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Highlights

- IFN-α induces ISGF3 components in NK cells by an auto-regulatory feedforward loop
- Some IFNAR-dependent transcripts in MCMV-activated NK cells are STAT1 independent
- ISGF3 components are non-redundant in promoting MCMV-driven NK cell expansion
- IRF9 shields NK cells from apoptosis by regulating cell surface molecule expression

In Brief

Using RNA-seq and ChIP-seq, Geary et al. investigate the impacts of type I interferon on NK cells during MCMV infection and demonstrate crucial and non-redundant roles for STAT1, STAT2, and IRF9 in promoting cytotoxicity and survival of antiviral NK cells.
Non-redundant ISGF3 Components Promote NK Cell Survival in an Auto-regulatory Manner during Viral Infection

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SUMMARY

Natural killer (NK) cells are innate lymphocytes that possess adaptive features, including antigen-specific clonal expansion and long-lived memory responses. Although previous work demonstrated that type I interferon (IFN) signaling is crucial for NK cell expansion and memory cell formation following mouse cytomegalovirus (MCMV) infection, the global transcriptional mechanisms underlying type I IFN-mediated responses remained to be determined. Here, we demonstrate that among the suite of transcripts induced in activated NK cells, IFN-α is necessary and sufficient to promote expression of its downstream transcription factors STAT1, STAT2, and IRF9, via an auto-regulatory, feedforward loop. Similar to STAT1 deficiency, we show that STAT2- or IRF9-deficient NK cells are defective in their ability to expand following MCMV infection, in part because of diminished survival rather than an inability to proliferate. Thus, our findings demonstrate that individual ISGF3 components are crucial cell-autonomous and non-redundant regulators of the NK cell response to viral infection.

INTRODUCTION

Natural killer (NK) cells are innate lymphocytes that are critical for tumor immunosurveillance and control of herpesvirus infections. NK cell activation is determined by the balance of germline-encoded inhibitory receptors, which recognize major histocompatibility complex (MHC) class I (i.e., “self”), and activating receptors that recognize stressed or infected cells, often in the context of diminished MHC class I (i.e., “missing self”) (Lanier, 2008). Upon activation, NK cells rapidly proliferate and release pro-inflammatory cytokines such as IFN-γ and cytotoxic molecules (granzyme B) to lyse transformed or infected target cells (Sun and Lanier, 2011). Although they have traditionally been classified as part of the innate immune system, NK cells are now appreciated to share many features with adaptive lymphocytes, including clonal expansion, longevity, and robust recall responses (Geary and Sun, 2017). One of the most well characterized models of “adaptive” NK cell responses occurs during mouse cytomegalovirus (MCMV) infection. The MCMV-encoded glycoprotein m157 is specifically recognized by the Ly49H receptor expressed on a subset of NK cells in C57BL/6 mice, and receptor-ligand engagement drives rapid NK cell proliferation and effector functions that provide resistance to MCMV (Arase et al., 2002; Brown et al., 2001; Daniels et al., 2001; Dokun et al., 2001; Smith et al., 2002). Following their rapid expansion, virus-specific NK cell effectors contract to form a long-lived pool of memory cells that exhibit enhanced effector functions upon secondary challenge (Sun et al., 2009).

