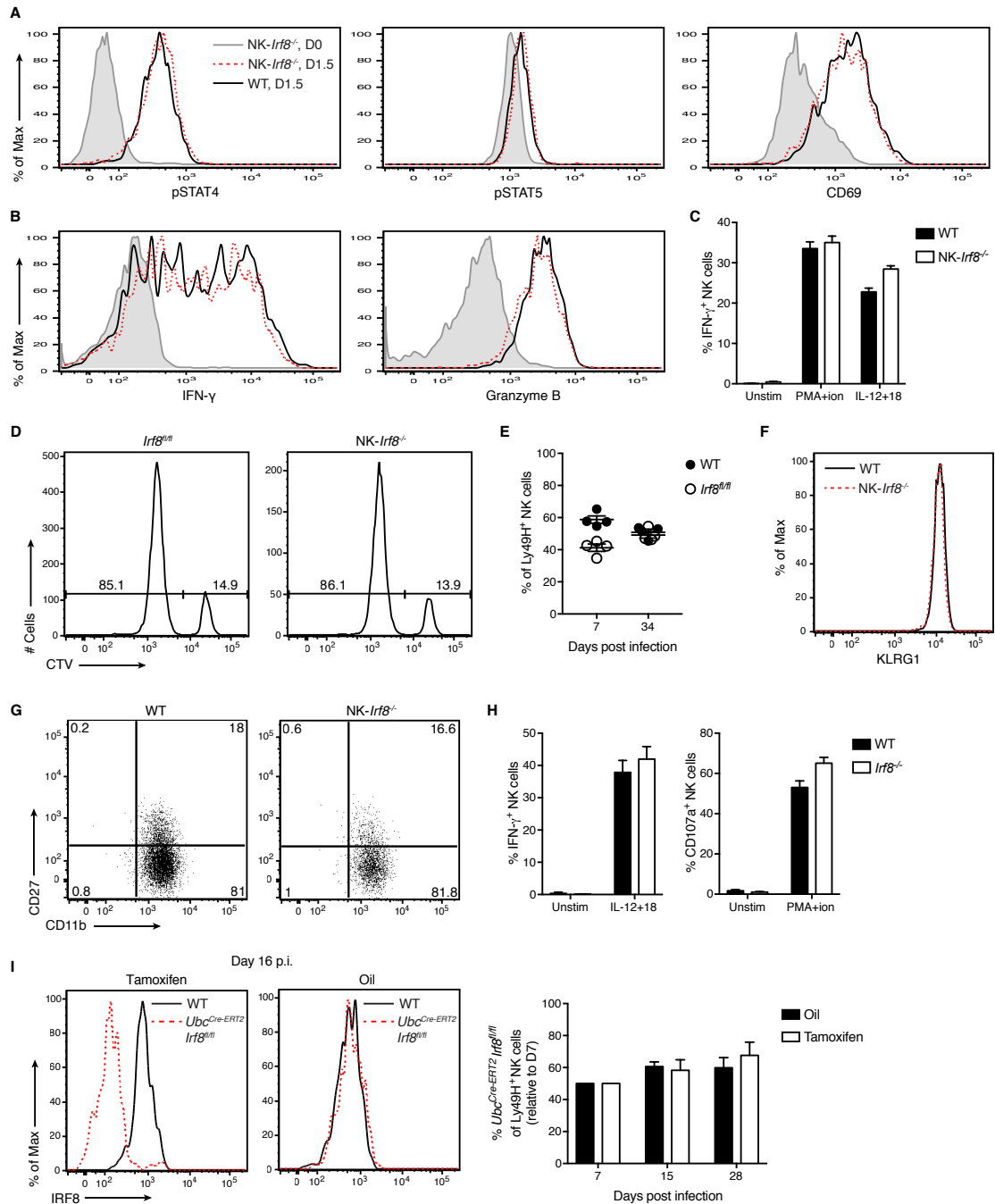
### **3. NK Cells Require IRF8 for Virus-Driven Expansion<sup>2</sup>**

MCMV-induced NK cell activation results in rapid production and release of cytolytic molecules (e.g. perforin, granzyme B) and proinflammatory cytokines (e.g. IFN- $\gamma$ ), resulting in lysis of infected cells and establishment of an antiviral state, as well as antigen-specific clonal proliferation of the Ly49H<sup>+</sup> NK cell subset (Sun and Lanier, 2011). Given the poor protective capacity of NK-*Irf8*<sup>-/-</sup> NK cells, we sought to delineate which of these effector functions was regulated by IRF8. WT and NK-*Irf8*<sup>-/-</sup> NK cells demonstrate comparable proinflammatory cytokine-dependent activation and IFN- $\gamma$

production during MCMV infection *in vivo* (Figures 10A and 10B), as well as following cytokine, or PMA and ionomycin stimulation *in vitro* (Figure 10C). Compared to WT cells, granzyme B production was similarly unaffected in NK-*Irf8*<sup>-/-</sup> NK cells (Figure 10B), resulting in normal *in vivo* elimination of m157-expressing target cells (Figure 10D).

To test the role for IRF8 in MCMV-driven NK cell expansion, we adoptively co-transferred WT and NK-*Irf8*<sup>-/-</sup> NK cells at equal numbers into Ly49H-deficient recipient mice (*Klra8*<sup>-/-</sup>), infected the recipients with MCMV, and tracked the transferred effector Ly49H<sup>+</sup> NK cell response in the recipients (Figure 11A). In contrast to WT NK cells, which expanded robustly and reached peak numbers at day 7 PI, NK-*Irf8*<sup>-/-</sup> NK cells were significantly reduced at this time point (Figure 11B). However, NK cells from Cre-negative littermate control mice expanded comparably to WT cells (Figure 10E). The defective expansion of NK-*Irf8*<sup>-/-</sup> NK cells was observed as early as day 3 PI, and was consistent across various organs (Figure 11C), excluding impaired trafficking as an explanation for the observed phenotype. However, despite the poor expansion, IRF8 deficiency did not impair the terminal maturation of NK cells during infection (Figures 10F and 10G).

Furthermore, a severely diminished pool of NK-*Irf8*<sup>-/-</sup> memory NK cells was observed at day 30 PI compared to WT memory NK cells (Figures 11B and 11D). Although reduced in number, IRF8-deficient memory NK cells were functional upon re-stimulation (Figure 10H). To determine whether IRF8 was required for adaptive NK cell



**Figure 10. IRF8 Is Dispensable for NK Cell Activation, Effector Function, and Memory Maintenance<sup>2</sup>**

(A and B) Mixed bone marrow chimeric mice harboring both WT and *NK-Irf8<sup>-/-</sup>* NK cells were infected with MCMV. (A) Representative histograms of pSTAT4 (left), pSTAT5 (middle), and CD69 expression (right) in splenic Ly49H<sup>+</sup> *NK-Irf8<sup>-/-</sup>* NK cells from uninfected (gray line) and infected (red dashed line) chimeric mice, and WT NK cells from infected chimeric mice (black line) at day 1.5 PI (B) Expression of intracellular IFN- $\gamma$  (left) and Granzyme B (right) at day 1.5 PI Data are representative of 3 mice.

(C) Percentage of splenic NK cells from mixed bone marrow chimeric mice producing IFN- $\gamma$  following 4 hour *ex vivo* stimulation with PMA and ionomycin or IL-12 and IL-18. Data are representative of two independent experiments with 4-5 mice per experiment.

(D) Splenocytes from WT (CTV<sup>lo</sup>) and m157-Tg (CTV<sup>hi</sup>) mice were labeled with different concentrations of CTV and transferred in equal numbers into NK-*Irf8*<sup>-/-</sup> and littermate control *Irf8*<sup>fl/fl</sup> mice. Representative histograms of CTV<sup>+</sup> cells in the spleen at day 1 following transfer. Data are representative of 3-4 mice per group.

(E) Experimental design as in Figure 11A, except transferring cells from CD45.2 *Irf8*<sup>fl/fl</sup> mice. The percentage of WT and *Irf8*<sup>fl/fl</sup> NK cells within transferred Ly49H<sup>+</sup> NK cells in blood at indicated days post infection. Data are representative of three independent experiments with 4-5 mice per experiment.

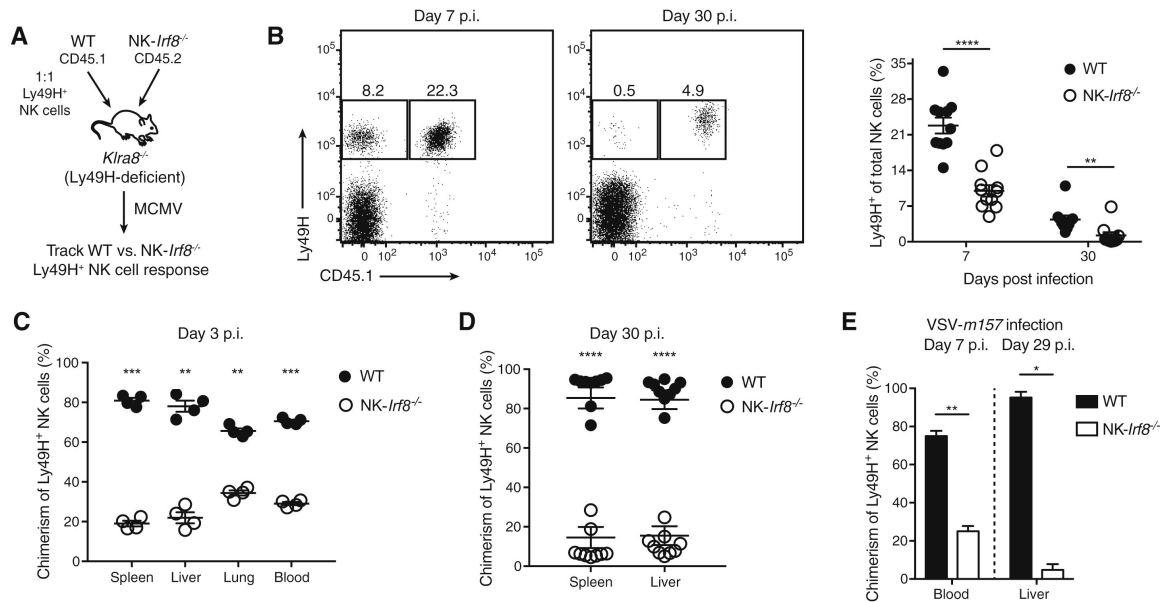
(F and G) Experimental design as in Figure 11A. Representative flow plots of KLRG1 expression (F) or CD11b and CD27 co-expression (G) on WT and NK-*Irf8*<sup>-/-</sup> Ly49H<sup>+</sup> NK cells in blood at day 7 PI. Data are representative of four independent experiments with 4-5 mice per experiment.

(H) Experimental design as in Figure 11A. Percentage of memory WT and *Irf8*<sup>-/-</sup> Ly49H<sup>+</sup> NK cells (day 30 PI) producing IFN- $\gamma$  (left panel) or degranulating (right panel) following 4 hour *ex vivo* stimulation with IL-12 and IL-18 or PMA and ionomycin, respectively. Data are representative of two independent experiments with 3 mice per experiment.

(I) Splenocytes from WT (CD45.1) and *Ubc*<sup>Cre-ERT2</sup> *Irf8*<sup>fl/fl</sup> mice (CD45.2), mixed to achieve equal numbers of Ly49H<sup>+</sup> KLRG1<sup>lo</sup> NK cells, were co-transferred into *Klra8*<sup>-/-</sup> recipients 1 day prior to MCMV infection. On days 7-9 PI, recipients were treated with either tamoxifen in corn oil or with corn oil control. Representative histograms of IRF8 expression in WT and *Ubc*<sup>Cre-ERT2</sup> *Irf8*<sup>fl/fl</sup> Ly49H<sup>+</sup> NK cells from blood of tamoxifen- and oil-treated mice at day 16 PI (left and middle panels, respectively). Relative to pre-treatment (day 7 PI), the percentage of *Ubc*<sup>Cre-ERT2</sup> *Irf8*<sup>fl/fl</sup> NK cells within transferred Ly49H<sup>+</sup> NK cells in blood of tamoxifen- or oil-treated recipients at indicated time points (right panel). Data are representative of two independent experiments with 3-4 mice per treatment group per experiment.

Data are presented as the mean  $\pm$  SEM.

<sup>2</sup>Originally published in *Immunity*. Adams, N.M., Lau, C.M., Fan, X., Rapp, M., Geary, C.D., Weizman, O.E., Diaz-Salazar, C., and Sun, J.C. 2018. Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity*. 48: 1172-1182. Copyright © [2018] Elsevier, Inc. Reproduced with permission of Elsevier, Inc via Copyright Clearance Center. <https://doi.org/10.1016/j.immuni.2018.04.018>



**Figure 11. IRF8 Is Required for NK Cell Expansion during MCMV Infection<sup>2</sup>**

(A) Experimental schematic. Splenocytes from WT mice (CD45.1) and *NK-Irf8*<sup>-/-</sup> mice (CD45.2), mixed to achieve equal numbers of Ly49H<sup>+</sup> KLRG1<sup>lo</sup> NK cells, were adoptively co-transferred into *Klr8*<sup>-/-</sup> recipients 1 day before MCMV infection.

(B) Representative flow plots gated on NK cells in blood at PI day 7 (left) and PI day 30 (middle). The percentage of WT and *NK-Irf8*<sup>-/-</sup> Ly49H<sup>+</sup> NK cells within the total NK cells in blood was quantified at the indicated time points (right). Data are representative of four independent experiments each with four to five mice.

(C) The percentage of WT and *NK-Irf8*<sup>-/-</sup> NK cells within transferred Ly49H<sup>+</sup> NK cells in various peripheral organs at PI day 3. Data are representative of two independent experiments each with four to five mice.

(D) The same as in (C), except in the spleen and liver at PI day 30. Data are representative of four independent experiments each with four to five mice.

(E) Experimental design as in (A), except that recipient mice were infected with VSV-*m157*. Shown are the percentages of WT and *NK-Irf8*<sup>-/-</sup> NK cells within transferred Ly49H<sup>+</sup> NK cells in blood at PI day 7 and in liver at PI day 29. Data are pooled from two independent experiments.

Groups were compared with an unpaired, two-tailed Student's t test (B) or against a hypothetical value of 50 with a one-sample t test (C–E). Data are presented as the mean ± SEM. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001.

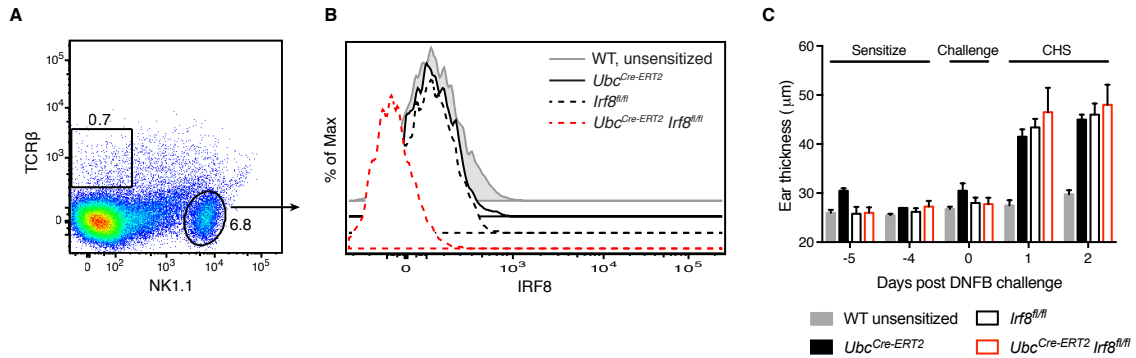
<sup>2</sup>Originally published in *Immunity*. Adams, N.M., Lau, C.M., Fan, X., Rapp, M., Geary, C.D., Weizman, O.E., Diaz-Salazar, C., and Sun, J.C. 2018. Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity*. 48: 1172-1182. Copyright © [2018] Elsevier, Inc. Reproduced with permission of Elsevier, Inc via Copyright Clearance Center. <https://doi.org/10.1016/j.immuni.2018.04.018>

responses against other viruses, we infected mice with recombinant vesicular stomatitis virus engineered to express the MCMV *m157* gene (VSV-*m157*). During VSV-*m157* infection, NK-*Irf8*<sup>-/-</sup> NK cells were similarly outcompeted by WT NK cells, and ultimately formed a smaller pool of memory NK cells (Figure 11E), suggesting that the requirement for IRF8 in antigen-specific NK cell expansion is not limited to MCMV infection. However, IRF8 was not required for hapten-specific NK cell-mediated contact hypersensitivity responses (Figure 12), suggesting IRF8 only plays a critical role during infectious priming, but not non-infectious priming, of adaptive NK cell responses.

To address whether IRF8 directly maintained virus-specific memory NK cell numbers, we generated a mouse model in which the floxed exon 2 of *Irf8* can be excised by a tamoxifen-inducible Cre cassette driven by the ubiquitin promoter (*Ubc*<sup>Cre-ERT2</sup> *Irf8*<sup>fl/fl</sup>). In our adoptive co-transfer system, recipient mice were treated with either tamoxifen or oil just prior to the contraction phase (treatment days 7-9 PI), resulting in successful deletion of *Irf8* in tamoxifen-, but not oil-treated, *Ubc*<sup>Cre-ERT2</sup> *Irf8*<sup>fl/fl</sup> NK cells (Figure 10I). Inducible deletion of *Irf8* after NK cell expansion did not affect formation of the memory NK cell pool, as *Ubc*<sup>Cre-ERT2</sup> *Irf8*<sup>fl/fl</sup> memory NK cells were found at similar proportions in the blood of tamoxifen- and oil-treated mice (Figure 10I). Thus, IRF8 is essential in driving the expansion, but not the memory maintenance of virus-specific NK cells.

NK cells also undergo homeostatic proliferation in a lymphopenic environment, thought to be driven by availability of common  $\gamma$ -chain-dependent cytokines





**Figure 12. IRF8 Is Dispensable for Hapten-Specific Contact Hypersensitivity (CHS) Responses<sup>2</sup>**

All mice received a 3-day course of tamoxifen treatment prior to sensitization, and anti-CD8 $\alpha$  and anti-CD4 depleting antibody treatment prior to both sensitization and challenge. Mice were sensitized on consecutive days by applying 0.5% DNFB to the abdominal skin, and challenged 4 days later by applying 0.45% DNFB to the ear. One group (gray filled) was not sensitized as a control.

(A) Representative flow plots showing successful T cell depletion in blood at day 2 post DNFB challenge.

(B) Representative histograms of IRF8 expression in NK cells from blood of indicated experimental groups at day 2 post DNFB challenge.

(C) Ear thickness measured at indicated time points prior to and after DNFB sensitization and challenge.

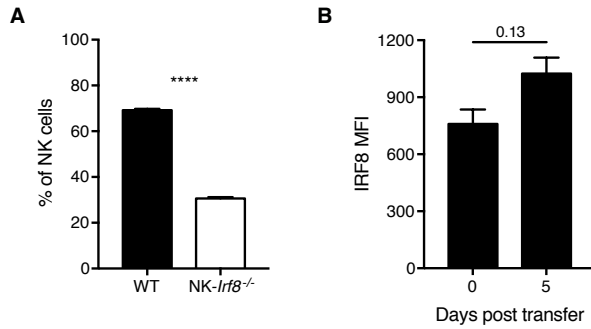
Data are representative of 4 mice per group. Data are presented as the mean  $\pm$  SEM.

<sup>2</sup>Originally published in *Immunity*. Adams, N.M., Lau, C.M., Fan, X., Rapp, M., Geary, C.D., Weizman, O.E., Diaz-Salazar, C., and Sun, J.C. 2018. Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity*. 48: 1172-1182. Copyright © [2018] Elsevier, Inc. Reproduced with permission of Elsevier, Inc via Copyright Clearance Center. <https://doi.org/10.1016/j.immuni.2018.04.018>

(Sun et al., 2011). NK-*Irf8*<sup>-/-</sup> NK cells were found to be defective in homeostatic proliferation following adoptive transfer into *Rag2*<sup>-/-</sup> *IL2rg*<sup>-/-</sup> mice, which lack both innate and adaptive lymphocytes (Figure 13A). Consistent with common  $\gamma$ -chain-dependent IRF8 expression in CD8<sup>+</sup> T cells (Miyagawa et al., 2012) and human NK cells (Mace et al., 2017), IRF8 abundance trended upwards in lymphopenia-driven NK cells (Figure 13B). These findings collectively indicate that IRF8 is specifically required to support robust expansion of mature NK cell numbers during infection and homeostatic proliferation, whereas redundant mechanisms exist for NK cell development or homeostatic maintenance, which necessitate modest cell turnover.

#### **4. IRF8 Promotes Proliferation through Direct Regulation of *Zbtb32***

To investigate pathways regulated by IRF8 that may be promoting NK cell expansion, we performed comparative transcriptome analysis by RNA-seq on purified WT and NK-*Irf8*<sup>-/-</sup> NK cells derived from MCMV-infected and -uninfected mixed bone marrow chimeric mice (Figure 14A). Consistent with the reported ability of IRF8 to act as a transcriptional activator or repressor depending on its binding partner (Tamura et al., 2015), 323 transcripts were downregulated, and 379 transcripts were upregulated in NK-*Irf8*<sup>-/-</sup> NK cells at day 4 PI compared to WT NK cells. Classification of differentially expressed genes into PANTHER pathways revealed a transcriptional program consistent with dysregulated cell cycle control, DNA replication, and metabolism in NK-*Irf8*<sup>-/-</sup> NK cells (Figure 15A). Because these gene sets trended towards being comparatively enriched in



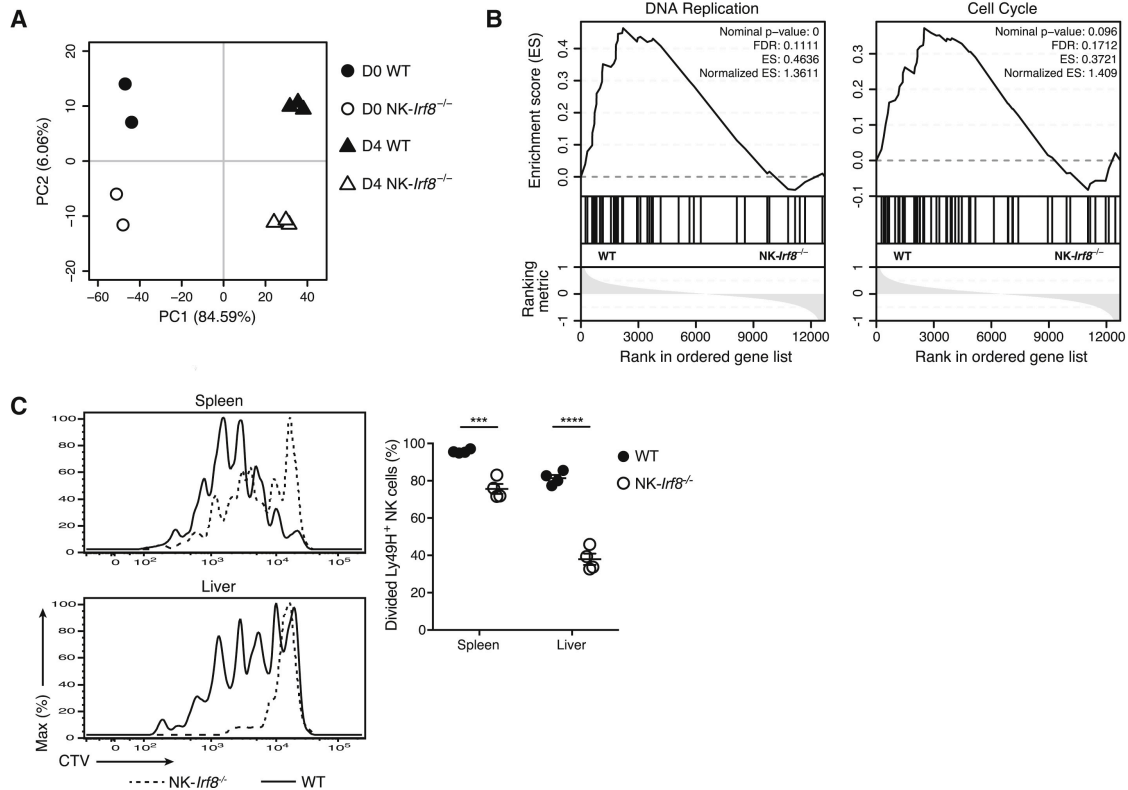
### Figure 13. IRF8 Is Required for Lymphopenia-Driven NK Cell Proliferation<sup>2</sup>

(A) Splenocytes from WT (CD45.1) and NK-*Irf8*<sup>-/-</sup> mice (CD45.2), mixed to achieve equal NK cell numbers, were co-transferred into *Rag2*<sup>-/-</sup> *IL2rg*<sup>-/-</sup> mice. Shown are the relative percentages of adoptively transferred WT versus NK-*Irf8*<sup>-/-</sup> NK cells in blood at day 5 post transfer.

(B) IRF8 MFI in WT NK cells from blood of WT mice and day 5 post transfer into *Rag2*<sup>-/-</sup> *IL2rg*<sup>-/-</sup> mice.

Data are representative of two independent experiments with 4-5 mice per experiment. Groups were compared against a hypothetical value of 50 using a one sample t test (A), or time points were compared against each other using an unpaired, two-tailed Student's t test. Data are presented as the mean ± SEM. \*\*\*\* p < 0.0001.

<sup>2</sup>Originally published in *Immunity*. Adams, N.M., Lau, C.M., Fan, X., Rapp, M., Geary, C.D., Weizman, O.E., Diaz-Salazar, C., and Sun, J.C. 2018. Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity*. 48: 1172-1182. Copyright © [2018] Elsevier, Inc. Reproduced with permission of Elsevier, Inc via Copyright Clearance Center. <https://doi.org/10.1016/j.immuni.2018.04.018>

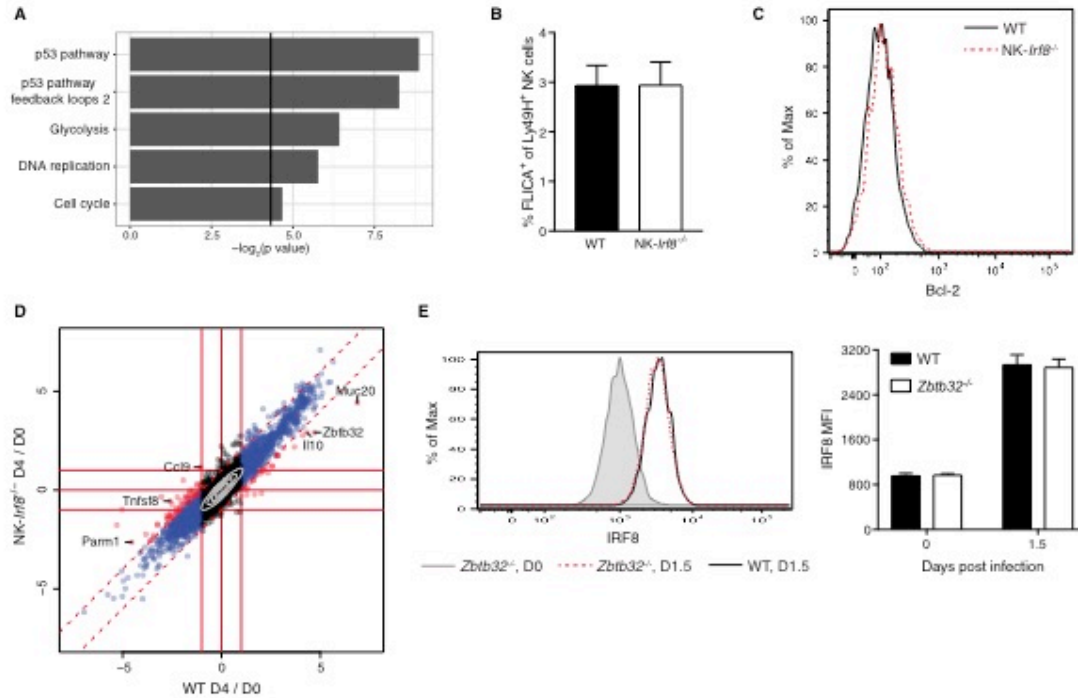


## Figure 14. IRF8 Drives a Transcriptional Program that Promotes NK Cell Proliferation<sup>2</sup>

(A and B) Mixed-bone-marrow chimeric mice harboring both WT and *NK-Irf8*<sup>-/-</sup> NK cells were infected with MCMV. Splenic Ly49H<sup>+</sup> WT and *NK-Irf8*<sup>-/-</sup> NK cells were sorted for RNA-seq at PI days 0 and 4 (two to three replicates per genotype and time point). (A) Principal-component analysis of RNA-seq data used the top 2,000 genes with the highest variance. Each dot represents an RNA sample from a single mouse. (B) Gene-set enrichment analysis of RNA-seq data at PI day 4. Genes within each given gene set are derived from significantly overrepresented PANTHER pathways and have expression above a minimal threshold according to the distribution of all genes.

(C) Experimental design as in Figure 11A, except that NK cells were labeled with CTV before adoptive co-transfer. Left: representative histograms of CTV in splenic and hepatic WT and *NK-Irf8*<sup>-/-</sup> Ly49H<sup>+</sup> NK cells at PI day 3. Right: quantification of Ly49H<sup>+</sup> NK cells that divided at least once. Data are representative of two independent experiments each with four mice. Groups were compared with an unpaired, two-tailed Student's t test. Data are presented as the mean ± SEM. \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

<sup>2</sup>Originally published in *Immunity*. Adams, N.M., Lau, C.M., Fan, X., Rapp, M., Geary, C.D., Weizman, O.E., Diaz-Salazar, C., and Sun, J.C. 2018. Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity*. 48: 1172-1182. Copyright © [2018] Elsevier, Inc. Reproduced with permission of Elsevier, Inc via Copyright Clearance Center. <https://doi.org/10.1016/j.immuni.2018.04.018>



**Figure 15. Pathways and Genes Dysregulated in NK-Irf8<sup>-/-</sup> NK Cells during MCMV Infection<sup>2</sup>**

(A) Experimental design as in Figure 14A. Gene ontology analysis of differential PANTHER pathways at day 4 PI. Their respective p values are shown. Line indicates  $p = 0.05$ .

(B) Experimental design as in Figure 11A. Percent of splenic WT and NK-Irf8<sup>-/-</sup> Ly49H<sup>+</sup> NK cells staining positive for FLICA (marking activated caspases) at day 3.5 PI. Data are representative of 4 mice.

(C) Mixed bone marrow chimeric mice harboring both WT and NK-Irf8<sup>-/-</sup> NK cells were infected with MCMV. Representative histograms of Bcl-2 expression in WT (black line) and NK-Irf8<sup>-/-</sup> (red dashed line) splenic Ly49H<sup>+</sup> NK cells at day 7 PI are shown. Data are representative of 3 mice.

(D) Relative change in expression (D4/D0) of all "expressed" genes in NK-Irf8<sup>-/-</sup> versus WT NK cells.  $|WT_{\text{Log2FC}}| < 1$  (black dots),  $|WT_{\text{Log2FC}}| \geq 1$  (blue and red dots),  $WT_{\text{Log2FC}} - NK-Irf8^{-/-}_{\text{Log2FC}} \geq 1$  or  $WT_{\text{Log2FC}} - NK-Irf8^{-/-}_{\text{Log2FC}} \leq -1$  (red dots).

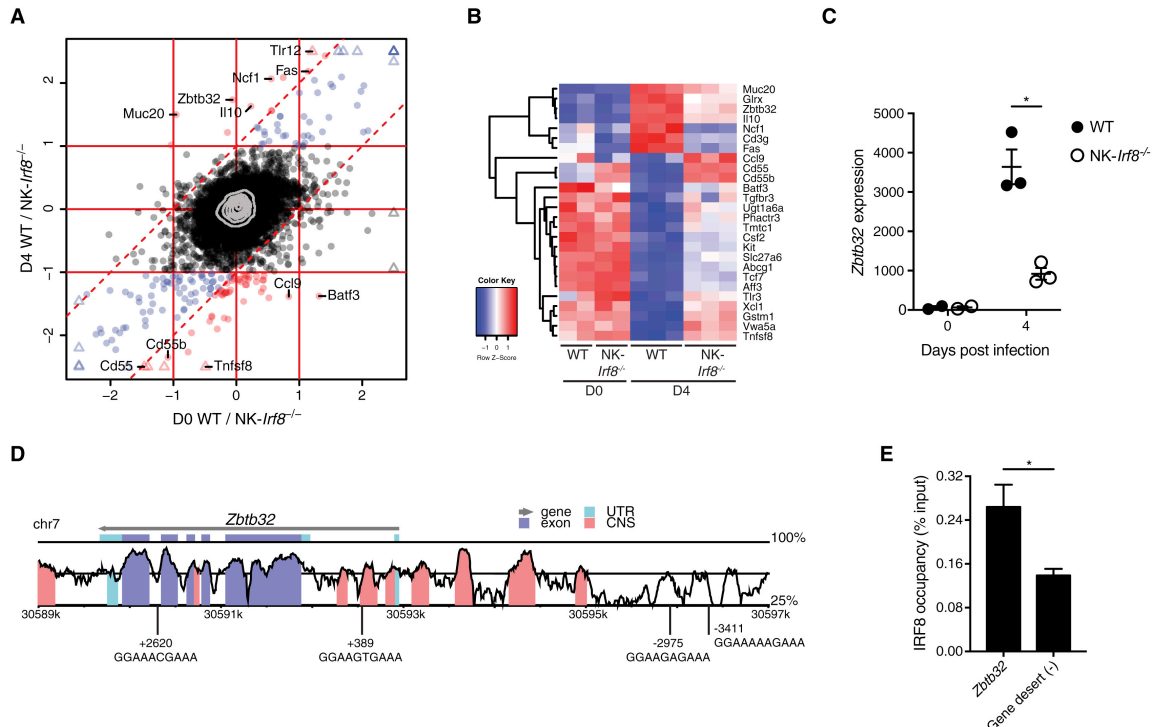
(E) Mixed bone marrow chimeric mice harboring both WT and Zbtb32<sup>-/-</sup> NK cells were infected with MCMV. Representative histograms of IRF8 expression in splenic Ly49H<sup>+</sup> Zbtb32<sup>-/-</sup> NK cells from uninfected (gray line) and infected (red dashed line) chimeric mice, and WT NK cells from infected chimeric mice (black line) at day 1.5 PI are shown (left panel). Quantification of IRF8 MFI in both genotypes prior to and at day 1.5 PI (right panel). Data are representative of two independent experiments with 2-6 mice per time point per experiment.

Data are presented as the mean  $\pm$  SEM.

<sup>2</sup>Originally published in *Immunity*. Adams, N.M., Lau, C.M., Fan, X., Rapp, M., Geary, C.D., Weizman, O.E., Diaz-Salazar, C., and Sun, J.C. 2018. Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity*. 48: 1172-1182. Copyright © [2018] Elsevier, Inc. Reproduced with permission of Elsevier, Inc via Copyright Clearance Center. <https://doi.org/10.1016/j.immuni.2018.04.018>

WT NK cells (Figure 14B), we tested whether cell proliferation was defective in NK-*Irf8*<sup>-/-</sup> NK cells. Indeed, analysis of WT and NK-*Irf8*<sup>-/-</sup> NK cells labeled with Cell Trace Violet (CTV) prior to adoptive co-transfer and MCMV infection confirmed that NK-*Irf8*<sup>-/-</sup> NK cells fail to divide efficiently (Figure 14C). In contrast, NK-*Irf8*<sup>-/-</sup> NK cells exhibited no evidence of enhanced caspase activation, and normal expression of the pro-survival protein Bcl-2 during MCMV-driven expansion (Figures 15B and 15C). Collectively, these data indicate that IRF8 is essential for inducing a broad transcriptional program that primarily serves to promote MCMV-driven NK cell proliferation.

We next sought to pinpoint key transcriptional targets of IRF8 that promote NK cell proliferation during infection *in vivo*. Given that there exist baseline transcriptional differences between WT and NK-*Irf8*<sup>-/-</sup> NK cells (Figure 14A), yet no NK cell developmental phenotype in the absence of IRF8 (Figure 6), we reasoned that infection-specific transcriptional differences would likely best explain the impaired proliferation of NK-*Irf8*<sup>-/-</sup> NK cells. Our RNA-seq analysis identified a subset of genes that were differentially upregulated or downregulated in NK-*Irf8*<sup>-/-</sup> NK cells in an infection-specific manner (Figures 16A, 16B, and 15D). One such gene was *Zbtb32*, which was more highly induced in WT than in NK-*Irf8*<sup>-/-</sup> NK cells during MCMV infection (Figures 16A-16C). *Zbtb32* is a Broad complex, Tramtrack, Bric à Brac and Zinc Finger (BTB-ZF) transcription factor previously reported to be essential for the proliferative burst of MCMV-specific NK cells (Beaulieu et al., 2014). *In silico* analysis identified multiple IRF8 binding sites that fall in moderate-to-highly conserved non-coding sequence (CNS) regions within the *Zbtb32* locus (Figure 16D), some of which are bound by IRF8 in other immune cells (Langlais et al., 2016; Marquis et al., 2011; Shin et al., 2011). Moreover,



**Figure 16. IRF8 Directly Regulates the Pro-proliferative Factor *Zbtb32*<sup>2</sup>**

(A–C) Experimental design as in Figure 14A. (A) Relative expression of all “expressed” genes in WT versus *NK-Irf8*<sup>-/-</sup> NK cells on PI day 4 compared with before infection. Red or blue dots denote  $|D4_{\text{Log2FC}}| \geq 1$ , and red dots specifically denote  $D4_{\text{Log2FC}} - D0_{\text{Log2FC}} \geq 1$  or  $D4_{\text{Log2FC}} - D0_{\text{Log2FC}} \leq -1$ , i.e., genes that showed greater differential expression between WT and *NK-Irf8*<sup>-/-</sup> NK cells at PI day 4 (D4) than at PI day 0 (D0). Triangles signify genes that were coerced onto the plot boundary. (B) Heatmap and hierarchical clustering of selected genes. Replicates are shown in order. Selected genes showed greater differential expression in WT cells than in *NK-Irf8*<sup>-/-</sup> NK cells and at D4 more than at D0. (C) RNA-seq reads mapping to the *Zbtb32* locus. The p value was calculated in DESeq2 and adjusted for testing multiple hypotheses.

(D) The position and sequence of putative IRF8 binding sites are shown in relation to the *Zbtb32* promoter. Traces show the percentage of conservation between mouse (mm10) and human (hg19) genomes (UCSC Genome Browser). Conserved non-coding sequences (CNSs) are defined as having >70% homology over >100 bp. The location relative to the TSS and the sequence of the putative IRF8 motif are listed for each putative IRF8 binding site.

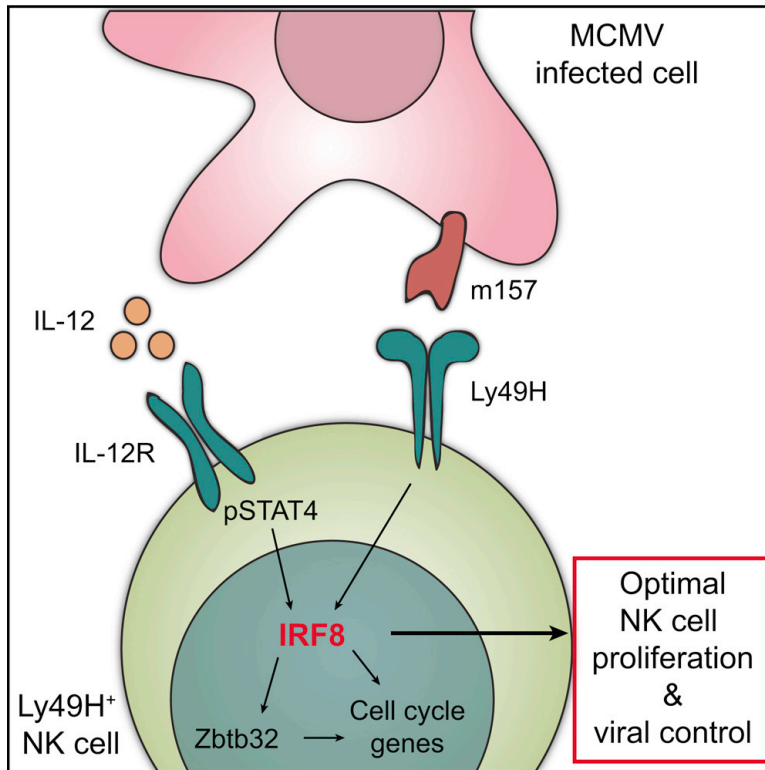
(E) Sorted splenic NK cells were stimulated with IL-2, IL-12, IL-15, and IL-18 for 16 hr. IRF8-DNA complexes were immunoprecipitated and then subjected to qPCR for amplification across a putative IRF8 binding site 2,975 bp upstream of the *Zbtb32* promoter. A gene desert ~50 kb upstream of the *Foxp3* locus served as a negative control for IRF8 binding. Data are representative of two independent experiments. Samples were compared with an unpaired, two-tailed Student’s t test.