Previous studies have demonstrated that pro-inflammatory cytokine signals, particularly IL-12 and type I interferon (IFN), are crucial for NK cell expansion and memory formation (Madera et al., 2016; Sun et al., 2012). Canonical type I IFN signaling requires TYK2 and JAK1-mediated phosphorylation of STAT1 and STAT2, which then form a heterotrimer with IRF9. This complex, termed ISGF3, translocates to the nucleus, where it binds to IFN-stimulated response elements (ISRE) to promote expression of hundreds of IFN-stimulated genes (ISGs) encoding proteins with antiviral functions (Ivashkiv and Donlin, 2014). It is thought that specificity for the ISRE sequence is provided by IRF9, while additional DNA contacts with STAT1 and STAT2 stabilize the interaction, and STAT2 provides the transcriptional activation domain (Bluyssen and Levy, 1997; Levy et al., 1989; Qureshi et al., 1995; Veals et al., 1993; Wesoly et al., 2007). However, there is increasing evidence that alternative complexes, containing unphosphorylated STATs or different combinations of STATs with or without IRF9, also form in response to type I IFN stimulation and may contribute to the pleiotropic biological effects of IFNs (reviewed in Fink and Grandvaux, 2013; Majoros et al., 2017). The biological importance of these alternative pathways has been demonstrated by studies showing that Stat2−/− or Irf9−/− mice were more resistant to certain viral infections.
than Stat1−/− mice (Hofer et al., 2012; Perry et al., 2011) and that STAT1-independent transcription of some ISGs can be driven by STAT2 or IRF9 (Luker et al., 2001; Perry et al., 2011). Thus, we sought to better understand the global transcriptional mechanisms underlying type I IFN-mediated responses of NK cells during viral infection and whether these pathways are directly controlled by canonical ISGF3 signaling.

**RESULTS AND DISCUSSION**

**Type I IFN Signaling in NK Cells Induces Auto-regulation of ISGF3 Components**

To investigate the genes induced by type I IFNs in NK cells, we performed comparative transcriptome analysis by RNA-sequencing (RNA-seq) on purified NK cells that were stimulated with IFN-α or left unstimulated (media alone). The vast majority of transcriptional changes were induced by IFN stimulation, and the most highly induced genes included many canonical ISGs, such as Ifit3, Rsad2, and Isg15 (Figure 1A). Among the genes significantly induced by IFN-α in NK cells, chromatin immunoprecipitation sequencing (ChIP-seq) demonstrated that 32% were bound by STAT1. Interestingly, the most highly differentially expressed genes were not preferentially STAT1 bound (Figure 1B). Among the top STAT1-bound, IFN-α-induced transcription factors were several known to be important for the NK cell response to MCMV, such as Runx3 (Rapp et al., 2017) and Tbx21 (Madera et al., 2018), as well as all three ISGF3 components (Figure 1C). Analysis of the individual Stat1, Stat2, and Irf9 loci confirmed that NK cells exposed to IFN-α significantly induced transcription of these genes (Figure 1D). Furthermore, STAT1 targeted the promoters of all three ISGF3 components (Figure 1E). Thus, we propose that STAT1 (and other ISGF3 components) may operate in an auto-regulatory manner in NK cells during viral infection.

**NK Cells Demonstrate IFN-Induced, but STAT1-Independent, Transcriptional Changes during Viral Infection**

To determine if all genes induced during type I IFN exposure were dependent on STAT1 signaling for their expression in vivo, we performed RNA-seq on wild-type (WT), Ifnar1−/−, and Stat1−/− Ly49H+ NK cells from MCMV-infected and uninfected mixed bone marrow chimeric mice (mBMCs) (Figure 2A). We observed 872 differentially expressed genes between WT and Ifnar1−/− NK cells (Figure 2B) and 838 differentially expressed genes between WT and Stat1−/− NK cells (Figure 2C) at day 1.5 post-infection (Pi). Most of the many significant changes were canonical ISGs (e.g., Ifi7, Rtp4, Mx1) that were induced ex vivo and common between both knockouts. Interestingly, the majority of differentially expressed genes did not overlap with our list of genes induced by IFN-α ex vivo. Importantly, we found that after MCMV infection, expression of all three ISGF3 components was upregulated in WT Ly49H+ NK cells, but this increase was almost completely abolished in the absence of STAT1 (Figure 2D) or the type I IFN receptor (Figure S1A). These findings are consistent with the auto-regulatory mechanism proposed above and also suggest that ISGF3 components operate in a feedforward manner to promote their own transcription.