Data are presented as the mean  $\pm$  SEM. \*p < 0.05.



<sup>2</sup>Originally published in *Immunity*. Adams, N.M., Lau, C.M., Fan, X., Rapp, M., Geary, C.D., Weizman, O.E., Diaz-Salazar, C., and Sun, J.C. 2018. Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity*. 48: 1172-1182. Copyright © [2018] Elsevier, Inc. Reproduced with permission of Elsevier, Inc via Copyright Clearance Center. <https://doi.org/10.1016/j.immuni.2018.04.018>

IRF8 ChIP followed by qPCR revealed enrichment of *Zbtb32* promoter DNA (compared to gene desert following immunoprecipitation with  $\alpha$ -IRF8 antibody), confirming that *Zbtb32* is a direct target of IRF8-mediated transcriptional activation (Figure 16E). However, during MCMV infection, IRF8 induction in NK cells was found to be independent of *Zbtb32* (Figure 15E). Given that the *Zbtb32* locus is IRF8-bound (Figure 16E) and that *Zbtb32* expression is IRF8-dependent (Figures 16A-16C), we thus propose a model wherein IRF8 functions upstream of *Zbtb32* to drive antiviral NK cell proliferation (Figure 17).



**Figure 17. Graphical Abstract for Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response<sup>2</sup>**

<sup>2</sup>Originally published in *Immunity*. Adams, N.M., Lau, C.M., Fan, X., Rapp, M., Geary, C.D., Weizman, O.E., Diaz-Salazar, C., and Sun, J.C. 2018. Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity*. 48: 1172-1182. Copyright © [2018] Elsevier, Inc. Reproduced with permission of Elsevier, Inc via Copyright Clearance Center. <https://doi.org/10.1016/j.immuni.2018.04.018>

### III. Discussion<sup>2</sup>

A growing body of literature supports the paradigm that NK cells in mice, primates, and humans undergo antigen-specific responses to viral pathogens (Adams et al., 2016). Adaptive NK cell responses include the prolific expansion and memory formation of virus-specific NK cell subsets (Sun et al., 2009), yet the transcriptional control of these processes is poorly understood. Here, we have revealed that the transcription factor IRF8 is dynamically regulated in antigen-specific NK cells during viral infection and is essential for NK cell-mediated immunity against MCMV. In contrast to its canonical regulation throughout the immune system by IFN- $\gamma$  (Driggers et al., 1990), we herein describe that the proinflammatory cytokine IL-12 and the activating receptor Ly49H represent the critical signals for maximal IRF8 upregulation in NK cells early during MCMV infection. Given that IL-12, but not IFN- $\gamma$ , is required for MCMV-driven NK cell expansion (Sun et al., 2012), IL-12-dependent regulation uniquely positions IRF8 to exert a functional role over antiviral NK cell responses.

Notably, *Irf8*<sup>-/-</sup> mice have greater viral susceptibility than do NK-*Irf8*<sup>-/-</sup> mice. Several studies have delineated a role for IRF8 in the development of monocytes, pDCs, and cDC1s, which are all affected in *Irf8*<sup>-/-</sup> mice (Aliberti et al., 2003; Schiavoni et al., 2002). Loss of IRF8-dependent CD8 $\alpha$ <sup>+</sup> cDC1s in *Irf8*<sup>-/-</sup> mice has been demonstrated to impair IL-12 production in a variety of pathogen models (Giese et al., 1997; Schariton-Kersten et al., 1997). During early MCMV infection, IRF8-dependent cDC1s are the major producer of IL-12 (Rapp et al., 2017), and IL-12 signaling is critical for programming adaptive NK cell responses to MCMV infection (Sun et al., 2012). Thus,

the striking mortality of *Irf8*<sup>-/-</sup> mice during MCMV infection is likely a combination of both cell-extrinsic (impaired NK cell priming due to low systemic IL-12 in the absence of cDC1s) and cell-intrinsic (impaired IRF8-dependent NK cell proliferative burst) functions for IRF8.

During MCMV infection, we observed that IRF8 drives a transcriptional program consistent with cell cycle progression, which supports NK cell clonal proliferation such that NK cells can mount a maximal effector response. Our data suggest that the output of the IRF8 transcriptional program is likely mediated in part by IRF8 directly binding to the *Zbtb32* locus to promote expression of Zbtb32, a pro-proliferative transcription factor previously reported to be essential for controlling the proliferative burst of virus-specific NK cells (Beaulieu et al., 2014), thus elucidating a previously uncharacterized relationship between these two transcription factors. Given its function, Zbtb32 is tightly regulated by NK cells during both homeostasis and MCMV infection. Naïve NK cells do not express Zbtb32, but NK cells transiently upregulate *Zbtb32* nearly 100-fold during MCMV infection in a manner similarly dependent on IL-12 signaling (Beaulieu et al., 2014). Maximal Zbtb32 induction is critical, as *Zbtb32* hemizyosity is insufficient to drive NK cell expansion during viral infection (Beaulieu et al., 2014). Because STAT4 itself also directly regulates *Zbtb32* expression, this may explain why only an incomplete, yet not abrogated, *Zbtb32* upregulation was observed in the absence of IRF8. Because additional transcription factors may also be regulating *Zbtb32* transcription (either individually or in concert with STAT4 and IRF8), it will be important to determine the complete mechanisms that ensure a robust IL-12 and STAT4 effector program within activated NK cells.

Despite the requirement for IRF8 in NK cell antiviral immunity, we observed no impact of IRF8 deficiency on NK cell development. This observation appears inconsistent with findings in patients with biallelic *IRF8* mutations across 3 unrelated families who exhibit impaired terminal maturation and diminished overall numbers of NK cells (Mace et al., 2017). Perhaps this discrepancy can be ascribed to our use of a specific deletion of *Irf8* in NK cells of transgenic mice, whereas NK cells in these patients developed in the presence of hematopoietic and stromal cells that also bear the *IRF8* mutations. Despite carrying biallelic *IRF8* mutations, the NK cell deficient patient had normal amounts of IRF8 protein, which translocated normally to the nucleus and demonstrated unaffected transcriptional activity dependent on PU.1 and IRF1, two of its canonical binding partners (Mace et al., 2017). Thus, the mutant IRF8 protein in humans may have adopted an altered function within the NK cell population rather than representing a nonfunctional protein or the ablated gene we investigated in mice.

In our study, we have delineated a dichotomous requirement for IRF8 in NK cells that appears to be dependent on the degree of cell turnover, as IRF8 was required for MCMV- and lymphopenia-driven proliferation, but dispensable for development and homeostatic maintenance. However, it remains unclear how IRF8 is targeted for transcriptional activity during different cellular states. Recent studies have found that dynamic epigenetic changes accompany CD8<sup>+</sup> T cell differentiation during bacterial and viral infection, and can modulate the availability of transcription factor binding sites (Pauken et al., 2016; Russ et al., 2014; Scharer et al., 2013; Scott-Browne et al., 2016; Yu et al., 2017). Furthermore, due to structural divergences in its IRF domain (Escalante et al., 2002), IRF8 requires a DNA-binding partner (e.g. Ets, AP-1, or other IRF family

members) to bind DNA with high affinity (Tamura et al., 2008). It is unknown whether IRF8 utilizes the same binding partners in NK cells as it does in other hematopoietic cells, yet the availability or modification of binding partners secondary to different NK cell states may dictate the set of genes regulated by IRF8. Indeed, this mechanism has been described in macrophages wherein the ability of IRF8 to bind alternative DNA sequences after LPS or IFN- $\gamma$  stimulation is dependent on stimulus-inducible partners (Kuwata et al., 2002; Mancino et al., 2015). Further understanding of the complete molecular mechanisms that regulate IRF8 transcriptional activity will offer valuable insights into how antigen-specific NK cells generate robust proliferative responses to viral infection.

In summary, our work highlights the importance of IRF8 in orchestrating adaptive NK cell antiviral responses. Because proinflammatory cytokines are necessary and sufficient for IRF8 upregulation in NK cells, it will be of interest to test whether IRF8 is similarly induced and required for the function of other innate lymphoid cell (ILC) lineages, which lack antigen receptors and are thought to initiate antimicrobial responses by sensing cytokines, alarmins, and inflammatory mediators (Sonnenberg and Artis, 2015). Analogous to expansion of mouse Ly49H<sup>+</sup> NK cells during MCMV infection, HCMV-seropositive individuals exhibit an expanded and long-lived population of NKG2C<sup>+</sup> NK cells (Gumá et al., 2004; Hendricks et al., 2014; Lopez-Vergès et al., 2011) that are capable of recall responses (Foley et al., 2012a). Whether these adaptive NK cell responses in humans similarly require IRF8 during HCMV infection remains to be determined. Nevertheless, our results uncover molecular events that provide insight into

the upstream regulation and downstream transcriptional program of IRF8, and shed light on ways to modulate NK cell antiviral immunity for therapeutic benefit.



## IV. Materials and Methods<sup>2</sup>

### 1. Experimental Model and Subject Details<sup>2</sup>

#### *Mice*

All mice used in this study were housed and bred under specific pathogen-free conditions at Memorial Sloan Kettering Cancer Center, and handled in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). The following mouse strains were used in this study: C57BL/6 (CD45.2), B6.SJL (CD45.1), B6 CD45.1xCD45.2, *Irf8*<sup>-/-</sup>, *Irf8*<sup>fl/fl</sup>, *Nkp46*<sup>iCre</sup> (Narni-Mancinelli et al., 2011), *Nkp46*<sup>iCre</sup> *Irf8*<sup>fl/fl</sup>, *Ubc*<sup>Cre-ERT2</sup>, *Ubc*<sup>Cre-ERT2</sup> *Irf8*<sup>fl/fl</sup>, *Klra8*<sup>-/-</sup> (Ly49H-deficient) (Fodil-Cornu et al., 2008), *Ifngr1*<sup>-/-</sup>, *Il12rb2*<sup>-/-</sup>, *Stat4*<sup>-/-</sup>, *Ifnar1*<sup>-/-</sup>, *Stat1*<sup>-/-</sup>, *Il18r1*<sup>-/-</sup>, m157-Tg (Tripathy et al., 2008), *Rag2*<sup>-/-</sup> *IL2rg*<sup>-/-</sup>, and *Zbtb32*<sup>-/-</sup> (Hirahara et al., 2008). Experiments were conducted using age- and gender-matched mice in accordance with approved institutional protocols.

#### *Virus*

MCMV (Smith strain) was serially passaged through BALB/c hosts three times, and then salivary gland viral stocks were prepared with a dounce homogenizer for dissociating the salivary glands of infected mice 3 weeks after infection. Recombinant VSV-*m157* was made by cloning the coding sequence for the MCMV glycoprotein m157 into the parental VSV Indiana strain provided by K. Schluns (MD Anderson) (Firth et al., 2013).

## 2. Method Details<sup>2</sup>

### *Mixed Bone Marrow Chimeras*

Mixed bone marrow chimeric mice were generated by lethally irradiating (900 cGy) host CD45.1xCD45.2 animals and reconstituting them with a 1:1 mixture of bone marrow cells from WT (CD45.1) and genetic-deficient (CD45.2) donor mice. Hosts were co-injected with anti-NK1.1 (clone PK136) to deplete any residual donor or host mature NK cells. CD45.1<sup>+</sup>CD45.2<sup>+</sup> host NK cells were excluded from all analyses.

### *In Vivo Virus Infection*

Adoptive co-transfer studies were performed by transferring splenocytes from WT (CD45.1) and genetic-deficient (CD45.2) mice, mixed to achieve equal numbers of Ly49H<sup>+</sup> KLRG1<sup>lo</sup> NK cells, into *Klra8*<sup>-/-</sup> recipients 1 day prior to MCMV infection. Recipient mice in adoptive co-transfer studies were infected either with MCMV by i.p. injection of  $7.5 \times 10^2$  plaque-forming units (PFU) in 0.5 mL or with VSV-*m157* by i.v. injection of  $1 \times 10^7$  PFU in 0.2 mL. Mixed bone marrow chimeric mice and mice in survival experiments received  $7.5 \times 10^3$  PFU or  $4 \times 10^4$  PFU MCMV respectively in 0.5 mL by i.p. injection.

In some experiments, recipient mice were treated for 3 consecutive days with 4 mg/day of tamoxifen (Sigma) dissolved in 0.2 mL corn oil or with 0.2 mL corn oil control (Sigma) by oral gavage, beginning on day 7 after infection.

### *Hapten Contact Hypersensitivity*

To study NK cell-mediated contact hypersensitivity responses, experimental mice were depleted of circulating T cells with 100 µg anti-CD8α (Bio X Cell, clone 2.43) and 400 µg anti-CD4 (Bio X Cell, clone GK1.5) depletion antibodies 3 days prior to sensitization as well as on the days of sensitization and challenge. Mice were sensitized on consecutive days by treating the shaved abdominal skin with 20 µL of 0.5% 1-fluoro-2,4-dinitrobenzene (DNFB, Sigma) in a 4:1 mixture of acetone (Fisher Scientific) to olive oil (Sigma). Mice were challenged 4 days later by treating both surfaces of the left ear with 10 µL of 0.45% DNFB in a 4:1 acetone to olive oil mixture. Both surfaces of the contralateral ear were treated with 10 µL of a 4:1 acetone to olive oil mixture (vehicle) as a control. Ear thickness was measured using a Käfer dial thickness gauge (Long Island Indicator Service).

### *Virus Quantification*

MCMV viral titers were determined as previously described (Johnson et al., 2016). DNA was isolated from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen). Following isolation, the DNA concentration was measured using Nanodrop for each sample, and 3 µL was added into a mastermix containing iQ SYBR Green (Bio-Rad) and primers specific to MCMV IE-1 DNA (forward: TCGCCCATCGTTTCGAGA, reverse: TCTCGTAGGTCCACTGACGGA). Copy number was determined by comparing C<sub>q</sub> values to a standard curve of known dilutions of an MCMV plasmid and normalizing relative to total DNA content.

### *Lymphocyte Isolation*

Spleens were dissociated using glass slides and filtered through a 100- $\mu$ m strainer. To isolate lymphocytes from liver, the tissue was physically dissociated using a glass tissue homogenizer and purified using a discontinuous gradient of 40% over 60% Percoll. To isolate cells from the lung, the tissue was physically dissociated using scissors and incubated for 30 minutes in digest solution (1 mg/mL type D collagenase in RPMI supplemented with 5% fetal calf serum, 1% L-glutamine, 1% penicillin-streptomycin, and 10 mM HEPES). Resulting dissociated tissue was passed through 100- $\mu$ m strainers, centrifuged, and lymphocytes were removed from the supernatant. To isolate bone marrow lymphocytes, cleaned femur and tibia bones were ground with mortar and pestle, and the resulting solution filtered through a 100- $\mu$ m strainer. Red blood cells in spleen, liver, lung, and bone marrow were lysed using ACK lysis buffer.

### *Flow Cytometry and Cell Sorting*

Cell surface staining of single-cell suspensions from various organs was performed using fluorophore-conjugated antibodies (BD Biosciences, eBioscience, BioLegend, Tonbo Biosciences, Cell Signaling Technology). Intracellular staining was performed by fixing and permeabilizing with the eBioscience Foxp3 Transcription Factor Staining Set (Thermo Fisher) for staining intranuclear proteins and cytokines, or with formaldehyde and methanol for staining phosphorylated STAT proteins.

Flow cytometry and cell sorting were performed on the LSR II and Aria II cytometers (BD Biosciences), respectively. Data were analyzed with FlowJo software (Tree Star). Flow cytometry of lymphocytes was performed using the following fluorophore-conjugated antibodies: CD3 $\epsilon$  (17A2), TCR $\beta$  (H57-597), CD19 (6D5), F4/80 (BM8.1), NK1.1 (PK136), NKp46 (29A1.4), Ly49H (3D10), CD45.1 (A20), CD45.2 (104), CD45 (30-F11), IRF8 (V3GYWCH), CD11b (M1/70), CD27 (LG.7F9), KLRG1 (2F1), Ly49D (4E5), Ly49A (YE1/48.10.6), Ly49C/I (5E6), STAT4 pY693 (38/p-Stat4), STAT5 pY694 (C11C5), F(ab')<sub>2</sub> Rabbit IgG (polyclonal), CD69 (H1.2F3), Granzyme B (GB11), IFN- $\gamma$  (XMG1.2), CD107a (1D4B), CD8 $\alpha$  (53-6.7), and Bcl-2 (3F11).

Apoptosis was evaluated by caspase activity staining using the carboxyfluorescein FLICA poly caspase assay kit (Bio-Rad). NK cell proliferation was analyzed by labeling cells with 5  $\mu$ M CellTrace Violet (CTV, Thermo Fisher) prior to transfer, and CTV labeling was performed according to manufacturer protocol.

#### *Ex Vivo Stimulation of Lymphocytes*

Approximately 10<sup>6</sup> spleen lymphocytes were stimulated for 4 hours in RPMI containing 10% fetal bovine serum with 20 ng/mL recombinant mouse IL-12 (R&D Systems) plus 10 ng/mL IL-18 (MBL) or 50 ng/mL PMA (Sigma) plus 500 ng/mL Ionomycin (Sigma). Cells were cultured in media alone as a negative control.

### *Chromatin Immunoprecipitation and Sequencing*

5-10 x 10<sup>6</sup> NK cells (TCR $\beta$ <sup>-</sup>CD3 $\epsilon$ <sup>-</sup>CD19<sup>-</sup>F4/80<sup>-</sup>NK1.1<sup>+</sup>) were first enriched from spleens of pooled C57BL/6 mice by negative selection over BioMag goat anti-rat IgG beads (Qiagen) coated with rat anti-mouse CD8 $\alpha$ , CD4, CD19, and Ter-119 antibodies (Bio X Cell, clones 2.43, GK1.5, 1D3, and TER-119 respectively), sorted to high purity, and incubated with or without 20 ng/mL IL-12 and 10 ng/mL IL-18 (as well as 10 ng/mL IL-2 and 10 ng/mL IL-15 for IRF8 ChIP, R&D Systems). NK cells were stimulated for 30 min (H3K4me3 ChIP) or 16 hours (STAT4 and IRF8 ChIP). DNA and proteins were cross-linked for 7.5 minutes using 0.75% formaldehyde. ChIP was performed as previously described (Zheng et al., 2007), using 10  $\mu$ g of rabbit polyclonal anti-STAT4 antibody (Santa Cruz sc-486, clone C-20), 1  $\mu$ g of rabbit polyclonal anti-trimethyl Histone H3 (Lys4) antibody (H3K4me3, Millipore 07473), or 8  $\mu$ g of goat polyclonal anti-IRF8 antibody (Santa Cruz sc-6058, clone C-19), followed by qPCR or Illumina next-generation sequencing. qPCR primers for IRF8 ChIP include: *Zbtb32* (forward: TACGGCGATCATCCCTCCTT, reverse: AGAGCATCATCTCCCTAGCG), and gene desert 50kB upstream of *Foxp3* (forward: TAGCCAGAAGCTGGAAAGAAGCCA, reverse: TGATACCCTCCAGGTCCAACCATT). After determining the Ct value, percent input was calculated as  $100 \times 2^{(Ct_{\text{adjusted input}} - Ct_{\text{target}})}$ , where the Ct<sup>input</sup> was adjusted from 5% to 100% by subtracting log<sub>2</sub> 20 from input Ct values.

### *RNA Sequencing*

RNA was isolated from sorted cell populations using TRIzol (Thermo Fisher) and total RNA was amplified using the SMART-seq V4 Ultra Low Input RNA kit (Clontech). Subsequently, 10 ng of amplified cDNA was used to prepare Illumina HiSeq libraries with the Kapa DNA library preparation chemistry (Kapa Biosystems) using 8 cycles of PCR. Samples were barcoded and run on Hiseq 2500 1T, in a 50bp/50bp paired-end run, using the TruSeq SBS Kit v3 (Illumina).

### *ATAC Sequencing*

ATAC-seq libraries were prepared as previously described (Buenrostro et al., 2013). Briefly, fresh cells were washed in cold PBS and lysed. Transposition occurred at 42°C for 45 minutes. DNA was purified using the MinElute PCR purification kit (Qiagen) and amplified for 5 cycles. Additional PCR cycles were evaluated by real time PCR. Final product was cleaned by Ampure Beads at a 1.5x ratio. Libraries were sequenced on a Hiseq 2500 1T in a 50bp/50bp paired-end run, using the TruSeq SBS Kit v3 (Illumina).

## **3. Quantification and Statistical Analysis<sup>2</sup>**

### *ChIP Sequencing Analysis*

Analysis of ChIP-seq data was performed as previously described (Rapp et al., 2017).

## *RNA and ATAC Sequencing Analysis*

For both RNA-seq and ATAC-seq, paired-end reads were trimmed for adaptors and removal of low quality reads using Trimmomatic (v.0.36) (Bolger et al., 2014). Trimmed reads were mapped to the *Mus musculus* genome (mm10 assembly) using Bowtie2 (v2.2.9) (Langmead and Salzberg, 2012). For RNA-seq, read counts for features exons were generated using the summarizeOverlaps function from the GenomicAlignments package (v1.10.1) (Lawrence et al., 2013). Differential analyses were executed with DESeq2 (v1.14.1) (Love et al., 2014) using the UCSC ensGene model as a reference annotation. Gene tracks were generated by converting BAM files to bigWig files using bedtools2 (v.2.26.0) (Quinlan and Hall, 2010) and UCSC's bedGraphToBigWig (v.4) (Kent et al., 2010) and visualized using the Gviz R package (v.1.18.2) (Hahne and Ivanek, 2016). All tracks show genomic coordinates in megabase pairs on the x-axis and normalized tag counts on the y-axis.

The distribution of read counts across all genes was bimodal. The assumption that this corresponded to “expressed” and “non-expressed” genes was supported by examination of marker genes known to be expressed or not expressed in NK cells. The local minimum between the two peaks was chosen to be the threshold for expression.

Gene set enrichment analysis (GSEA) was performed using the v3.0 software running default parameters (Subramanian et al., 2005) (<http://www.broad.mit.edu/gsea/>), and with genes from significantly overrepresented PANTHER pathways. GSEA plots were replotted into a vector graphics format using the ReplotGSEA script by Thomas Kuilman (<https://github.com/PeeperLab/Rtoolbox/blob/master/R/ReplotGSEA.R>).



Plots of fold change vs. fold change (Figures 16A and 15D) were used to identify candidate genes of interest (Figure 16B), with the assumption that genes that are insufficiently upregulated or downregulated in NK-*Irf8*<sup>-/-</sup> NK cells at D4 may account for the D4-specific phenotype observed, despite there also being baseline transcriptional differences between NK-*Irf8*<sup>-/-</sup> and WT NK cells (Figure 14A). The first plot (Figure 16A) examined genes differentially expressed ( $|\log_2 \text{fold change}| \geq 1$ ) between WT and NK-*Irf8*<sup>-/-</sup> NK cells at D4 (red or blue-colored genes), then further identified a subset of these DE genes for which the magnitude of the difference between WT and NK-*Irf8*<sup>-/-</sup> is larger on D4 than at D0 ( $D4_{\text{Log2FC[WT/NK-}Irf8^{-/-}]} - D0_{\text{Log2FC[WT/NK-}Irf8^{-/-}]} \geq 1$  or  $D4_{\text{Log2FC[WT/NK-}Irf8^{-/-}]} - D0_{\text{Log2FC[WT/NK-}Irf8^{-/-}]} \leq -1$ ). Similarly, a separate fold change plot (Figure 15D) examined genes that are differentially expressed in WT NK cells on D4 vs. D0, and then honed in on a subset of these genes for which the magnitude of the difference between D4 and D0 is larger in WT than in NK-*Irf8*<sup>-/-</sup> NK cells ( $WT_{\text{Log2FC[D4/D0]}} - NK-*Irf8*^{-/-}_{\text{Log2FC[D4/D0]}} \geq 1$  or  $WT_{\text{Log2FC[D4/D0]}} - NK-*Irf8*^{-/-}_{\text{Log2FC[D4/D0]}} \leq -1$ ). Genes which satisfied both of these analyses, and which showed statistical significance ( $p_{\text{adj}} < 0.05$ ) in their respective initial differential expression analyses, excluding pseudogenes and genes with ambiguous annotation, are plotted as a heatmap (Figure 16B).

### *Statistical Analyses*

For graphs, data are shown as mean  $\pm$  SEM, and unless otherwise indicated, statistical differences were evaluated using a two-tailed unpaired Student's t test, assuming equal sample variance. Statistical differences in survival were determined by Gehan-Breslow-

Wilcoxon Test analysis.  $p < 0.05$  was considered significant. Graphs were produced and statistical analyses were performed using GraphPad Prism.

#### **4. Data and Software Availability<sup>2</sup>**

ChIP-seq, RNA-seq (timecourse), and ATAC-seq datasets are available in the Gene Expression Omnibus (GEO) repository as a SuperSeries under accession code GSE106139. RNA-seq data comparing WT and NK-*Irf8*<sup>-/-</sup> NK cells before and after MCMV infection are available in GEO under accession code GSE112948.

# **Chapter 3: Cytomegalovirus Infection Drives Avidity Selection of Natural Killer Cells<sup>3</sup>**

## **I. Introduction<sup>3</sup>**

### **1. Selection of Adaptive Lymphocytes during an Infectious Challenge**

A fundamental feature of the adaptive immune system is that it is equipped to recognize a nearly infinite array of potential antigens (Goldrath and Bevan, 1999). However, the high diversity of the adaptive antigen receptor repertoire is limited by niche size and constant cell turnover, such that the frequency of naive lymphocytes specific to a certain antigen is extremely low (Goldrath and Bevan, 1999; Jenkins et al., 2010). Similarly, during an infectious challenge, the diversity of the effector lymphocyte population is shaped by the repertoire of presented antigens and competition for these antigens, wherein lymphocytes receive proliferative and survival signals proportional to their responsiveness to a given epitope (Kedl et al., 2003; Kedl et al., 2000; Oberle et al., 2016). The process of affinity maturation, whereby T and B cells bearing antigen receptors (TCR and BCR, respectively) with optimal affinity to the relevant antigen undergo preferential expansion, shapes the immune response such that cells with the greatest protective potential to the host are most likely to survive and provide immunity upon pathogen re-encounter.

During the primary immune response, competition for antigen and resources results in outgrowth of CD8<sup>+</sup> T cells bearing TCRs that recognize their cognate antigen with optimal affinity (Day et al., 2007; Trautmann et al., 2005). B cells undergo a similar selection process. However, unlike T cells, which express a rearranged but

developmentally fixed antigen receptor sequence, B cells actively tune the affinity of their individual BCR in the dark zone of germinal centers via somatic hypermutation mediated by the DNA-editing enzyme activation-induced cytidine deaminase (AID) (Kim et al., 1981; Küppers et al., 1993; Muramatsu et al., 1999). B cells then migrate to the light zone to sample antigens presented by follicular DCs (MacLennan, 1994), where those bearing BCRs with highest affinity for antigen preferentially receive signals for survival and proliferation, leading to B cell affinity maturation (MacLennan, 1994). During the secondary or recall immune response, memory T cells as a population continue to undergo selection processes. Upon reinfection of previously primed mice with *Listeria monocytogenes*, T cell affinity maturation is observed, in which the diversity of the CD8<sup>+</sup> T cell TCR V $\beta$  repertoire specific for the listeriolysin (LLO)<sub>91-99</sub> epitope becomes restricted, suggesting that secondary CD8<sup>+</sup> T cell expansion is a selective process (Busch and Pamer, 1999). Concomitantly, activated and memory T cells undergo dynamic cellular membrane changes, including increased cholesterol content and enhanced clustering of TCR and co-receptors (Slifka and Whitton, 2001). These changes are independent of TCR affinity, but endow T cells with enhanced sensitivity for antigen by optimizing the signal transduction machinery (i.e. functional avidity maturation) (von Essen et al., 2012; Viganò et al., 2012). Thus, at both the individual cell and population levels, and during both primary and secondary immune responses, the adaptive immune system evolves by selecting lymphocytes best able to combat the specific pathogens encountered.

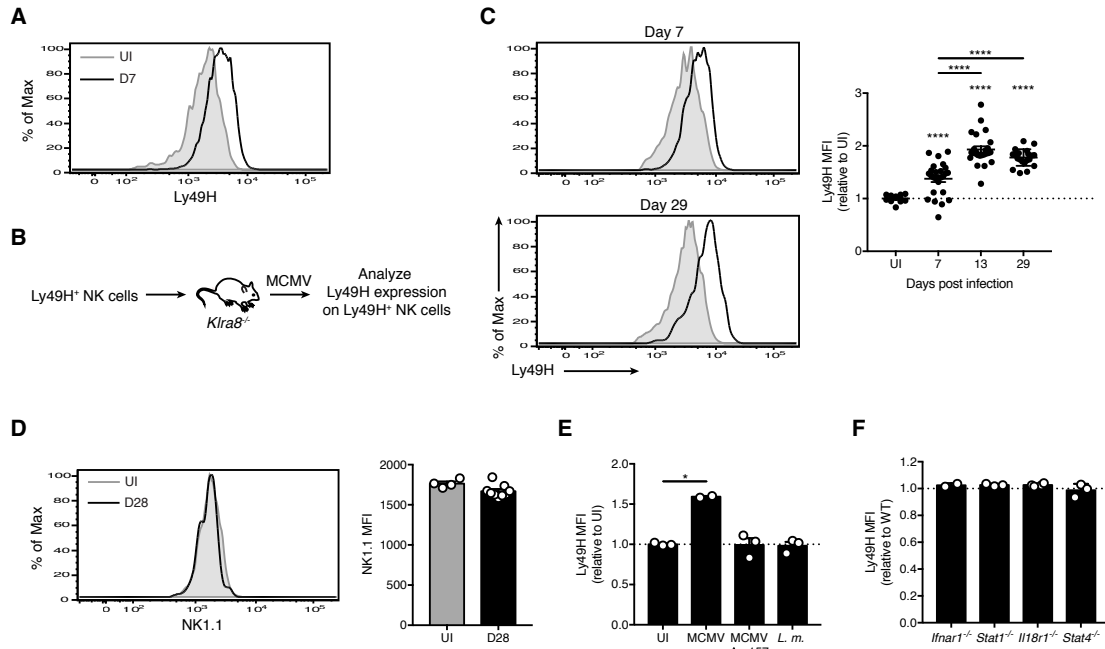
## **2. Heterogeneity of NK Cell “Adaptive” Responses**

NK cells are innate lymphocytes that play a critical, dominant role in herpesvirus control in both mice and humans (Biron et al., 1989; Bukowski et al., 1985; Etzioni et al., 2005). In addition to their traditional characterization as an arm of the innate immune system, NK cells exhibit “adaptive” antiviral responses to MCMV, demonstrating robust clonal proliferation and subsequent contraction of the effector NK cell pool to establish a long-lived population of memory NK cells with enhanced functionality and protective capacity following reinfection with MCMV (Sun et al., 2009). In mice, this adaptive response is driven by recognition of MCMV-encoded m157, a major histocompatibility complex (MHC) class I-like glycoprotein expressed on the infected cell surface, by a subset of NK cells bearing the activating receptor Ly49H (Arase et al., 2002; Daniels et al., 2001; Dokun et al., 2001; Sun et al., 2009). NK cells express a combination of germline-encoded activating and inhibitory receptors in random distribution, and a subset of naïve NK cells in C57BL/6 mice expresses Ly49H (Lanier, 2005). Some groups have reported heterogeneity within the naïve Ly49H<sup>+</sup> NK cell pool, where preferential expansion of unlicensed NK cells, NK cells with a history of recombination-activating gene (RAG) expression, and NK cells that lack expression of KLRG1 or the inhibitory receptor NK cell receptor (NKR)-P1B was described (Kamimura and Lanier, 2015; Karo et al., 2014; Orr et al., 2010). However, whether Ly49H<sup>+</sup> NK cells undergo selection during generation of the effector and memory pool on the basis of their avidity for m157 remains unresolved. Here, we interrogated the effect that heterogeneity in antigen receptor expression on Ly49H<sup>+</sup> NK cells has on their functional responses, and provide evidence that NK cells undergo avidity selection during MCMV infection.

## II. Results<sup>3</sup>

### 1. MCMV Glycoprotein m157 Drives Higher Ly49H Expression within the Antiviral NK Cell Pool<sup>3</sup>

To investigate whether viral infection modulates the overall avidity of the virus-specific NK cell pool, we infected wild-type (WT) mice with MCMV and observed increased levels of Ly49H receptor on Ly49H<sup>+</sup> NK cells (Figures 18A and 19A). To definitively track these antigen-experienced NK cells into the memory phase of the response, we adoptively transferred splenocytes, containing Ly49H<sup>+</sup> NK cells, into Ly49H-deficient recipients (*Klra8*<sup>-/-</sup> mice) (Figure 18B). Since the endogenous NK cells in *Klra8*<sup>-/-</sup> mice cannot engage m157 (Fodil-Cornu et al., 2008), only the transferred Ly49H<sup>+</sup> NK cells will undergo m157-dependent clonal proliferation following MCMV infection (Sun et al., 2009). Compared with the naïve Ly49H<sup>+</sup> NK pool, MCMV-driven effector and memory Ly49H<sup>+</sup> NK cells displayed higher Ly49H expression (Figure 18C). Ly49H expression was even greater on the effector NK cell pool as it contracts between days 7 and 13 PI, and remained high on day 29 PI (Figure 18C). In contrast, expression of NK1.1, which recognizes the MCMV-encoded protein m12 (Aguilar et al., 2017), remained unchanged on memory NK cells (Figure 18D), indicating that the sustained change in the distribution of Ly49H expression within the antiviral NK cell pool may be unique among NK cell activating receptors during MCMV infection. Neither infection with MCMV lacking m157 (MCMV- $\Delta$ m157) nor with *Listeria monocytogenes* affected Ly49H expression on Ly49H<sup>+</sup> NK cells (Figure 18E), indicating that m157 is required for this process. During MCMV infection, NK cells are exposed not only to infected cells displaying m157, but also to a proinflammatory environment rich in IL-12 and type I interferon



**Figure 18. MCMV-Driven Changes in Ly49H Expression within the Antiviral NK Cell Pool Are m157-Dependent<sup>3</sup>**

(A) Representative histograms of Ly49H expression on splenic Ly49H<sup>+</sup> NK cells (gating strategy in Fig. S1A) from uninfected (UI) and MCMV-infected WT mice at day 7 PI (D7). Data are representative of at least five independent experiments with 5-10 mice per experiment.

(B and C) Experimental schematic (B). WT splenocytes were adoptively transferred into *Klra8*<sup>-/-</sup> recipients, and the phenotype of Ly49H<sup>+</sup> NK cells tracked following infection of the recipients with MCMV. (C) Representative histograms of Ly49H expression on Ly49H<sup>+</sup> NK cells from blood of uninfected (UI) WT mice (shaded gray) versus at day 7 PI (top left) and day 29 PI (bottom left). Quantification of Ly49H MFI on Ly49H<sup>+</sup> NK cells at indicated timepoints PI relative to UI WT mice bled on the same day (right). Data are representative of three independent experiments with 10-25 mice per experiment.

(D) As in (C), representative histograms (left) and MFI (right) of NK1.1 on Ly49H<sup>+</sup> NK cells from blood of UI WT mice and at day 28 PI. Data are representative of at least three independent experiments with 3-9 mice per experiment.

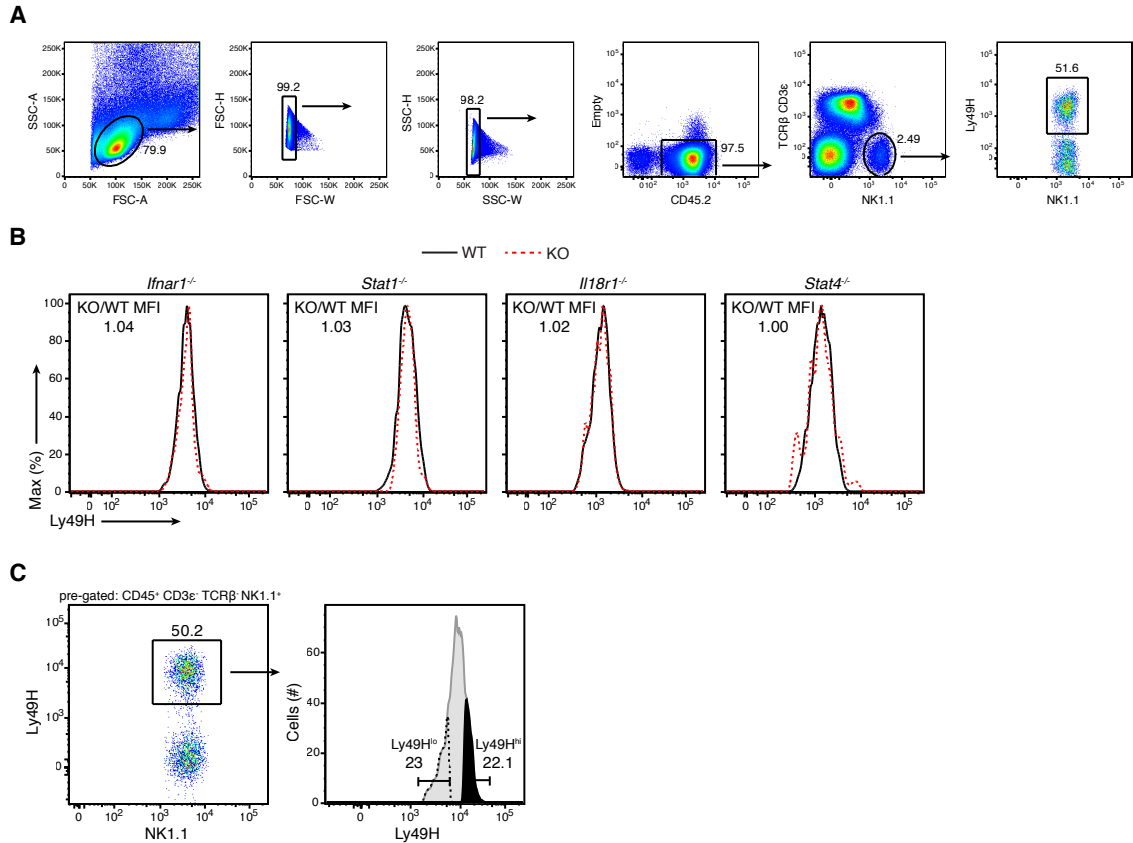
(E) WT mice were infected with MCMV, MCMV- $\Delta$ m157, *Listeria monocytogenes* (*L.m.*) or uninfected (UI). Data is represented as Ly49H MFI on Ly49H<sup>+</sup> NK cells from blood at day 7 PI for indicated infections relative to uninfected. Data are representative of two independent experiments with 2-5 mice per group.

(F) As in (B), except splenocytes from congenically marked WT and indicated knockout (KO) mice were adoptively co-transferred. Data is represented as Ly49H MFI on KO Ly49H<sup>+</sup> NK cells relative to WT from blood at day 7 PI. Data are representative of two to four independent experiments with 2-5 mice per group. Groups with a ratio < 1 were compared against a hypothetical value of 1 using a one sample t test.

Groups were compared using an unpaired, two-tailed Student's t test (D) or against a hypothetical value of 1 using a one sample t test (E, F). In (C), each timepoint was compared against a hypothetical value of 1 using a one sample t test, and against each other using a paired two-tailed t test. Data are presented as the mean  $\pm$  SEM. \*  $p < 0.05$ ; \*\*\*\*  $p < 0.0001$ .

<sup>3</sup>The material herein has been submitted for publication in a modified format. Adams, N.M., Geary, C.D., Santosa, E.K., Lumaquin, D., Le Ludec, J.B., Sottile, R., van der Ploeg, K., Hsu, J., Whitlock, B.M., Jackson, B.T., Weizman, O.E., Huse, M., Hsu, K.C., and Sun, J.C. 2019. Cytomegalovirus Infection Drives Avidity Selection of Natural Killer Cells. *Immunity. in press.*





### Figure 19. Gating Strategy for Mouse Ly49H<sup>+</sup> NK Cells<sup>3</sup>

(A) Representative flow plots from spleen of uninfected WT mouse, illustrating the gating strategy and sorting strategy for analysis and purification of mouse Ly49H<sup>+</sup> NK cells.