Although the transcriptional profile of IFNAR1- and STAT1-deficient NK cells appeared similar at the global level, direct comparison of genes revealed that fewer than 60% of the differentially expressed transcripts were common to both knockouts relative to WT NK cells (Figures 2E and S1B–S1E). The dysregulated genes unique to Stat1−/− NK cells could be due to downstream influences of other cytokine signaling pathways that have been proposed to induce phosphorylation of STAT1 (e.g., IFN-γ or IL-21); however, the transcriptional changes unique to Ifnar1−/− NK cells suggest that non-canonical type I IFN signaling pathways may be active in NK cells during viral infection.

**Individual ISGF3 Components Promote Clonal Expansion of Antiviral NK Cells in a Cell-Autonomous and Non-redundant Manner**

This noncongruent transcriptomic profile of Ifnar1−/− versus Stat1−/− NK cells led us to hypothesize that IRF9, and potentially STAT2, may also play important, non-redundant roles in the response of NK cells to MCMV infection. Because both Ifnar1−/− and Stat1−/− mice are highly susceptible to MCMV (Gil et al., 2001), we determined whether IRF9 is also required for host control of MCMV infection. We challenged WT and Irf9−/− mice with MCMV and found that Irf9−/− mice were unable to control the infection, resulting in higher viral titers in the blood (Figure S2A) and early death (Figure S2B).

To more directly interrogate the importance of IRF9 in the expansion of MCMV-specific NK cells, we co-transferred equal numbers of WT (CD45.1) and Irf9−/− NK cells to expand the pool of effector cells (Dokun et al., 2001; Sun and Lanier, 2011). To test the role of IRF9 in the expansion of MCMV-specific NK cells, we co-transferred equal numbers of WT (CD45.1) and Irf9−/− (CD45.2) Ly49H+ NK cells into Ly49H-deficient recipients, and following MCMV infection, we tracked the expansion of the transferred Ly49H+ NK cells (Figure 3A). In contrast to WT NK cells, which underwent robust expansion, IRF9-deficient NK cells expanded modestly and represented a smaller proportion of the effector and memory NK cell pools (Figures 3B and 3C). STAT2-deficient NK cells showed a similar defect in expansion and contribution to the memory cell pool after MCMV infection (Figures 3D and 3E). Together, these findings demonstrate a non-redundant requirement for all ISGF3 components in promoting NK cell expansion following viral infection.

As has been previously reported for Ifnar1−/− mice (Guan et al., 2014; Mizutani et al., 2012), we observed minor defects in NK cell development in the global absence of IRF9 and STAT2 (Figures S3A–S3C; data not shown). To confirm that the expansion defects we observed were due to cell-intrinsic roles for IRF9 and STAT2 during MCMV infection, we generated mBMC by reconstituting irradiated mice with bone marrow from Irf9−/− or Stat2−/− and WT mice. In this setting, both IRF9- and STAT2-deficient NK cells repopulated the mice with similar efficiency to WT NK cells (Figure S3D; data not shown), and the knockout cells that arose were phenotypically indistinguishable from WT NK cells (Figure S3E; data not shown), suggesting that the developmental defects observed in NK cells
directly isolated from the *Irf9*−/− or *Stat2*−/− mice were cell extrinsic. When WT and IRF9- or STAT2-deficient cells were adoptively transferred from mBMC into Ly49H-deficient mice, we observed a similar expansion defect in knockout NK cells following MCMV infection (data not shown).

A major function of NK cells during viral infection is to produce pro-inflammatory cytokines such as IFN-γ in addition to mediating cytotoxicity. In uninfected mice, WT and *Irf9*−/− NK cells showed comparable IFN-γ production and degranulation after ex vivo stimulation (Figure S3F; data not shown) and killed m157-expressing target cells similarly (Figure S3G), suggesting that IRF9 does not play a critical role in the acquisition of NK cell effector functions during development. However, during MCMV infection, *Irf9*−/− NK cells failed to upregulate granzyme B to the same extent as WT cells (Figure 3F), although they expressed similar levels of IFN-γ (Figure 3G) and activation markers (Figures 3H and 3I). Thus, defects in expansion and expression of cytolytic molecules by *Irf9*−/− NK cells likely
contribute to the increased susceptibility of \( \text{Irf9}^{-/-} \) mice to MCMV infection. However, because \( \text{Irf9}^{-/-} \) NK cells can still produce IFN-\( \gamma \), this may afford the \( \text{Irf9}^{-/-} \) mice some degree of protection.