(B) Representative histograms of Ly49H expression on WT (black line) and indicated genetically-deficient (KO, red dashed line) Ly49H<sup>+</sup> NK cells from Figure 18F. For each histogram, the corresponding ratio of Ly49H MFI on KO compared to WT Ly49H<sup>+</sup> NK cells is shown.

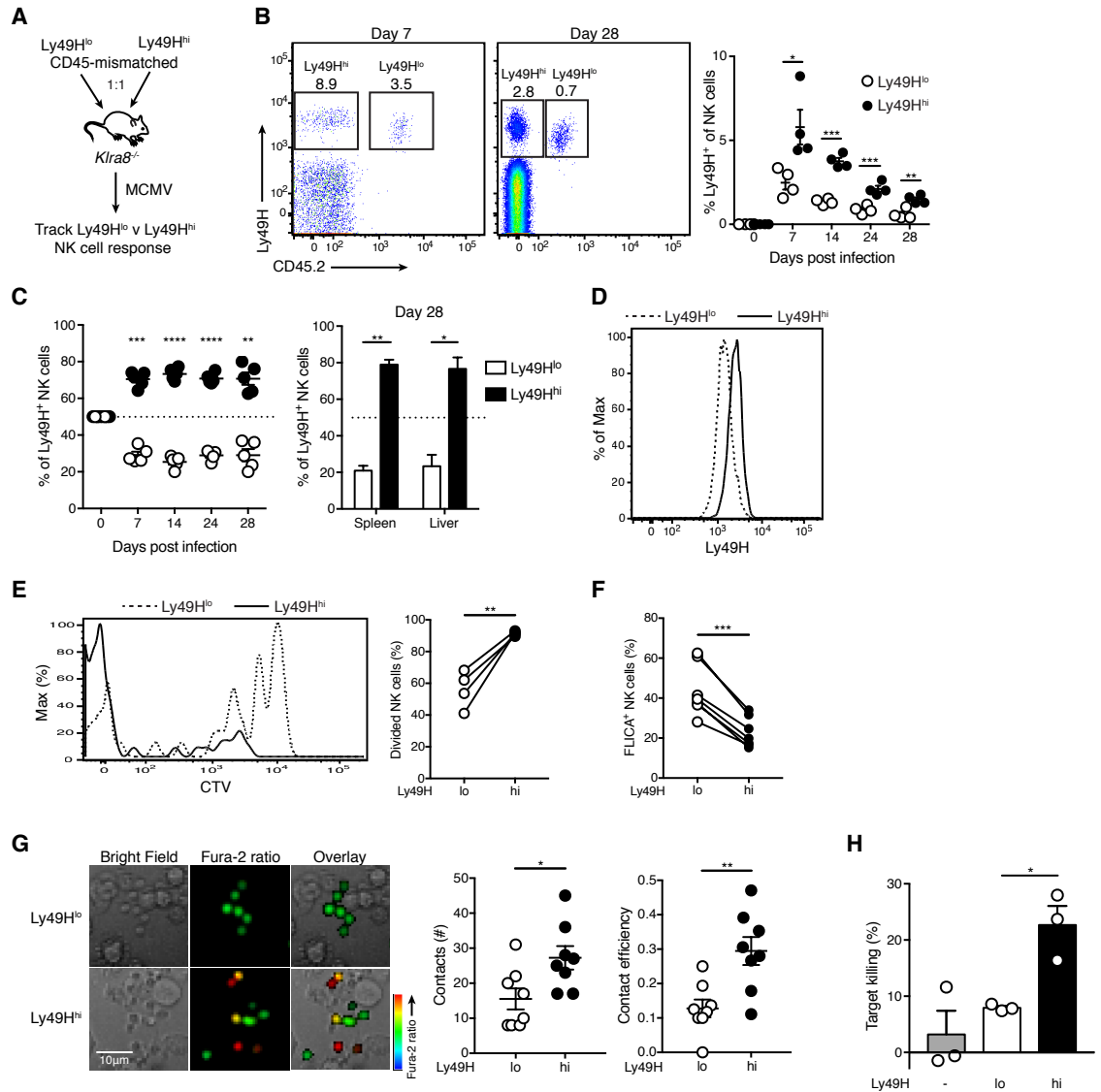
(C) Representative flow plots from spleen of uninfected WT mouse, illustrating the gating and sorting strategy for Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells, defined as Ly49H<sup>+</sup> NK cells in the bottom or top ~20-25% by Ly49H MFI, respectively.

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(Biron and Tarrio, 2015), which has been shown to support the proliferative burst and memory formation of Ly49H<sup>+</sup> NK cells (Madera et al., 2016; Madera and Sun, 2015; Sun et al., 2012). However, NK cells unresponsive to IL-12 (*Stat4*<sup>-/-</sup>), IL-18 (*Il18r1*<sup>-/-</sup>), and type I IFN (*Ifnar1*<sup>-/-</sup> and *Stat1*<sup>-/-</sup>) expressed Ly49H comparably to WT effector NK cells following MCMV infection (Figures 18F and 19B), suggesting that these proinflammatory cytokines are individually dispensable for the MCMV-driven avidity changes. Thus, MCMV infection elicits an m157-dependent change in the distribution of Ly49H expression on effector and memory Ly49H<sup>+</sup> NK cells.

## **2. Avidity Selection Shapes the Effector and Memory Ly49H<sup>+</sup> NK Cell Pool during MCMV Infection<sup>3</sup>**

As the naïve Ly49H<sup>+</sup> NK cell pool has a broad distribution in Ly49H expression (over 1-log spread in Ly49H MFI, Figure 18A), we reasoned that one explanation for a population increase in Ly49H is the preferential expansion of Ly49H<sup>+</sup> NK cells with greater baseline Ly49H expression over Ly49H<sup>+</sup> NK cells expressing less of this receptor (i.e. avidity selection). To directly test this hypothesis, we purified Ly49H<sup>+</sup> NK cells with different Ly49H receptor abundance (Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup>, defined as Ly49H<sup>+</sup> NK cells in the bottom or top ~20% of Ly49H MFI, respectively) from congenically distinct WT mice and adoptively co-transferred equal numbers of Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells into Ly49H-deficient recipients (Figures 20A and 19C). Following MCMV infection of the recipients, the activation and expansion of the co-transferred NK cell populations were



**Figure 20.  $Ly49H^{+}$  NK Cells Undergo Avidity Selection During MCMV Infection<sup>3</sup>**

(A-D) Experimental schematic (A). Equal numbers of splenic  $Ly49H^{lo}$  and  $Ly49H^{hi}$  NK cells, purified from congenically distinct WT mice, were adoptively co-transferred into  $Klra8^{-/-}$  recipients, and these two NK cell responses tracked following infection of the recipients with MCMV. (B) Representative flow plots gated on NK cells from blood at day 7 PI (left) and spleen at day 28 PI (middle). Quantification of percent  $Ly49H^{lo}$  and  $Ly49H^{hi}$  NK cells within total NK cells in blood at indicated timepoints (right). (C) The percentage of  $Ly49H^{lo}$  and  $Ly49H^{hi}$  NK cells within transferred  $Ly49H^{+}$  NK cells in blood at indicated timepoints (left) and in indicated organs at day 28 PI (right). (D) Representative histograms of Ly49H expression on  $Ly49H^{lo}$  (dashed line) and  $Ly49H^{hi}$  (black line) NK cells from blood at day 7 PI. Data are representative of two independent experiments with 3-5 mice per experiment.

(E) WT splenocytes were labeled with CTV and adoptively transferred into congenically distinct WT mice 1 day prior to MCMV infection. Representative histograms of CTV in splenic Ly49H<sup>lo</sup> (dashed line) and Ly49H<sup>hi</sup> (black line) NK cells at day 7 PI (left). Quantification of indicated NK cell populations that have divided at least once (right). Data are representative of three independent experiments with 4 mice per experiment.

(F) Percent of splenic Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells staining positive for FLICA (marking activated caspases) at day 4 PI. Data are pooled from two independent experiments with 3-4 mice per experiment.

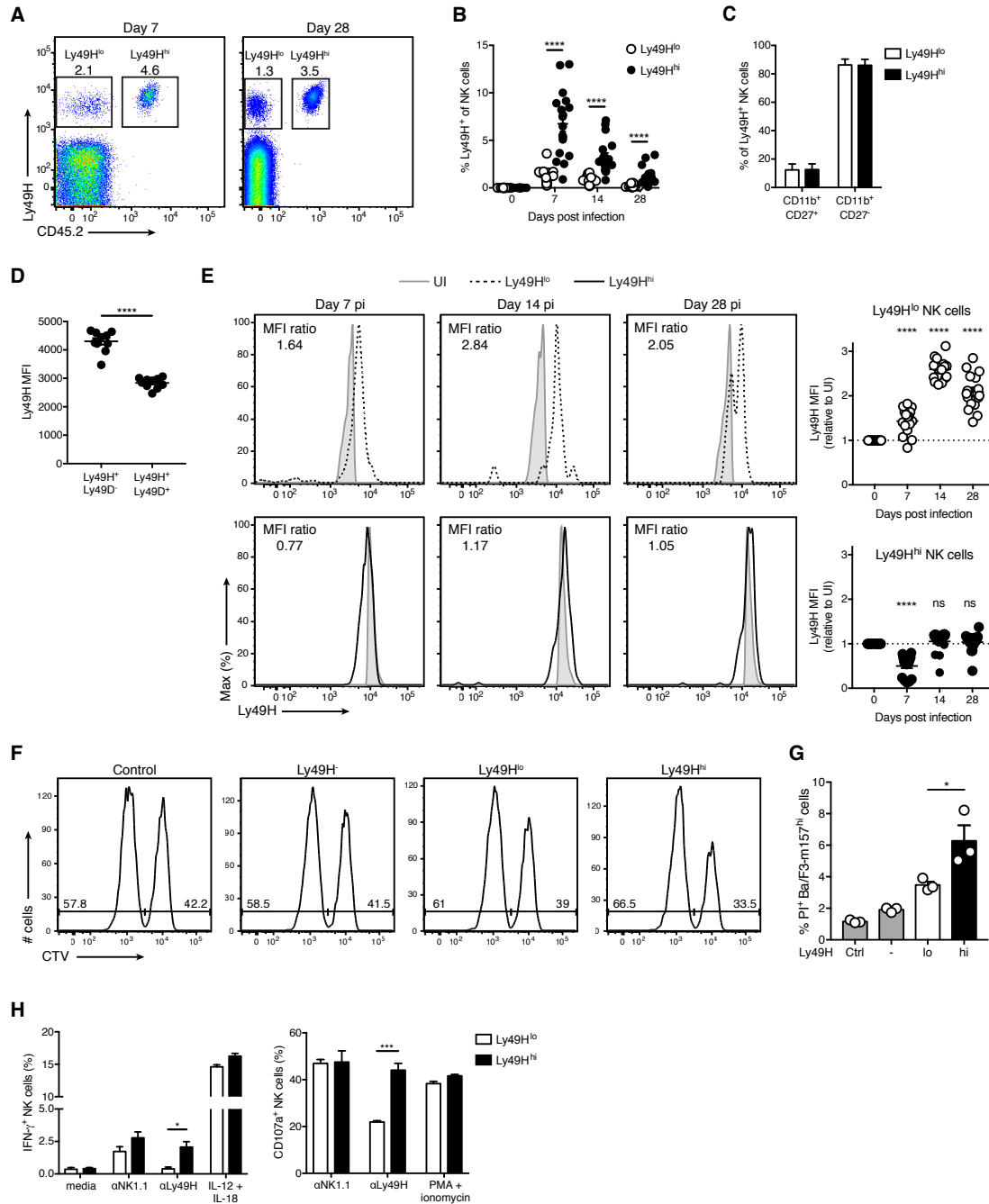
(G) Purified splenic Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells were loaded with Fura-2AM and co-cultured with Ba/F3-m157 target cells. Live-cell imaging was then performed. Representative images from the time-lapse analysis (left). Quantification of the number of contacts between NK cells and target cells (middle) and contact efficiency (right), defined as the proportion of target cell contacts made by NK cells that resulted in an NK cell Ca<sup>2+</sup> flux (change in NK cell color from green to yellow or red). Data are representative of two experiments with 4 replicates per group per experiment.

(H) Purified splenic Ly49H<sup>lo</sup>, Ly49H<sup>hi</sup>, and Ly49H<sup>hi</sup> NK cells were incubated with Ba/F3-m157 target cells (CTV<sup>hi</sup>) and Ba/F3 control cells (CTV<sup>lo</sup>) at 10:1:1 ratio (effector:target:control) for 6 hours. Quantification of target cell killing by indicated NK cell populations compared to control wells lacking NK cells. Data are representative of three independent experiments with 3 replicates per group per experiment. Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells were compared using an unpaired, two-tailed Student's t test.

Groups were compared using a paired, two-tailed t test (B, E, F), an unpaired, two-tailed Student's t test (G) or against a hypothetical value of 50 using a one sample t test (C). Data are presented as the mean ± SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

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analyzed longitudinally in response to the same antigenic and inflammatory milieu (Figure 20A). Although both Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells underwent clonal expansion and memory formation (Figures 20B and 20C), the magnitude of their response differed. Ly49H<sup>hi</sup> NK cells notably expanded more robustly and outnumbered the Ly49H<sup>lo</sup> NK cells at 7 days PI (Figures 20B and 20C), the peak of the antiviral NK cell response to MCMV, regardless of congenic markers used (Figure 21A). Furthermore, Ly49H<sup>hi</sup> NK cells preferentially contributed to the memory NK cell pool in peripheral blood, as well as in various organs, at 28 days PI (Figures 20B and 20C). The ratio of Ly49H<sup>hi</sup> to Ly49H<sup>lo</sup> NK cells did not diverge further during contraction (Figure 20C), suggesting the greater abundance of Ly49H<sup>hi</sup> memory NK cells was due to their advantage during expansion. Even when purified Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells were transferred into separate animals to exclude competition of the two groups within the same host, Ly49H<sup>hi</sup> NK cells produced a larger effector and memory population than did Ly49H<sup>lo</sup> NK cells (Figure 21B), indicating that NK cell avidity for viral ligand regulates intrinsic aspects of the NK cell antiviral response. Despite their differential expansion, Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells matured similarly (Figure 21C). Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells retained their relative differences in Ly49H expression following infection (Figure 20D), suggesting that Ly49H expression was clonally maintained. Interestingly, we observed that naïve Ly49H<sup>+</sup> NK cells co-expressing the DAP12-dependent activating receptor Ly49D exhibited moderately reduced Ly49H abundance (Figure 21D). Thus, Ly49D expression levels may contribute towards establishing heterogeneity in Ly49H expression within the naïve NK cell pool.



**Figure 21. Ly49H<sup>hi</sup> NK cells Exhibit Heightened Ly49H-Dependent Responses<sup>3</sup>**

(A) Experimental design as in Figure 20A. Representative flow plots gated on NK cells from blood at day 7 PI (left) and spleen at day 28 PI (right). Shown is an example in which Ly49H<sup>lo</sup> (CD45.1) and Ly49H<sup>hi</sup> (CD45.2) NK cells bear the opposite congenic markers to those used in Figure 20B.

(B) *Klra8*<sup>-/-</sup> recipients received either purified splenic Ly49H<sup>lo</sup> or Ly49H<sup>hi</sup> NK cells and were infected with MCMV. Quantification of percent Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells within total NK cells in blood at indicated timepoints. Data are pooled from three

(Ly49H<sup>lo</sup>) or four (Ly49H<sup>hi</sup>) independent experiments with 3-8 mice per group per experiment.

(C) Experimental design as in Figure 20A. Percentage of Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells from blood within the CD11b<sup>+</sup>CD27<sup>+</sup> or CD11b<sup>+</sup>CD27<sup>-</sup> (terminally mature) subsets at day 7 PI. Data are representative of two independent experiments with 3-5 mice per experiment.

(D) Quantification of Ly49H MFI on Ly49H<sup>+</sup>Ly49D<sup>-</sup> and Ly49H<sup>+</sup>Ly49D<sup>+</sup> NK cells from blood of WT mice. Data are representative of three independent experiments with 3-9 mice per experiment.

(E) Experimental design as in Figure 21B. Representative histograms (left three panels) of Ly49H expression on transferred Ly49H<sup>lo</sup> (dashed line, top row) or Ly49H<sup>hi</sup> (black line, bottom row) NK cells from blood compared with the bottom or top 20%, respectively, of Ly49H-expressing naïve NK cells from uninfected WT mice (shaded gray histograms). Quantification of Ly49H MFI on Ly49H<sup>lo</sup> or Ly49H<sup>hi</sup> NK cells at indicated timepoints relative to their corresponding naïve NK cell populations (right panels). Data are pooled from three (Ly49H<sup>lo</sup>) or four (Ly49H<sup>hi</sup>) independent experiments with 3-8 mice per group per experiment.

(F and G) Experimental design as in Figure 20H. (F) Representative histograms of target and control cell percentages within live cells following co-culture with the indicated NK cell populations. (G) Percentage of propidium iodide (PI)-staining target cells among total CTV<sup>hi</sup> cells following co-culture with the indicated NK cell populations. Data are representative of three independent experiments with 3 replicates per group per experiment. Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells were compared using an unpaired, two-tailed Student's t test.

(H) Percentage of purified splenic Ly49H<sup>lo</sup> or Ly49H<sup>hi</sup> NK cells producing IFN- $\gamma$  (left) or degranulating (right) following 4 hour *ex vivo* culture with the indicated stimuli. Data are representative of two independent experiments with 4 replicates per stimulation group per experiment. Groups were compared using an unpaired, two-tailed Student's t test and corrected for testing multiple hypotheses.

Groups were compared using an unpaired, two-tailed Student's t test (B), a paired, two-tailed t test (C, D), or against a hypothetical value of 1 using a one sample t test (E). Data are presented as the mean  $\pm$  SEM. ns, not significant; \* p < 0.05; \*\*\* p < 0.0001; \*\*\*\* p < 0.0001.

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To determine whether avidity maturation (i.e. upregulation of Ly49H at the cellular level) and avidity selection were occurring concomitantly, we longitudinally tracked the Ly49H MFI on purified Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells, which we compared with pre-infection expression levels. Interestingly, we observed that Ly49H<sup>lo</sup> NK cells increased their expression of Ly49H to a greater degree (relative to the bottom 20% of Ly49H-expressing naïve NK cells), most notably during the contraction phase, with Ly49H expression approaching 2- to 3-fold the pre-infection level of Ly49H<sup>lo</sup> NK cells (Figure 21E). In contrast, Ly49H<sup>hi</sup> NK cells did not considerably increase their Ly49H expression (relative to the top 20% of Ly49H-expressing naïve NK cells) (Figure 21E), suggesting there may be an “upper limit” for the expression of this activating receptor on NK cells.

To investigate the mechanism behind differential expansion of Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells, we transferred bulk splenocytes labeled with CTV into WT mice. Following MCMV infection, Ly49H<sup>hi</sup> NK cells divided more efficiently (Figure 20E), illustrating greater proliferation of these cells. Furthermore, during MCMV-driven expansion, Ly49H<sup>lo</sup> NK cells demonstrated greater caspase activity (as assessed by FLICA staining) than did Ly49H<sup>hi</sup> NK cells (Figure 20F), indicating higher levels of Ly49H<sup>lo</sup> NK cell death. Collectively, these data suggest that NK cell avidity regulates the proliferative capacity and survival of a given Ly49H-expressing NK cell during MCMV infection, which underlies the greater expansion and subsequent memory pool establishment of Ly49H<sup>hi</sup> NK cells.



### 3. Differential m157-Mediated Activation within the Ly49H<sup>+</sup> NK Cell Population<sup>3</sup>

These data raised the possibility that the amount of Ly49H receptor imparts NK cells with a differential capacity for engagement with infected cells (i.e. avidity) or differential magnitude of Ly49H-dependent signaling. To test this hypothesis, we performed live cell imaging of purified Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells co-cultured with Ba/F3 cells expressing m157 (Ba/F3-m157). Ly49H receptor ligation results in phosphorylation of its adaptor DAP12 within an immunoreceptor tyrosine-based activation motif (ITAM), which recruits and activates Syk and zeta chain of T cell receptor associated protein kinase 70 (ZAP70), initiating their signaling cascades and resulting in increased intracellular calcium (Lanier, 2008). Thus, to visualize productive target cell encounters, NK cells were labeled with the cell permeable Fura-2 calcium indicator prior to co-culture in order to detect activation-induced Ca<sup>2+</sup> flux. Not only did Ly49H<sup>hi</sup> NK cells make more contacts with Ba/F3-m157 cells than did Ly49H<sup>lo</sup> NK cells, but also a greater percentage of Ly49H<sup>hi</sup> NK cell contacts resulted in robust NK cell activation and Ca<sup>2+</sup> flux (Figure 20G). These results suggest that Ly49H<sup>hi</sup> NK cells may be preferentially recruited into the antiviral response.

Given that NK cell cytotoxicity requires activating receptor engagement, we speculated that NK cell cytotoxic function is dependent on avidity for cognate ligand. To assess this, we co-cultured purified NK cells with varying degrees of Ly49H expression (Ly49H<sup>-</sup>, Ly49H<sup>lo</sup>, and Ly49H<sup>hi</sup>) with equal numbers of target Ba/F3-m157 and control Ba/F3 cells labeled differentially with CTV. As expected, Ly49H<sup>-</sup> cells did not alter the original target-to-control cell ratio compared to wells receiving no NK cells (Figures 20H

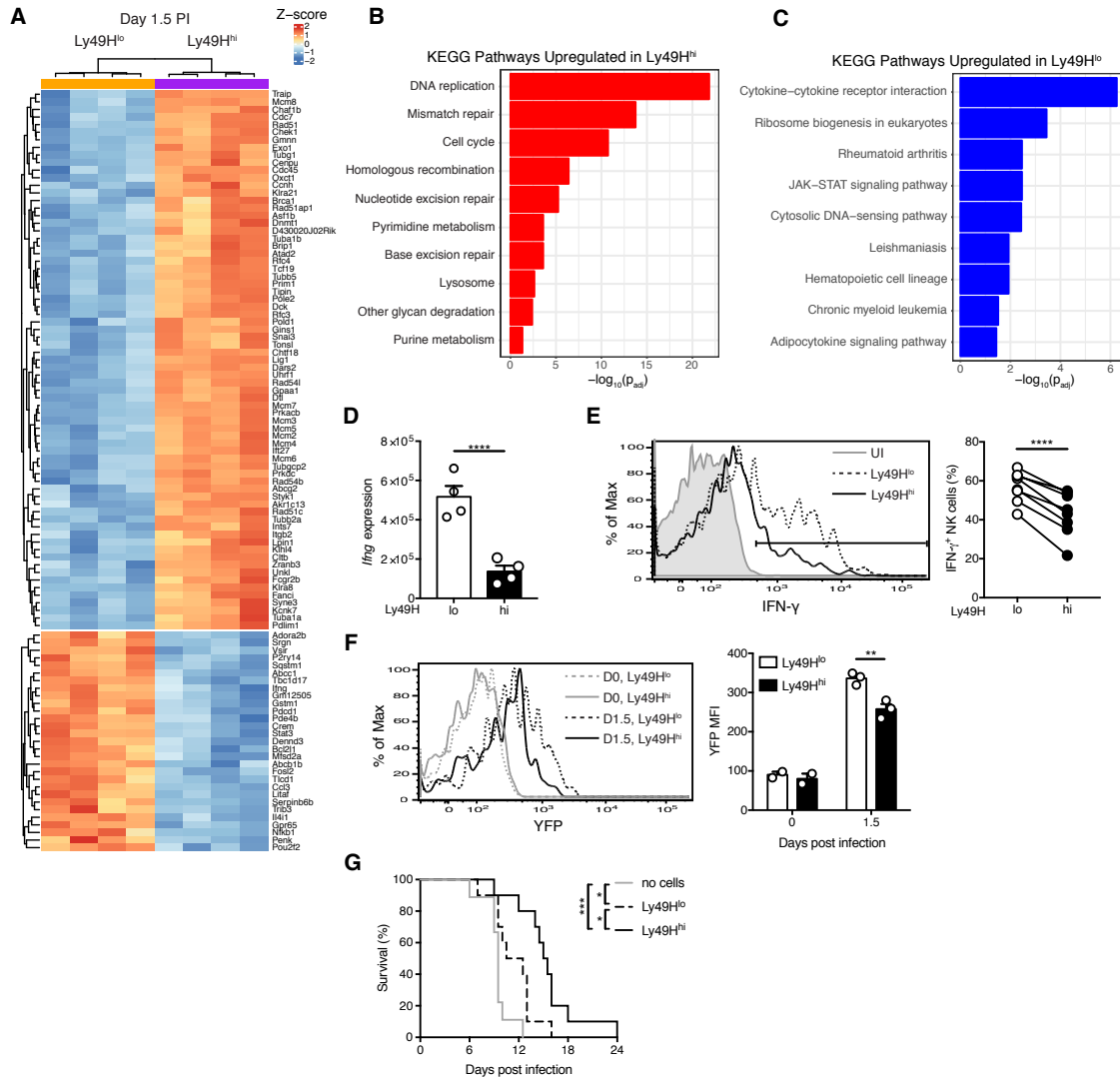
and 21F), indicating their failure to mediate Ly49H-dependent cytotoxicity. Although Ly49H<sup>lo</sup> cells were capable of killing Ba/F3-m157 target cells, Ly49H<sup>hi</sup> cells possessed the most potent cytotoxic function (Figures 20H, 21F and 21G). The enhanced functionality of Ly49H<sup>hi</sup> NK cells was strictly Ly49H-dependent, as a greater percentage of Ly49H<sup>hi</sup> NK cells produced IFN- $\gamma$  and degranulated only in response to Ly49H ligation *ex vivo*, but not NK1.1 ligation or nonspecific activation with PMA and ionomycin (Figure 21H), excluding a developmentally-determined general heightened reactivity of these cells. Collectively, these data indicate that higher Ly49H expression precipitates greater Ly49H-dependent activation and effector function (i.e. functional avidity) in the presence of cells expressing cognate ligand.

#### **4. Differential Effector Functions within the Ly49H<sup>+</sup> NK Cell Population Are Specified During Early MCMV Infection<sup>3</sup>**

The detection of viral ligand-bearing infected cells by Ly49H<sup>+</sup> NK cells is an essential signal for NK cell-mediated host protection, yet MCMV also induces a highly inflammatory environment rich in IL-12, IL-18, and type I interferon (Biron and Tarrio, 2015), which can lead to nonspecific activation of NK cells (Dokun et al., 2001). Although circulating levels of most proinflammatory cytokines decline within 2 days of the onset of MCMV infection, they play an essential role in promoting NK cell effector function (e.g. IFN- $\gamma$  production) and programming their adaptive responses (Biron and Tarrio, 2015; Madera et al., 2016; Madera and Sun, 2015; Sun et al., 2012). Thus, to come to a more nuanced and comprehensive understanding of how NK cell avidity

regulates NK cell responses to MCMV-induced signals, we performed comparative transcriptome analysis by RNA-seq on purified splenic Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells during early MCMV infection (day 1.5 PI). Surprisingly, we found that a large number of genes were differentially expressed ( $p_{\text{adj}} < 0.05$ ) between these two NK cell populations, with 1232 transcripts upregulated and 709 transcripts downregulated in Ly49H<sup>hi</sup> compared with Ly49H<sup>lo</sup> NK cells, the top 100 of which are shown in Figure 22A. As a validation of our experimental design, *Klra8*, encoding for Ly49H, was the most differentially expressed gene ranked by p-value. Classification of differentially expressed genes into Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways revealed divergent properties between these two NK cell populations. Ly49H<sup>hi</sup> NK cells demonstrated a transcriptional program enriched for genes responsible for cell cycle control, DNA replication, and maintenance of genome integrity (Figures 22B and 23A), consistent with the observed proliferative advantage of these cells (Figure 20E). In contrast, genes upregulated in Ly49H<sup>lo</sup> NK cells were primarily involved with cytokine and Janus kinase (JAK)-STAT signaling (Figures 22C and 23B). The extent and nature of the observed transcriptional differences underscore the diversity within the Ly49H<sup>+</sup> NK cell compartment and the divergence of their cellular activities within the earliest days of MCMV infection.

To validate and extend our RNA-seq results, we focused on several differentially expressed genes notable for their known roles in NK cell biology and for their regulation by proinflammatory cytokines. Transcript (*Il2ra*) and protein (CD25) levels of the high-affinity  $\alpha$  subunit of the IL-2 receptor were both more robustly upregulated in Ly49H<sup>lo</sup> compared with Ly49H<sup>hi</sup> NK cells during MCMV infection (Figures 23C and 23D).



### Figure 22. Ly49H<sup>lo</sup> NK Cells Produce More IFN-γ During Early MCMV Infection<sup>3</sup>

(A-D) Splenic Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells were sorted for RNA-seq at day 1.5 PI (4 replicates per population). (A) Heat map and hierarchical clustering of top 100 differentially expressed genes by p-value. (B-C) Gene ontology analysis of differential KEGG pathways for genes significantly ( $p_{adj} < 0.05$ ) upregulated in Ly49H<sup>hi</sup> NK cells (B) and upregulated in Ly49H<sup>lo</sup> NK cells (C). Their respective p values are shown. (D) Quantification of RNA-seq reads mapping to the *Ifng* locus. P value was calculated in DESeq2 and adjusted for testing multiple hypotheses.

(E) Representative histograms of intracellular IFN-γ expression in splenic Ly49H<sup>+</sup> NK cells from uninfected (UI) and MCMV-infected WT mice at day 1.5 PI (left). Quantification of percent IFN-γ<sup>+</sup> NK cells within indicated NK cell populations (right). Data are representative of at least five independent experiments with 3-15 mice per experiment.

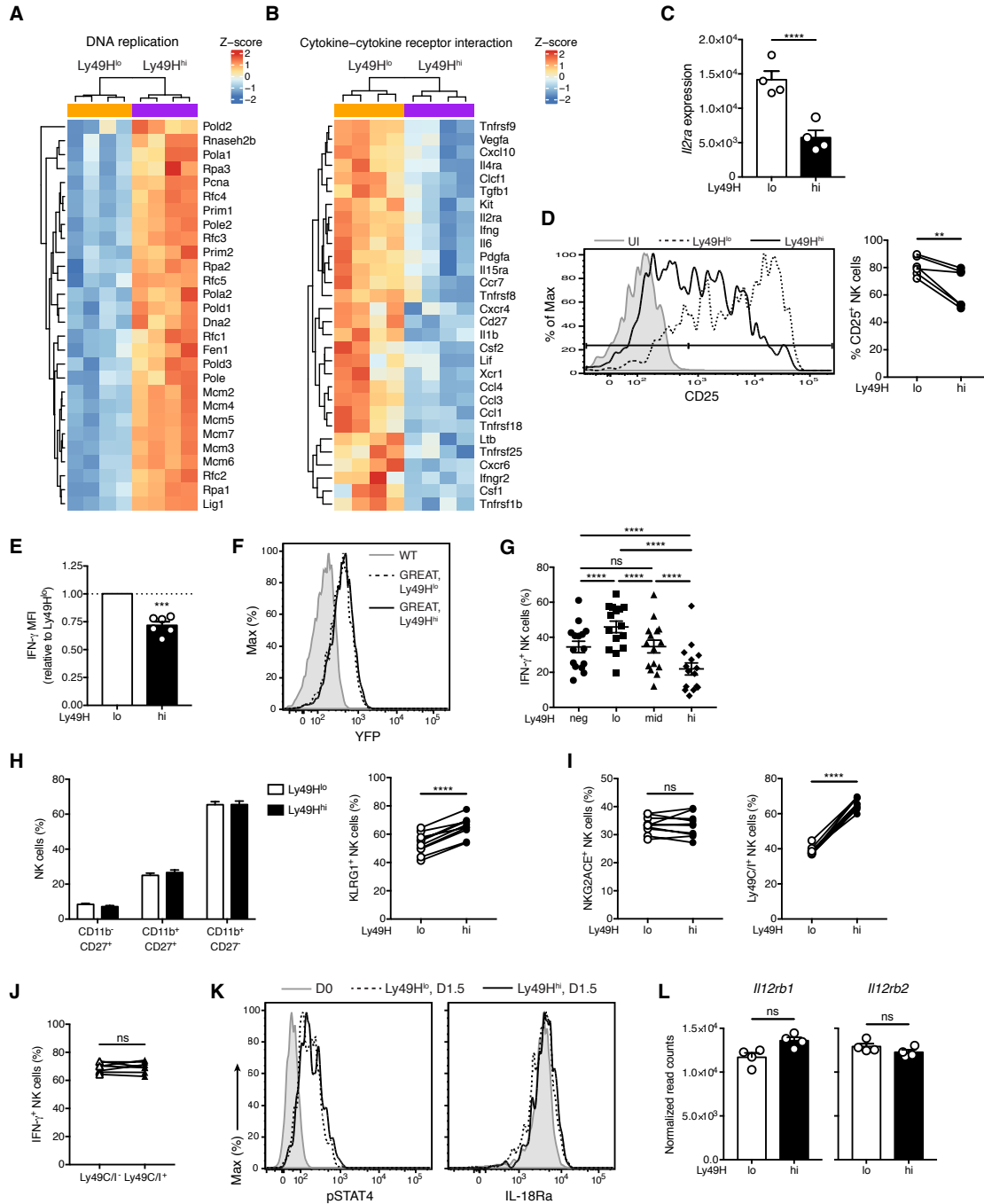
(F) As in (E), except uninfected or MCMV-infected *Ifng*-IRES-YFP reporter mice at day 1.5 PI. Representative histograms (left) and quantification of YFP MFI (right) before and

after MCMV infection. Data are representative of two independent experiments with 2-3 mice per time point per experiment.

(G) Kaplan-Meier survival curves of *Rag2*<sup>-/-</sup> *Il2rg*<sup>-/-</sup> mice that received either no cells (gray line), 50,000 purified Ly49H<sup>lo</sup> NK cells (black dashed line), or 50,000 purified Ly49H<sup>hi</sup> NK cells (black line) 2 days prior to MCMV infection. Data are pooled from two independent experiments with 4-5 mice per group per experiment.

Groups were compared using a paired, two-tailed t test (E, F) or the Log-rank (Mantel-Cox) test with correction for testing multiple hypothesis (G). Data are presented as the mean ± SEM. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001.

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**Figure 23. Ly49H Expression Specifies NK Cell Effector Function during Early MCMV Infection<sup>3</sup>**

(A-C) Experimental design as in Figures 22A-D. (A-B) Heat map and hierarchical clustering of differential KEGG pathway genes found to be significantly upregulated in Ly49H<sup>hi</sup> NK cells (DNA replication, A) or Ly49H<sup>lo</sup> NK cells (cytokine-cytokine receptor interaction, B) at day 1.5 PI. (C) RNA-seq reads mapping to the *Il2ra* locus. P value was calculated in DESeq2 and adjusted for testing multiple hypotheses.

(D) Representative histograms of CD25 expression in splenic Ly49H<sup>+</sup> NK cells from uninfected (UI) and MCMV-infected WT mice at day 1.5 PI (left). Quantification of the percentage of CD25<sup>+</sup> NK cells within indicated NK cell populations (right). Data are representative of three independent experiments with 6-15 mice per experiment.

(E) Experimental design as in Figure 22E. IFN- $\gamma$  MFI on IFN- $\gamma$ <sup>+</sup> Ly49H<sup>hi</sup> NK cells relative to IFN- $\gamma$  Ly49H<sup>lo</sup> NK cells at day 1.5 PI. Data are representative of at least five independent experiments with 3-15 mice per experiment.

(F) Representative histograms of YFP expression in naïve Ly49H<sup>lo</sup> (black dashed line) or Ly49H<sup>hi</sup> (black line) NK cells from blood of *Ifng*-IRES-YFP reporter mice. YFP MFI in Ly49H<sup>+</sup> NK cells from blood of WT mice (gray line) was used as a control. Data are representative of two independent experiments with 5 mice per experiment.

(G) Experimental design as in Figure 22E. Quantification of percent IFN- $\gamma$ <sup>+</sup> NK cells within indicated NK cell populations. Ly49H<sup>mid</sup> NK cells are all Ly49H<sup>+</sup> NK cells with Ly49H expression between that of Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells. Data are representative of at least five independent experiments with 3-15 mice per experiment.

(H) Percentage of naïve Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells from blood that fall within each NK cell maturation stage, defined by expression of CD11b and CD27 (left), and that express KLRG1 (right). Data are representative of at least three independent experiments with 5-10 mice per experiment.

(I) Percentage of naïve Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells from blood expressing the licensed (in C57BL/6 mice) inhibitory receptors NKG2A (left) and Ly49C/I (right). Data are representative of three independent experiments with 5-10 mice per experiment.

(J) Percentage of splenic Ly49C/I<sup>-</sup> and Ly49C/I<sup>+</sup> cells (within Ly49H<sup>+</sup> NK cells) producing IFN- $\gamma$  at day 1.5 PI.

(K) Representative histograms of intracellular pSTAT4 (left) and surface IL-18Ra (right) expression on splenic Ly49H<sup>+</sup> NK cells from uninfected (D0) and MCMV-infected WT mice at day 1.5 PI. Data are representative of two independent experiments with 3-6 mice per experiment.

(L) Experimental design as in Figures 22A-D. Quantification of RNA-seq reads mapping to the *I112rb1* (left) and *I112rb2* (right) loci. P values were calculated in DESeq2 and corrected for testing multiple hypotheses.

Groups were compared using a paired, two-tailed t test (D, H, I, J), against a hypothetical value of 1 using a one sample t test (E), or an RM one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (G). Data are presented as the mean  $\pm$  SEM. ns, not significant; \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001.

<sup>3</sup>The material herein has been submitted for publication in a modified format. Adams, N.M., Geary, C.D., Santosa, E.K., Lumaquin, D., Le Luduec, J.B., Sottile, R., van der Ploeg, K., Hsu, J., Whitlock, B.M., Jackson, B.T., Weizman, O.E., Huse, M., Hsu, K.C., and Sun, J.C. 2019. Cytomegalovirus Infection Drives Avidity Selection of Natural Killer Cells. *Immunity*. *in press*.

Another intriguing gene was *Ifng* (encoding IFN- $\gamma$ ), transcripts of which were detected in much higher quantities in Ly49H<sup>lo</sup> compared with Ly49H<sup>hi</sup> NK cells (Figure 22D). Consistent with the transcript analysis, we observed that a greater percentage of Ly49H<sup>lo</sup> NK cells produced IFN- $\gamma$  during MCMV infection (Figure 22E), and Ly49H<sup>lo</sup> NK cells produced more IFN- $\gamma$  than Ly49H<sup>hi</sup> NK cells on a per-cell basis (Figure 23E). Using *Ifng*-IRES (internal ribosome entry site)-YFP (yellow fluorescent protein) reporter mice (herein designated interferon-gamma reporter with endogenous polyA transcript “GREAT” mice), we also detected greater upregulation of *Ifng* transcript in Ly49H<sup>lo</sup> NK cells during MCMV infection (Figure 22F). However, prior to infection, there were no differences between naïve Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells in baseline *Ifng* transcript (Figures 22F and 23F) or their ability to produce IFN- $\gamma$  when stimulated with IL-12 and IL-18 *ex vivo* (Figure 21H), suggesting that infection-specific differences in the regulation of the *Ifng* locus accounted for the observed phenotype. Although bulk Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells are thought to express similar levels of IFN- $\gamma$  at this early time point (Dokun et al., 2001), this “population average” conceals considerable heterogeneity within the Ly49H<sup>hi</sup> NK cell compartment. Within Ly49H<sup>hi</sup> NK cells, greater Ly49H expression yielded a lower frequency of IFN- $\gamma$  production (Figure 23G). Thus, the extent of Ly49H signaling *in vivo* during MCMV infection can modify NK cell effector properties, even at low levels of Ly49H ligation.

The superior IFN- $\gamma$  production by Ly49H<sup>lo</sup> NK cells could also not be explained by increased maturity of these cells. Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells had similar CD11b/CD27 maturation profiles despite modest differences in KLRG1 (Figure 23H), and similar proportions of both NK cell populations expressed NKG2A (Figure 23I), one



of the inhibitory receptors (along with Ly49C/I) licensed in C57BL/6 mice. A greater proportion of Ly49H<sup>hi</sup> NK cells expressed Ly49C/I (Figure 23I), yet consistent with an earlier report (Orr et al., 2010), expression of Ly49C/I had no effect on IFN- $\gamma$  production *in vivo* during MCMV infection (Figure 23J). Thus, the post-infection functional and transcriptional differences between Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells are likely due to infection-specific, rather than developmental, differences.