**IRF9 Promotes Survival but not Proliferation of NK Cells following Viral Infection**

To determine how IRF9 deficiency impaired NK cell expansion, we performed comparative transcriptome analysis by RNA-seq on purified WT and \( \text{Irf9}^{-/-} \) Ly49H+ NK cells from mBMC 4 days post-MCMV infection, a time point immediately after \( \text{Irf9} \) expression peaks in WT cells (Figure 2D). The absence of IRF9 altered the expression of 1,008 genes (Figure 4A). Although similar numbers of genes were induced and repressed by IRF9, the most significant changes occurred in genes found to be downregulated in \( \text{Irf9}^{-/-} \) cells relative to WT cells, and many of these were canonical ISGs (Figure 4A). Pathway analysis showed dysregulation of IFN signaling pathways (Figure S4A) but also revealed alterations in apoptosis signaling and lymphocyte activation pathways (Figure 4B). Labeling NK cells with the division-tracking dye CTV prior to adoptive transfer confirmed that IRF9 was dispensable for MCMV-driven NK cell proliferation (Figure 4C). In contrast, FLICA staining (to detect activated caspases) showed evidence of greater apoptosis in \( \text{Irf9}^{-/-} \) NK cells (Figure 4D). Together, these findings suggest that IRF9 is required for NK cell survival, but not proliferation, following MCMV infection.

Previous studies have shown that IFNAR1-deficient lymphocytes are susceptible to killing by activated NK cells after viral infection because of lower expression of MHC I and higher expression of ligands for NK cell activating receptors (Crouse et al., 2014; Madera et al., 2016; Xu et al., 2014). Consistent
Figure 3. Non-redundant and Cell-Intrinsic Requirement for ISGF3 Components for the Optimal Expansion of Antiviral NK Cells

(A) Experimental schematic. Equal numbers of splenic Ly49H+ KLRG1lo NK cells from WT (CD45.1) and Irf9−/− or Stat2−/− mice (CD45.2) were co-transferred into Ly49H-deficient recipients prior to infection with MCMV.

(B) Absolute percentages of adoptively transferred WT versus Irf9−/− Ly49H+ NK cells measured in peripheral blood at the indicated times following MCMV infection or inferred from transfer (day 0).

(C) Relative percentage of transferred WT and Irf9−/− Ly49H+ NK cells in indicated organs at day 28 PI.

(D) As in (B), except that NK cells were transferred from WT and Stat2−/− mice.

(E) As in (C), except that NK cells were transferred from WT and Stat2−/− mice.

(F) Expression of intracellular granzyme B in splenic WT and Irf9−/− Ly49H+ NK cells from mBMC animals on day 2 PI with MCMV.

(G) As in (F), except showing IFN-γ.

(H and I) KLRG1 (H) and CD27 and CD11b (I) expression were assessed in the blood 7 days PI with MCMV following co-adoptive transfer of WT and Irf9−/− cells, as in (B).

Data are representative of two (D–G) or three (B, C, H, and I) independent experiments with n = 3–5 mice per group. Symbols represent mean (B and D) or individual mice (C and E–G), and error bars show SEM (*p < 0.05).
Figure 4. IRF9 Is Required for Survival but Not Proliferation of NK Cells following Viral Infection

mBMC mice harboring both WT and Irf9−/− NK cells were infected with MCMV. Splenic Ly49H+ WT and Irf9−/− NK cells were sorted for RNA-seq at 4 days PI (n = 3 per genotype).

(A) Volcano plot of RNA-seq data showing differentially expressed genes with genes of interest labeled. Horizontal line indicates p = 0.05, and vertical lines show absolute log2 fold change = 1.

(B) Top five enriched pathways as calculated using the PANTHER database, including number of differentially expressed genes per pathway and names of representative genes. Bar plots depict −log10 p values calculated using goseq.

(C) As in Figure 3A, except NK cells were labeled with CTV prior to adoptive co-transfer. Left: representative histogram of CTV in splenic WT and Irf9−/− Ly49H+ NK cells at day 3 PI. Right: quantification of Ly49H+ NK cells that divided more than once.

(D) Pan-caspase activation in transferred Ly49H+ NK cell populations in the spleen at day 4 PI.

(E) Heatmap of row-normalized log2-transformed counts of differentially expressed genes with putative roles in NK cell activation or inhibition. Experimentally validated activating ligands are green and experimentally validated inhibitory ligands are red.

(F) WT and Irf9−/− NK cells were co-transferred into Ly49H-deficient recipients as in Figure 3A or transferred into Rag2−/− IL2rg−/− mice or Nkp46Cre R26DTA mice. Data are representative of three independent experiments with n = 2–5 mice per group. Symbols represent individual mice, and error bars show SEM (*p < 0.05).
with these findings, our RNA-seq analysis revealed that \textit{lrf9}^{-/-} NK cells also have dysregulated expression of known and putative ligands for activating and inhibitory NK cell receptors following MCMV infection (Figure 4E), which may contribute to overall susceptibility to cytotoxicity. Specifically, \textit{lrf9}^{-/-} NK cells showed higher expression of \textit{Clec2i}, which encodes \textit{Cir}-q, a ligand for the activating receptor \textit{NKRP1f} \cite{iizuka_2003}, and lower expression of several MHC class I molecules, including \textit{H2-t23}, encoding the MHC class Ib molecule Qa-1b, which is critical for protecting activated T cells from lysis by NKG2A+ NK cells \cite{lu_2007}. Furthermore, of the molecules affected by \textit{irf9} ablation, ChiP-seq showed that four of the nine genes had \textit{irf9} binding to their promoters in NK cells stimulated with IFN-\textalpha\ (Figure S4B), suggesting direct IRF9-mediated regulation of these loci.

Previous work demonstrated that depletion of NK cells or transfer into NK cell-deficient hosts was sufficient to rescue the expansion defect of Ifnar1^{-/-} T and NK cells \cite{Crouse_2014, madera_2016, xu_2014}. Likewise, we found that following transfer into lymphocyte-deficient Rag2^{-/-}Il2rg^{-/-} mice or NK cell-deficient (\textit{Nkpg46}^{Cre} \textit{R26DTA}) mice, \textit{lrf9}^{-/-} cells expanded comparably with WT NK cells following MCMV infection (Figure 4F). Although the co-transferred WT NK cells in this setting are likely capable of fratricide, we hypothesized that they are not present in sufficient numbers to mediate appreciable levels of killing. Furthermore, \textit{lrf9}^{-/-} NK cells showed higher levels of cell death relative to WT NK cells when co-incubated with high ratios of activated effector NK cells (Figure S4C). Thus, IRF9-dependent sensing of type I IFNs during viral infection is necessary to protect activated NK cells against elimination by neighboring (host) NK cells.

In summary, we identified the total set of genes induced by IFN-\textalpha\ stimulation of purified NK cells and found that a subset of these genes, including \textit{Stat1}, \textit{Stat2}, and \textit{lrf9}, were also induced during MCMV infection in a manner dependent on type I IFN signaling, suggesting an auto-regulatory loop in NK cells to support robust IFN-dependent antiviral responses. We used IRF9- and STAT2-deficient mice to demonstrate a non-redundant requirement for all three ISGF3 components in NK cell expansion and memory cell formation after MCMV. NK cells deficient in the type I IFN pathway proliferate normally but have dysregulated cell death pathways and altered expression of NK cell receptor ligands, making them susceptible to NK cell-mediated fratricide.