Despite Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells exhibiting differential capacity for IFN- $\gamma$  production at day 1.5 PI, they surprisingly displayed similar abundance of phosphorylated STAT4 (pSTAT4) (Figure 23K), which mediates IFN- $\gamma$  production downstream of IL-12 signaling. They also expressed comparable levels of the IL-18 receptor alpha (Figure 23K). (IL-18 signaling is known to enhance IFN- $\gamma$  production in synergy with IL-12 signaling) (Pien et al., 2000). Furthermore, transcripts encoding IL-12 receptor chains did not differ between the two populations (Figure 23L). Thus, Ly49H-mediated regulation of proinflammatory cytokine responsiveness is likely occurring further downstream of cytokine receptor expression and target phosphorylation.

To understand whether the functional differences between these two NK cell subsets have a physiological role in host defense, we compared protection of susceptible *Rag2<sup>-/-</sup> Il2rg<sup>-/-</sup>* mice by an equal number of purified Ly49H<sup>lo</sup> or Ly49H<sup>hi</sup> NK cells. We observed that although Ly49H<sup>lo</sup> NK cells were able to afford hosts some degree of protection against MCMV challenge (compared to mice that did not receive NK cells), Ly49H<sup>hi</sup> NK cells significantly extended the survival of susceptible hosts beyond that of Ly49H<sup>lo</sup> NK cells (Figure 22G). Collectively, these results reveal a division of labor

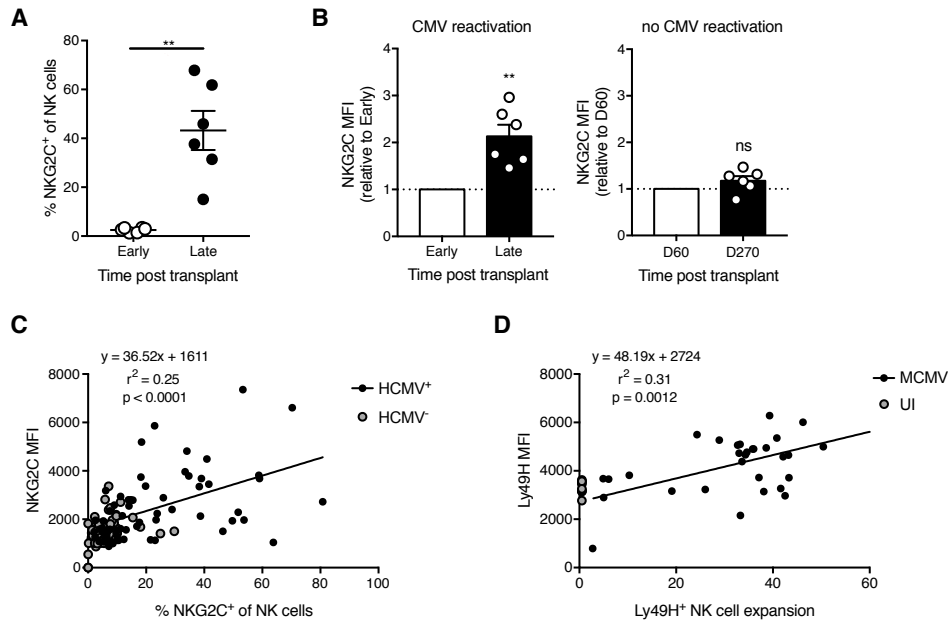
between these two NK cell populations specified in part by early transcriptional events. Whereas Ly49H<sup>hi</sup> NK cells excel in Ly49H-dependent effector responses, Ly49H<sup>lo</sup> cells possess a greater capacity for IFN- $\gamma$  production. Furthermore, these avidity-dependent functional differences between Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells regulate the protective antiviral responses mounted by NK cells.

### **5. Evidence for Avidity Selection of Human NK Cells during HCMV Infection<sup>3</sup>**

Analogous to the Ly49H<sup>+</sup> NK cell response against MCMV in mice, CD94/NKG2C<sup>+</sup> NK cells are expanded in the peripheral blood of the human cytomegalovirus (HCMV)-seropositive healthy human population (Gumá et al., 2004; Lopez-Vergès et al., 2011), as well as in recipients of solid organ and hematopoietic stem cell transplants (HSCT) who reactivate HCMV (Della Chiesa et al., 2012; Foley et al., 2012a; Horowitz et al., 2015; Lopez-Vergès et al., 2011; Muccio et al., 2016). HCMV-encoded UL40 peptides loaded onto the non-classical MHC class I molecule human leukocyte antigen (HLA)-E were recently shown to activate human NKG2C<sup>+</sup> NK cells in a peptide-specific manner (Hammer et al., 2018), analogous to the interaction between MCMV m157 and Ly49H in mice. Thus, to determine whether HCMV infection similarly modulates the overall avidity of the human NKG2C<sup>+</sup> NK cell pool, we analyzed NK cells from peripheral blood of T cell-depleted HSCT recipients. The transplant setting is a unique and relatively controlled setting in which the timing of HCMV infection (or reactivation in patients previously reported to be HCMV-seropositive) can be estimated, allowing for a longitudinal analysis of NK cells in the same patient. Consistent with previous reports

(Foley et al., 2012a; Horowitz et al., 2015; Muccio et al., 2016), we observed an expanded population of NKG2C<sup>+</sup> NK cells in HSCT patients following detection of HCMV (Figure 24A). Phenotypic analysis revealed higher NKG2C expression on the expanded NKG2C<sup>+</sup> NK cell population from HCMV-reactivating patients, but not on the small population of NKG2C<sup>+</sup> NK cells from patients who remain HCMV-seronegative post-transplant (Figure 24B). This evidence is supportive of the hypothesis that avidity selection of NK cells during CMV infection may be conserved between mouse and human.

We further examined a larger healthy human cohort, and observed that whereas healthy HCMV-seronegative donors had minimal variation in NKG2C expression and percentage of NKG2C<sup>+</sup> NK cells, HCMV-seropositive healthy donors displayed a positive linear relationship between NKG2C expression and percentage of NKG2C<sup>+</sup> NK cells (Figure 24C). Similarly, during the mouse Ly49H<sup>+</sup> NK cell response to MCMV, Ly49H expression strongly correlated with expansion of the Ly49H<sup>+</sup> population at the peak of the antiviral response (Figure 24D). These data support the idea that the degree of virus-driven NK cell clonal expansion and the extent of NK cell avidity selection are closely linked processes in both mice and humans.



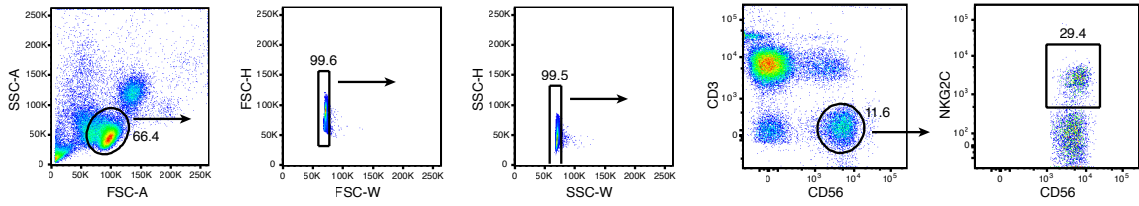
### Figure 24. HCMV Reactivation in HSCT Recipients Drives Higher NKG2C Expression on Human NKG2C<sup>+</sup> NK Cells<sup>3</sup>

(A and B) Blood was drawn from T cell-depleted HSCT recipients in the year following transplant (every ~30-90 days). HCMV infection status was determined by viral qPCR. “Early” and “Late” are paired samples from the same HCMV-reactivating recipients. “Early” refers to a range of timepoints between 15-60d post-transplant depending on the recipient. “Late” includes the window between 200d-1yr post-transplant. The data are presented as such to synchronize the timing of adaptive NKG2C<sup>+</sup> NK cell appearance, which is recipient-dependent. It is expansion of this NKG2C<sup>+</sup> NK cell population that dictates the observed kinetic profile of NKG2C MFI. (A) Percentage of NKG2C<sup>+</sup> NK cells among CD3<sup>+</sup>CD56<sup>+</sup> cells (gating strategy in Figure 25). (B) Quantification of NKG2C MFI on NKG2C<sup>+</sup> NK cells from transplant recipients who reactivate (left) or do not reactivate (right) HCMV. Groups were compared using a paired, two-tailed t test (A) or against a hypothetical value of 1 using a one sample t test (B). Data are presented as the mean ± SEM. ns, not significant; \*\* p < 0.01.

(C) Blood was drawn from healthy HCMV-seropositive and HCMV-seronegative donors. Correlation between NKG2C MFI and percentage of NKG2C<sup>+</sup> NK cells. Linear regression was performed on HCMV<sup>+</sup> samples. P value represents the likelihood of a non-zero slope.

(D) Experimental design as in Figure 18B. Correlation between Ly49H MFI and the Ly49H<sup>+</sup> NK cell expansion (i.e. percentage of Ly49H<sup>+</sup> NK cells among total NK cells) at day 7 PI. Uninfected mice were assigned 0% expansion. Linear regression was performed on MCMV-infected samples. P value represents the likelihood of a non-zero slope.

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**Figure 25. Gating Strategy for Human NKG2C<sup>+</sup> NK Cells<sup>3</sup>**

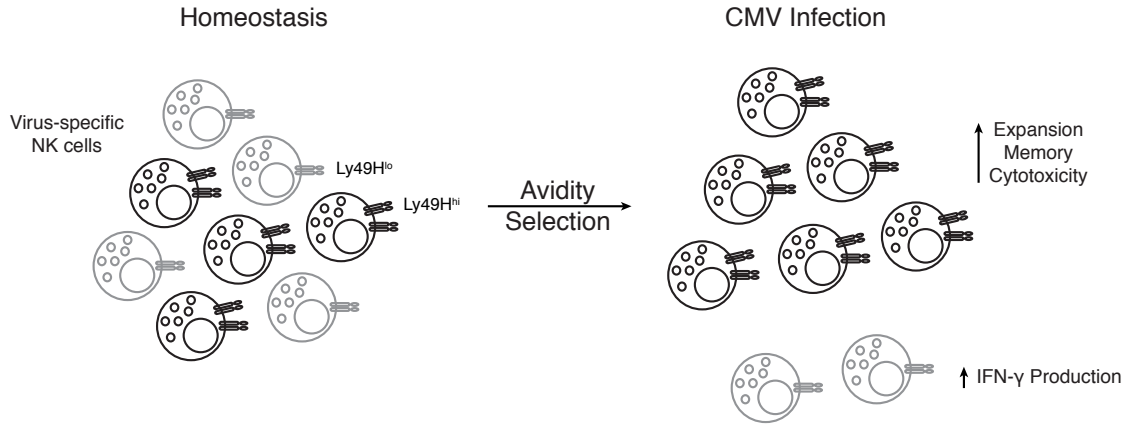
Representative flow plots from blood of HCMV-seropositive donor, illustrating the gating strategy for analysis of human NKG2C<sup>+</sup> NK cells.

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### III. Discussion<sup>3</sup>

Our study provides a mechanistic understanding of both the functional contribution of NK cells with a range of avidities for their viral ligand, and the dynamic selection of NK cell avidity during MCMV infection (Figure 26). The naïve Ly49H<sup>+</sup> NK cell compartment has evolved to possess considerable heterogeneity, not only in expression of a plethora of NK cell receptors (Lanier, 2005), but also in the expression level of Ly49H itself. Although NK cells of varying avidities are recruited during early MCMV infection, similar to CD8<sup>+</sup> T cells with a range of TCR affinities (Zehn et al., 2009), NK cells with higher avidity preferentially expand and dominate the later adaptive antiviral response. Concurrent with our studies using NK populations sorted on the basis of Ly49H surface density, Grassmann et al. used retrogenic color barcoding to track NK cell responses at a clonal level (accompanying manuscript). In accordance with our findings, these investigators also observed that Ly49H expression in individual NK cell clones closely correlated with the degree of their clonal expansion. Thus, the major conclusions from both of these complementary studies are in agreement.

This avidity selection process may contribute to the generation of a memory NK cell pool with heightened specificity for MCMV re-encounter (Sun et al., 2009), yet more limited in responsiveness to heterologous infection and bystander inflammation (Min-Oo and Lanier, 2014). Given that there is no evidence to suggest that NK cells can somatically alter the affinity of an individual germline-encoded NK cell receptor for its ligand, avidity selection represents an alternative evolutionary strategy to produce the



**Figure 26. Graphical Abstract for Cytomegalovirus Infection Drives Avidity Selection of Natural Killer Cells<sup>3</sup>**

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most specialized NK cells to combat a specific viral pathogen. Furthermore, it appears that effector and memory Ly49H<sup>lo</sup> NK cells express more Ly49H than they do as naïve NK cells, suggesting that avidity maturation (i.e. upregulation of Ly49H at the cellular level) and avidity selection may be occurring concomitantly.

Previous *in vivo* imaging studies have described that TCR transgenic CD8<sup>+</sup> T cell priming by cognate peptide-loaded DCs occurs in three distinct phases: 1) transient serial encounters with DCs, 2) stable contacts with DCs, and 3) CD8<sup>+</sup> T cell motility and proliferation (Mempel et al., 2004). Although a small number of TCR-pMHC interactions (Henrickson et al., 2008; Purbhoo et al., 2004), peptides with low affinity for TCR (Zehn et al., 2009), or short duration of antigen exposure (Kaech and Ahmed, 2001; van Stipdonk et al., 2001) can be sufficient for naïve CD8<sup>+</sup> T cell activation and proliferation into effectors, stable contacts between CD8<sup>+</sup> T cells and DCs are required for the full differentiation program. These stronger and longer interactions program CD8<sup>+</sup> T cell clonal expansion, and are critically dependent on the absolute number of TCR-pMHC interactions (Henrickson et al., 2008). Furthermore, high affinity TCR ligands and prolonged antigen exposure are thought to dictate the duration and magnitude, respectively, of the CD8<sup>+</sup> T cell expansion (Prlic et al., 2006; Zehn et al., 2009). Collectively, these studies delineate how the quantity, quality (i.e. strength), and duration of antigen exposure during CD8<sup>+</sup> T cell priming regulates their clonal expansion. Our data reveal that many of the requirements for naïve CD8<sup>+</sup> T cell and NK cell priming and clonal expansion are conserved. Although MCMV infection results in the activation of both Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells, these cells are transcriptionally distinct during the earliest days of infection. We speculate that the greater number of activating receptor-



cognate ligand interactions facilitates stronger or longer contacts between Ly49H<sup>hi</sup> NK cells with infected cells, which plays a fundamental role in programming the subsequent responses of these cells. As a consequence, Ly49H<sup>hi</sup> NK cells both proliferate and survive better during MCMV-driven expansion, contributing to their more robust adaptive responses and antiviral host protection.

Despite the greater potential of Ly49H<sup>hi</sup> NK cells for expansion and cytotoxicity, Ly49H<sup>lo</sup> NK cells are nevertheless the primary IFN- $\gamma$ -producing NK cells during the early effector response, suggesting a division of labor between these two populations of cells. One possibility is that Ly49H<sup>hi</sup> NK cells must allocate cellular and metabolic resources for the competing demands of both IFN- $\gamma$  secretion and robust expansion, or that strong Ly49H receptor ligation may at some level antagonize NK cell responsiveness to proinflammatory cytokines. Indeed, it has been demonstrated that MCMV-driven memory NK cells, which have greater Ly49H surface density, have diminished bystander responses to heterologous infection (Min-Oo and Lanier, 2014). Thus, strong activation through Ly49H precipitates both immediate and heritable changes in IFN- $\gamma$  production.

Furthermore, the expression level of Ly49H is also clonally maintained during MCMV-driven proliferation, as Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells maintain their relative differences in Ly49H expression following infection. Given that *Klra8* (encoding Ly49H) is the most differentially expressed gene ranked by p-value between Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells at day 1.5 PI, we hypothesize that expression of Ly49H is transcriptionally maintained during infection. Given that the effector function profile of an NK cell during MCMV infection hinges on its Ly49H expression, further investigation

into the full complement of factors (we provide evidence that Ly49D may be one such variable) that regulates and maintains Ly49H expression during development, homeostasis, and infection is warranted.

Similar to the selection of mouse NK cells possessing the highest surface density of Ly49H during MCMV infection, “adaptive” NKG2C<sup>+</sup> human NK cells from HCMV-seropositive healthy donors express more NKG2C (Gumá et al., 2004; Lopez-Vergès et al., 2011). Our data extends this observation, and we now demonstrate that the NKG2C<sup>+</sup> NK cell pool that arises in HSCT recipients following infection or reactivation of HCMV similarly expresses more NKG2C, suggesting that the increase in this population may be the result of avidity selection, a general mechanism in NK cell biology conserved between mouse and human. *NKG2C* zygosity has been reported to influence surface receptor levels and NKG2C<sup>+</sup> NK cell numbers in HCMV<sup>+</sup> subjects (Muntasell et al., 2013), and could contribute to the range in these parameters observed in our transplant patient cohort that reactivates HCMV. Future work is required to determine the molecular mechanisms behind avidity selection of human NK cells, and the function of NK cells with different avidities in human immunity. Nevertheless, our findings collectively indicate that avidity selection of NK cells may be a product of the unique and longstanding co-evolution of NK cells with CMV, and can inform vaccination strategies to elicit memory NK cells with the greatest avidity for viral ligands.

## IV. Materials and Methods<sup>3</sup>

### 1. Experimental Model and Subject Details<sup>3</sup>

#### *Mice*

All mice used in this study were housed and bred under specific pathogen-free conditions at Memorial Sloan Kettering Cancer Center, and handled in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). The following mouse strains were used in this study: C57BL/6 (CD45.2), B6.SJL (CD45.1), *Klra8*<sup>-/-</sup> (Ly49H-deficient) (Fodil-Cornu et al., 2008), *Stat4*<sup>-/-</sup>, *Il18r1*<sup>-/-</sup>, *Ifnar1*<sup>-/-</sup>, *Stat1*<sup>-/-</sup>, *Ifng*-IRES-YFP (GREAT), and *Rag2*<sup>-/-</sup> *Il2rg*<sup>-/-</sup> mice. Experiments were conducted using age- and gender-matched mice in accordance with approved institutional protocols.

#### *Primary Human Cells*

Peripheral blood was collected from healthy donors and patients undergoing allogeneic bone marrow transplantation at Memorial Sloan Kettering Cancer Center (MSKCC). All donors provided informed written consent for research, and the use of samples was approved by the MSKCC Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll purification, aliquoted, and stored in liquid nitrogen prior to experimentation.

## *Virus*

MCMV (Smith strain) was serially passaged through BALB/c hosts three times, and then salivary gland viral stocks were prepared with a dounce homogenizer for dissociating the salivary glands of infected mice 3 weeks after infection.

## *Bacteria*

Frozen stocks of *Listeria monocytogenes* were grown in brain-heart infusion (BHI) broth. Bacteria culture samples were grown to mid-log phase, measured by optical density (A600), and diluted in PBS for injection.

## **2. Method Details<sup>3</sup>**

### *In Vivo Virus Infection*

Adoptive co-transfer studies (both competitive and non-competitive) were performed by transferring  $10^5$  Ly49H<sup>lo</sup> NK cells and/or  $10^5$  Ly49H<sup>hi</sup> NK cells, purified from spleens of congenically distinct WT mice (CD45.1 or CD45.2), into *Klra8*<sup>-/-</sup> mice 1 day prior to MCMV infection. Recipient mice in adoptive co-transfer studies were infected with MCMV by i.p. injection of  $7.5 \times 10^2$  PFU in 0.5 mL.

Survival studies were performed by transferring either no cells,  $5 \times 10^4$  purified splenic Ly49H<sup>lo</sup> NK cells, or  $5 \times 10^4$  purified splenic Ly49H<sup>hi</sup> NK cells into *Rag2*<sup>-/-</sup> *Il2rg*<sup>-/-</sup>

mice 2 days prior to MCMV infection. Recipient mice in survival studies were infected with  $7.5 \times 10^3$  PFU MCMV in 0.5 mL by i.p. injection.

In several experiments, WT mice were directly infected with MCMV ( $7.5 \times 10^3$  PFU) or MCMV- $\Delta$ m157 ( $10^5$  PFU) in 0.5 mL by i.p. injection.

#### *In Vivo Bacterial Infection*

All mice were infected with priming doses equivalent to 2000-5000 colony-forming units (CFU) by tail vein injection.