It is well established that NK cell cytotoxicity is triggered by weighing the balance of activating and inhibitory inputs \cite{joncker_2009}. Although NK cells have been shown to tolerate a wide range of MHC class I levels \cite{brodin_2010, jonsson_2010}, their tolerance of low levels of MHC\textalpha\ is significantly reduced in inflammatory settings \cite{ludigs_2015, sun_2008}. Thus, it may not be surprising that subtle shifts in expression of a handful of cell surface molecules (activating and inhibitory ligands) would be causative of larger defects in the ability of ISGF3 component-deficient NK cells to expand following MCMV infection. We speculate that it is the combined impact of increased activating ligand and reduced MHC class I expression on proliferating \textit{lrf9}^{-/-} NK cells together with the heightened cytotoxic function of neighboring (WT host) NK cells that leads to fratricide during the antiviral response.

Previous studies have shown that expression of ISGF3 components increases following treatment of cells with IFNs or other cytokines and that this heightened expression can augment the induction of downstream ISGs \cite{ivashkiv_2014}. However, this evidence has primarily been demonstrated in cell lines or macrophages stimulated with IFN-\gamma; here, we demonstrate that type I IFN signaling in primary lymphocytes is both necessary and sufficient to induce \textit{Stat1}, \textit{Stat2}, and \textit{lrf9} expression \textit{in vitro} and \textit{in vivo} through an auto-regulatory mechanism. Such transcriptional auto-regulatory loops have been described across a range of systems and are important controllers of diverse processes ranging from bacterial pathogenicity \cite{zhang_2013} to neuronal development \cite{bai_2007} and cell cycle progression in eukaryotes \cite{johnson_1994}. That ISGF3 components in the mammalian immune system are also regulated in this manner likely allows lymphocytes to respond rapidly at the first sign of infection and highlights the importance of the type I IFN pathway in the host immune response.

Although our transcriptomic data were suggestive that non-canonical type I IFN signaling complexes might be activated in NK cells during viral infection (Figure 2E), adoptive transfer experiments with NK cells deficient in any member of the ISGF3 complex, or the upstream receptor, had a similar phenotype (i.e., an expansion defect due to fratricide). Thus, our data neither prove nor disprove the existence of non-canonical type I IFN signaling but demonstrate a critical, non-redundant function of individual ISGF3 components in antiviral NK cell defense. Furthermore, our study suggests that the dominant roles of the type I IFN pathway in NK cells during viral infection are to promote cytotoxicity (via upregulation of granzyme B) and protect against fratricide by proper regulation of cell surface receptors and ligands. Additionally, it is likely that other ISGs will have important functions in NK cells that are being masked by the overwhelming fratricide phenotype, and future studies are needed to address the potential roles of the ISGs identified in our \textit{ex vivo} or \textit{in vivo} experiments. A greater understanding of the molecular mechanisms underlying IFN signaling in activated lymphocytes will aid in our fight against a plethora of infectious diseases.

**STAR METHODS**

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SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.07.060.

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AUTHOR CONTRIBUTIONS
C.D.G., N.M.A., and S.V.G. performed experiments. C.D.G., C.K., C.M.L., Y.P., and C.S.L. analyzed the data. A.R.T. provided mice. C.D.G. and J.C.S. designed the study and wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing financial interests.

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### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-Mouse CD3ε (clone 17A2) | Tonbo Biosciences | Cat#25-0032; RRID:AB_2621619 |
| Anti-Mouse TCRβ (clone H57-597) | BioLegend | Cat#109220; RRID:AB_893624 |
| Anti-Mouse CD19 (clone 6D5) | BioLegend | Cat#115530; RRID:AB_830707 |
| Anti-Mouse F4/80 (clone BM8.1) | BioLegend | Cat#123117; RRID:AB_893489 |
| Anti-Mouse NK1.1 (clone PK136) | Tonbo Biosciences | Cat#65-5941; RRID:AB_2621910 |
| Anti-Mouse Nkp46 (clone 29A1.4) | BioLegend | Cat#137604; RRID:AB_2235755 |
| Anti-Mouse Ly49H (clone 3D10) | eBioscience | Cat#11-5886-81; RRID:AB_1257160 |
| Anti-Mouse CD45.1 (clone A20) | BioLegend | Cat#110729; RRID:AB_1134170 |
| Anti-Mouse CD45.2 (clone 104) | BioLegend | Cat#109821; RRID:AB_493730 |
| Anti-Mouse CD49b (clone Dx5) | BioLegend | Cat#108918; RRID:AB_2265144 |
| Annexin V | BioLegend | Cat#640943; RRID:AB_2616658 |
| Anti-Mouse/Human CD11b (clone M1/70) | BioLegend | Cat#101223; RRID:AB_755985 |
| Anti-CD27 (clone LG.7F9) | eBioscience | Cat#14-0271-81; RRID:AB_467182 |
| Anti-Mouse KLRG1 (clone 2F1) | Tonbo Biosciences | Cat#50-5893; RRID:AB_2621800 |
| Anti-Mouse Ly49D (clone 4E5) | BioLegend | Cat#138308; RRID:AB_10639939 |
| Anti-Mouse Ly49A (clone YE1/48.10.6) | BioLegend | Cat#116810; RRID:AB_572013 |
| Anti-Mouse Ly49C and Ly49H (clone SE6) | BD Biosciences | Cat#553277; RRID:AB_394751 |
| Anti-Mouse CD69 (clone H1.2F3) | BioLegend | Cat#104524; RRID:AB_2074979 |
| Anti-Human/Mouse Granzyme B (clone GB11) | BioLegend | Cat#515403; RRID:AB_2114575 |
| Anti-Mouse IFN gamma (clone XMG1.2) | BioLegend | Cat#20-7311; RRID:AB_2621616 |
| Anti-Mouse CD107a (clone 1D4B) | BioLegend | Cat#121611; RRID:AB_1732051 |
| Anti-NK-1.1 depletion antibody (clone PK136) | J. Sun (PI) | N/A |
| InVivoMab Anti-Mouse CD8α (NK cell enrichment, clone 2.43) | Bio X Cell | Cat#BE0061; RRID:AB_1125541 |
| InVivoMab Anti-Mouse CD4 (NK cell enrichment, clone G1.5) | Bio X Cell | Cat#BE0003-1; RRID:AB_1107636 |
| InVivoMab Anti-Mouse CD19 (NK cell enrichment, clone 1D3) | Bio X Cell | Cat#BE0150; RRID:AB_10949187 |
| InVivoMab Anti-Mouse Ter-119 (NK cell enrichment, clone TER-119) | Bio X Cell | Cat#BE0183; RRID:AB_10949625 |
| Anti-IRF9: ISGF-3gamma p48 (C-20) (ChIP, polyclonal) | Santa Cruz Biotechnology | Cat#sc-496; RRID:AB_2127709 |
| Anti-STAT1 (M-22) (ChIP, polyclonal) | Santa Cruz Biotechnology | Cat#sc-592; RRID:AB_632434 |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Recombinant Mouse IL-12 Protein | R&D Systems | Cat#419-ML |
| Recombinant Mouse IL-18 | MBL | Cat#B002-5 |
| Recombinant Mouse IL-2 Protein | R&D Systems | Cat#402-ML |
| Recombinant Mouse IFNα 1 Protein | R&D Systems | Cat#2105-1 |
| Phorbol 12-myristate 13-acetate (PMA) | Sigma-Aldrich | Cat#P8139 |
| Ionomycin calcium salt from Streptomyces conglobatus (Ionomycin) | Sigma-Aldrich | Cat#0634 |
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Critical Commercial Assays** |        |            |
| QIAamp DNA Blood Mini Kit | QIAGEN | Cat#51106 |
| TRizol Reagent | Thermo Fisher Scientific | Cat#15596026 |
| Foxp3 Transcription Factor Staining Buffer Set | Thermo Fisher Scientific | Cat#00-5523-00 |
| iQ SYBR Green Supermix | Bio-Rad | Cat#1708880 |
| BioMag Goat Anti-Rat IgG (NK cell enrichment) | QIAGEN | Cat#310107 |
| CellTrace Violet Cell Proliferation Kit | Thermo Fisher Scientific | Cat#C34557 |
| Fixable Viability Dye eFluor 506 | eBioscience | Cat#65-0866-18 |
| FAM FLICA Poly Caspase Kit | Bio-Rad | Cat#ICT092 |
| **Deposited Data** |        |            |
| Raw Data Files for RNA and ChIP Sequencing | NCBI Gene Expression Omnibus | GSE106139 |