#### *Lymphocyte Isolation*

Spleens were dissociated using glass slides and filtered through a 100- $\mu$ m strainer. To isolate lymphocytes from liver, the tissue was physically dissociated using a glass tissue homogenizer and purified using a discontinuous gradient of 40% over 60% Percoll. Red blood cells in blood, spleen, and liver were lysed using ACK lysis buffer.

#### *Flow Cytometry and Cell Sorting*

Cell surface staining of single-cell suspensions from various tissues was performed using fluorophore-conjugated antibodies (BD Biosciences, eBioscience, BioLegend, Tonbo Biosciences). Intracellular staining was performed by fixing and permeabilizing with the

eBioscience Foxp3 Transcription Factor Staining Set (Thermo Fisher) for staining intranuclear proteins and cytokines.

Flow cytometry and cell sorting were performed on the LSR II and Aria II cytometers (BD Biosciences), respectively. Data were analyzed with FlowJo software (Tree Star). Flow cytometry of lymphocytes was performed using the following fluorophore-conjugated antibodies: CD3 $\epsilon$  (17A2), TCR $\beta$  (H57-597), CD19 (ID3), F4/80 (BM8.1), NK1.1 (PK136), Ly49H (3D10), CD45.1 (A20), CD45.2 (104), CD11b (M1/70), CD27 (LG.3A10), KLRG1 (2F1), Ly49D (4E5), Ly49C/I (5E6), NKG2A/C/E (20D5), CD25 (PC61), IFN- $\gamma$  (XMG1.2), CD107a (1D4B), STAT4 pY693 (38/p-Stat4), IL-18Ra (P3TUNYA), and Fixable Viability Dye. Staining of human PBMCs was performed using: CD3 (UCHT1, BD Biosciences), CD56 (N901, Beckman Coulter), NKG2C (REA205, Miltenyi Biotec), and LIVE/DEAD as viability marker (Thermofisher).

Apoptosis was evaluated by caspase activity staining using the carboxyfluorescein FLICA poly caspase assay kit (Bio-Rad). NK cell proliferation was analyzed by labeling cells with 5  $\mu$ M CellTrace Violet (CTV, Thermo Fisher) prior to transfer, and CTV labeling was performed according to manufacturer protocol.

#### *Ex Vivo Stimulation of Lymphocytes*

$10^4$  purified splenic Ly49H<sup>lo</sup> or Ly49H<sup>hi</sup> NK cells were stimulated for 4 hours in RPMI containing 10% fetal bovine serum with 20 ng/mL recombinant mouse IL-12 (R&D

Systems) plus 10 ng/mL IL-18 (MBL), 10 ng/mL PMA (Sigma) plus 1  $\mu$ g/mL Ionomycin (Sigma), or 25  $\mu$ g/mL of plate-bound anti-mouse NK1.1 (PK136, Biolegend) or anti-mouse Ly49H (3D10, Biolegend). Cells were cultured in media alone as a negative control.

### *Ca<sup>2+</sup> Imaging of NK Cells*

Purified splenic NK cells (TCR $\beta$ <sup>-</sup>CD3 $\epsilon$ <sup>-</sup>CD19<sup>-</sup>F4/80<sup>-</sup>CD45<sup>+</sup>NK1.1<sup>+</sup>) with different Ly49H expression were loaded with 5  $\mu$ g/ml Fura-2AM for 30 min and transferred into colorless RPMI (no phenol red) containing 5% FCS.  $8 \times 10^4$  NK cells were mixed 1:4 with Ba/F3-m157 cells and imaged in 8-well chamber slides (Thermo Fisher Scientific) using an inverted fluorescence video microscope (IX-81; Olympus) fitted with a 20 $\times$ , 0.75 NA objective lens (Olympus). A Xe lamp (DG-4; Sutter Instrument) was used for fluorophore excitation, and data were collected on an electron-multiplying charge-coupled device camera (ImagEM; Hamamatsu Photonics). Time-lapse recordings were made using SlideBook software (Intelligent Imaging Innovations). One brightfield image and one Fura-2 image were taken every 20 s for 20 min.

### *Ex Vivo Killing Assay*

Ba/F3 control cells and Ba/F3-m157 target cells (Arase et al., 2002) were labeled differentially with Cell Trace Violet (CTV, Invitrogen), according to manufacturer protocol (Ba/F3, CTV<sup>lo</sup>; Ba/F3-m157, CTV<sup>hi</sup>).  $5 \times 10^3$  of each cell line were mixed with 5

$\times 10^4$  purified splenic NK cells with different Ly49H expression, or without NK cells (control condition). Effector and target cells were co-cultured for 6 hours at 37°C in RPMI-1640 containing 10% FBS. After 6 hours, cells were stained with propidium iodide prior to flow cytometry. The percentages of target cell killing were determined using the following formula, adapted from (Viant et al., 2017):  $100 - \left( \frac{(\% \text{ Ba/F3-m157 cells} / \% \text{ Ba/F3 cells})}{(\% \text{ Ba/F3-m157 cells} / \% \text{ Ba/F3 cells}) \text{ control}} \right) \times 100$ . For this formula, only CTV<sup>+</sup> cells within live cells were considered. The percentage of propidium iodide-staining target and control cells was determined from total CTV<sup>hi</sup> and CTV<sup>lo</sup> cells respectively.

### *RNA Sequencing*

Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells were sorted from the spleens of WT mice 1.5 days post MCMV infection. Spleens from three infected mice were pooled to achieve  $\sim 5 \times 10^4$  cells of each population (one paired replicate). RNA was isolated from sorted cell populations using TRIzol (Thermo Fisher) and total RNA was amplified using the SMART-seq V4 Ultra Low Input RNA kit (Clontech). Subsequently, 10 ng of amplified cDNA was used to prepare Illumina HiSeq libraries with the Kapa DNA library preparation chemistry (Kapa Biosystems) using 8 cycles of PCR. Samples were barcoded and run on HiSeq 2500 1T, in a 50bp/50bp paired-end run, using the TruSeq SBS Kit v3 (Illumina).



### 3. Quantification and Statistical Analysis<sup>3</sup>

#### *RNA Sequencing Analysis*

For RNA-seq, paired-end reads were trimmed for adaptors and removal of low-quality reads using Trimmomatic (v.0.36) (Bolger et al., 2014). Trimmed reads were mapped to the *Mus musculus* genome (mm10 assembly) and counted at transcript-level using quasi-mapping approach by Salmon (v0.10.2) (Patro et al., 2017). These transcript-level estimates were then summarized at gene-level using tximport (v1.9.12) (Soneson et al., 2015). Differential expression (DE) analysis was executed with DESeq2 (v1.12.17) (Love et al., 2014) using UCSC knownGene model as a reference annotation. Genes were considered to be differentially expressed between two groups (Ly49H<sup>hi</sup> versus Ly49H<sup>lo</sup>) if their adjusted p-values were less than 0.05. Gene Ontology analysis was performed using goseq (v1.33.0) (Young et al., 2010) on KEGG pathway database (KEGG.db v3.2.3) with a cutoff of log<sub>2</sub> fold change > 0 or < 0 for DE upregulated or downregulated genes, respectively.

#### *Statistical Analyses*

For graphs, data are shown as mean ± SEM, and unless otherwise indicated, statistical differences were evaluated using an unpaired, two-tailed Student's t test, assuming equal sample variance. For experiments in which Ly49H<sup>lo</sup> and Ly49H<sup>hi</sup> NK cells were compared within the same mouse, a paired, two-tailed t test was used. Statistical differences in survival were determined by the Log-rank (Mantel-Cox) test. p < 0.05 was

considered significant. Graphs were produced and statistical analyses were performed using GraphPad Prism.

## **Chapter 4: Discussion**

### **I. Evolution of NK Cell Recognition of CMV**

#### **1. CMV Evasion Strategies of the Host Immune System**

The antiviral NK cell responses we set out to characterize mechanistically in these studies are driven by a specific receptor-ligand interaction, namely that between the activating NK cell receptor Ly49H and the MCMV-encoded glycoprotein m157 expressed on infected cells (Arase et al., 2002; Smith et al., 2002). Given that the CMV life cycle is much shorter than that of a mouse, why would CMV encode within its genome a ligand that can promote its rejection? Furthermore, activating NK cell receptors are germline-encoded receptors, with no evidence of receptor rearrangement, and are primarily thought to recognize conserved molecular patterns on pathogens, host stress ligands, and self-MHC molecules (Lanier, 2005). Why would NK cells devote part of their receptor repertoire specifically to the recognition of CMV, and what does this say about the evolutionary role of NK cells in host protection against CMV?

Immune evasion is a primary aim of many pathogens as avoiding elimination is critical to persistence and subsequent dissemination to other hosts. CMV is a member of the  $\beta$ -herpesvirus family of viruses, and as such, contains a large double-stranded DNA genome. Much of their large genome is dedicated to preventing cells they infect from recognition by or activation of the host immune system (Sun and Lanier, 2009). During viral infection, CD8<sup>+</sup> T cells recognize viral-derived peptides presented on MHC class I of infected cells, leading to activation of CD8<sup>+</sup> T cells and mobilization of their cytotoxic function against the infected cells. In an attempt to circumvent this cytotoxic adaptive

lymphocyte response, both MCMV and HCMV encode a number of gene products that function as immunoevasins to interfere with many steps of the biosynthesis and expression of MHC class I molecules (Sun and Lanier, 2009). However, during their development, NK cells are “licensed” to kill cells lacking expression of self-MHC in the presence of an activating signal (Kim et al., 2005). Thus, in the event of MHC class I downregulation, infected cells should in theory become highly susceptible to NK cell-mediated killing via “missing self”. CMV has evolved two general strategies to evade NK cell activation, both of which hinge on shifting the fine balance between NK cell activation and inhibition: 1) interfering with NK cell activating pathways (e.g. by limiting expression of NKG2D ligands) or 2) delivering an alternative inhibitory signal to NK cells. This latter strategy is likely the basis for the evolution of the Ly49H-m157 interaction.

## **2. Examples of Host NK Cell Adaptation to CMV Evasion Strategies**

Given that self-MHC molecules are the ligands for inhibitory NK cell receptors, CMV evolved decoy proteins that mimic this interaction. The MCMV glycoprotein m157 is one such protein. In certain strains of mice (e.g. 129/J), m157 binds the inhibitory NK cell receptor Ly49I (Arase et al., 2002), which likely cripples the response of Ly49I-bearing NK cells. Studies interrogating the crystal structure of m157 demonstrated that it has a characteristic MHC-fold and binds to Ly49I with significantly higher affinity than classical Ly49-MHC interactions (Adams et al., 2007). Reconstructing the natural history of Ly49 and killer-cell immunoglobulin-like receptors (KIRs, the human equivalent of

the mouse Ly49) has illustrated the general principle that inhibitory NK cell receptors are ancestral and that activating NK cell receptors evolved from them (Abi-Rached and Parham, 2005). Thus, in certain strains of mice (e.g. C57BL/6), the activating NK cell receptor Ly49H likely evolved from Ly49I (Abi-Rached and Parham, 2005), and in doing so, retained the ability to bind m157 but not self-MHC. We now appreciate that Ly49H is the gene product encoded by the CMV-resistance gene *Cmv1* (now *Klra8*) that determines mouse strain susceptibility to MCMV infection (Brown et al., 2001; Lee et al., 2001; Scalzo et al., 1990; Scalzo et al., 1995).

MA/My mice provide yet another example of NK cells evolving their activating receptor repertoire to cope with MCMV-mediated maintenance of TCR-agnostic forms of MHC class I. NK cells in MA/My mice do not express Ly49H yet are still resistant to MCMV (Desrosiers et al., 2005). In this mouse strain, the activating NK cell receptor Ly49P recognizes the MCMV-encoded glycoprotein m04. There is evidence for m04 complexing with MHC class I in MCMV-infected cells and impairing antigen presentation independent of modulating MHC class I surface expression levels (Kavanagh et al., 2001a; Kavanagh et al., 2001b; Kleijnen et al., 1997; LoPiccolo et al., 2003; Lu et al., 2006; Wagner et al., 2002), suggesting that m04 may have evolved as a strategy to inhibit both NK cell and CD8<sup>+</sup> T cell responses. Ly49P recognition of m04 is dependent on host H2-D<sup>k</sup> expression (Desrosiers et al., 2005; Kielczewska et al., 2009), although the structure of the ligand complex has yet to be elucidated.

The current status of the evolutionary détente between HCMV and the human immune system has elements of both the attempted viral evasion strategies and host adaptation mechanisms in C57BL/6 and MA/My mice. It was recently shown that a

subset of human NK cells bearing the activating receptor NKG2C can recognize HCMV-encoded UL40 peptides loaded onto the non-classical MHC class I molecule HLA-E on infected cells (Hammer et al., 2018). Although not a viral decoy protein itself (as in the case of MCMV *m157*), UL40 peptides are presumed to maintain surface expression of HLA-E to inhibit NK cell responses (through NKG2A, the counterpart inhibitory receptor to the activating NKG2C) in concert with other HLA downregulation mechanisms. Collectively, these illustrative examples highlight the longstanding co-evolution between CMV and the mouse and human immune systems and suggest that in response to the strong selective pressure of CMV infection, some hosts have adapted accordingly.

### **3. Current Status of NK Cell-CMV Co-Evolution**

Comparison of MCMV strains isolated from wild mice reveals a great degree of polymorphism at the *m157* locus (Voigt et al., 2003). Furthermore, serial passage of MCMV through C57BL/6 mice leads to the development of *m157* mutations that result in *m157* no longer being detected by Ly49H (French et al., 2004; Voigt et al., 2003). In human patients, the HCMV strain best able to stimulate NKG2C<sup>+</sup> NK cells is found at very low frequency (~1%) in clinical isolates, and there is high sequence variability within the nonameric peptide of UL40 peptide that binds HLA-E (Hammer et al., 2018). Collectively, these data suggest that there is selection pressure against CMV expressing proteins that can stimulate activating NK cell receptors.

So if CMV does indeed have the capacity to mutate further to circumvent these host adaptations, why does it not? One possibility, perhaps a depressing one from the

perspective of the hosts it infects, is that right now, CMV is at a stand-off with its hosts. Right now, we could be living in just one snapshot of this evolutionary struggle, which does not preclude complete eradication of CMV or CMV overtaking its hosts in the future. However, a more likely explanation is that CMV needs the hosts it infects to propagate itself. This hypothesis is fitting with extensive epidemiologic data. Although CMV is a common congenital and opportunistic infection, in which cases serious complications or even death can occur, it does not cause life-threatening disease in healthy individuals (Griffiths et al., 2015). CMV infection rates are quite high in the general population, measuring about 60% of individuals from developed countries and almost 100% of individuals in developing countries (Griffiths et al., 2015). Furthermore, even after CMV infection is effectively cleared, it can establish latency in the host salivary gland. Collectively, its absent disease course, high prevalence, and long lifespan suggest that host survival may be the most beneficial outcome for CMV in that it maximizes CMV dissemination in the population.

Despite the benign clinical course in healthy individuals, patients with rare deficiencies in NK cell numbers or function suffer severe complications from herpesvirus infections (Orange, 2013), underscoring the critical role for NK cells in control of herpesvirus infection. Although the specificity for herpesvirus susceptibility is striking, it is nevertheless unlikely that the ancestral “NK cell-like” cell came to be to combat this family of viruses. All of the aforementioned examples of NK cell-mediated control of CMV are rooted in CMV attempting to evade the T cell response. Furthermore, the natural history of Ly49 and KIRs suggests that inhibitory NK cell receptors preceded activating receptors, and that activating receptors are short lived and more prone to be

subject to selection (Abi-Rached and Parham, 2005). The fact that NK cells now devote part of their receptor repertoire specifically to CMV recognition is likely a combination of three factors: 1) the high prevalence of CMV, 2) the evasion strategies CMV employs, and 3) poor host fitness after CMV infection in the absence of NK cells. As evolution plays out, it will be interesting to observe whether any of these three key CMV variables change and the subsequent effect on the NK cell receptor repertoire. Furthermore, as new pathogens enter the arena, it will be important to understand whether there is any association between protection and activating NK cell receptor phenotype.



## **II. Heterogeneity in Antiviral NK Cell Responses**

Since NK cells lack the ability for somatic rearrangement of their antigen receptors, the diversity of possible ligands recognized by NK cells is far more restricted than that of their adaptive lymphocyte cousins. Despite the limitations of antigen recognition by a germline encoded receptor repertoire, NK cells as a population are extraordinarily diverse as a result of the random distribution in their expression of activating and inhibitory receptors (Lanier, 2005). In humans, as opposed to in inbred mice, the diversity is further compounded by different KIR and HLA alleles. From the complexity of the NK cell receptor repertoire, it follows that even two NK cells expressing the same activating receptor may respond differently to signaling through that receptor. As discussed previously, MCMV decoy ligands are recognized by complementary NK cell activating and inhibitory receptors. However, the decoy ligands commonly bind inhibitory receptors with greater affinity (Adams et al., 2007). Thus, the fact that not all NK cells express the full complement of activating and inhibitory receptors ensures that there exists a subpopulation of NK cells in which the activating signals predominate. Furthermore, in the context of “adaptive” responses to MCMV infection, NK cell heterogeneity in the receptor repertoire manifests as cells with functionally distinct responses. Some groups have reported preferential expansion of unlicensed NK cells and NK cells that lack expression of KLRG1 or the inhibitory receptor NKR-P1B (Kamimura and Lanier, 2015; Orr et al., 2010; Rahim et al., 2016). Collectively, these data suggest that NK cell responses have evolved to be highly tunable in nature.

In our work, we have described yet another layer of heterogeneity in NK cell antiviral responses that is dependent on the expression level of the activating receptor

Ly49H in the context of MCMV infection of C57BL/6 mice. The distinct functional responses imparted by heterogeneity in Ly49H expression appears to be independent of other known mechanisms, including NK cell licensing and maturation. Rather, the amount of Ly49H signaling (a combination of its duration, magnitude, and quality), regulated by the surface Ly49H expression of an NK cell, drives distinct transcriptional and functional outputs of NK cells during antiviral host defense.

One major lingering question from these studies is how this heterogeneity in activating receptor expression level is established. We have preliminary evidence to suggest that this heterogeneity is linked intrinsically to the receptor repertoire of a given NK cell. NK cells that co-express the DAP12-dependent activating receptor Ly49D have less Ly49H. Since signaling downstream of Ly49H is also DAP12-dependent, one hypothesis that arises from this finding is that perhaps there is competition for signaling adapters or components in a naïve NK cell. Interestingly, although Ly49H<sup>hi</sup> cells are selected for in the effector NK cell pool during MCMV infection, Ly49H<sup>hi</sup> NK cells do not further increase their expression of Ly49H (in contrast to Ly49H<sup>lo</sup> NK cells that do). Together these data suggest that there may be an upper limit to the amount of activating receptor signaling that NK cells can receive. This is consistent with the extensive checks that NK cells put on unleashing their cytotoxic potential. Given their “natural” (i.e. without the requirement of prior target exposure) cytotoxicity, NK cells are programmed to kill only in the absence of self-MHC. Thus, we hypothesize that limiting the expression level of any one activating receptor may be another strategy to prevent unbridled NK cell responses.

### **III. Summary**

NK cells have long been known to contribute to antiviral defense, and more recently, facilitated by the discovery of a specific interactions between the activating NK cell receptor Ly49H and the MCMV ligand m157, shown to mount a response to cytomegalovirus infection that in many ways mimics the immune responses of their adaptive lymphocyte cousins. The results within this thesis describe one mechanism by which these “adaptive” NK cell responses are regulated at the transcriptional level, and how different subpopulations of antiviral NK cells have distinct functional outputs during CMV infection that contribute to the heterogeneous responses of these cells. In particular, the transcription factor IRF8 is induced in NK cells exposed to IL-12 in the inflammatory environment of MCMV infection, and supports the proliferation and expansion of virus-specific NK cells by controlling expression of a suite of cell cycle genes, including the known pro-proliferative factor Zbtb32. Furthermore, the expression level of their MCMV-recognition receptor Ly49H dictates the effector function of a given NK cell during infection. Those NK cells with greatest Ly49H expression are more cytotoxic and also proliferate and survive better during virus-driven expansion, yielding a larger effector and memory NK cell pool. In contrast, a greater proportion of “low avidity” NK cells produce IFN- $\gamma$ . These studies provide a framework for further investigation into the regulation and heterogeneity of antiviral NK cell responses and for harnessing the full cytotoxic function of these cells.

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