**Experimental Models: Cell Lines**

| Name | PI | RRID |
|------|----|------|
| Ba/F3 | L. Lanier | N/A |
| Ba/F3-m157 | L. Lanier (Voigt et al., 2003) | N/A |

**Experimental Models: Organisms/Strains**

| Name | PI | RRID |
|------|----|------|
| Mouse: WT or CD45.2: C57BL/6J | The Jackson Laboratory | Stock#000644; RRID:IMSR_JAX:000664 |
| Mouse: WT or CD45.1: B6.SJL-Ptprc<sup>+/−</sup> Pepc<sup>−/−</sup>/BoyJ | The Jackson Laboratory | Stock#002014; RRID:IMSR_JAX:002014 |
| Mouse: CD45.1xC45_2 | J. Sun (PI) | N/A |
| Mouse: Ifng<sup>−/−</sup> | A. Thomsen (Kimura et al., 1996) | N/A |
| Mouse: R26<sup>DTA</sup>; B6.129P2-Gt(ROSA)26Sortm1(DTA)J | The Jackson Laboratory | Stock#009669; RRID:IMSR_JAX:009669 |
| Mouse: Nkp46<sup>C<sub>Cre</sub></sup> | E. Vivier (Narni-Mancinelli et al., 2011) | N/A |
| Mouse: Nkp46<sup>C<sub>Cre</sub></sup> R26<sup>DTA</sup> | J. Sun (PI) | N/A |
| Mouse: Ifnar1<sup>−/−</sup>; B6(Cg)-Ifnar1<sup>tm1.2Ess/J</sup> | The Jackson Laboratory | Stock#028288; RRID:IMSR_JAX:028288 |
| Mouse: Stat1<sup>−/−</sup> | R. Schreiber (Meraz et al., 1996) | N/A |
| Mouse: Rag2<sup>−/−</sup>; IL2rg<sup>−/−</sup>; C129S4-Rag2<sup>tm1.1Hld</sup> Il2rg<sup>tm1.1Tkv/J</sup> | The Jackson Laboratory | Stock#014593; RRID:IMSR_JAX:014593 |
| Mouse: Kira8<sup>−/−</sup> or Ly49H-deficient | S. Vidal (Fodil-Cornu et al., 2008) | N/A |
| Mouse: Stat2<sup>−/−</sup>; B6.129-Stat2<sup>tm13Smed/J</sup> | The Jackson Laboratory | Stock#023309; RRID:IMSR_JAX:023309 |

**Oligonucleotides**

| Name | RRID |
|------|------|
| Primers against MCMV IE-1 (F: TCGCCCATCGTTTCGAGA, R: TCTCGTAGGTCCACTGACGGA) | Johnson et al., 2016 |

**Software and Algorithms**

| Name | RRID |
|------|------|
| DESeq2 (v.1.14.1) | Love et al., 2014 |
| Trimmomatic (v.0.36) | Bolger et al., 2014 |
| Bowtie2 (v2.2.9) | Langmead and Salzberg, 2012 |
| GenomicAlignments (v.1.10.1) | Lawrence et al., 2013 |
| bedtools2 (v2.26.0) | Quinlan and Hall, 2010 |
| bedGraphToBigWig (v.4) | Kent et al., 2010 |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Joseph Sun (sunj@mskcc.org).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
All mice used in this study were housed and bred under specific pathogen-free conditions at Memorial Sloan Kettering Cancer Center (MSKCC) in accordance with all guidelines of the Institutional Animal Care and Use Committee. This study used the following mouse strains, all on the C57BL/6 genetic background: C57BL/6 (CD45.2; The Jackson Laboratory), B6.SJL (CD45.1; Taconic), Ifnar1−/−/C0 (Müller et al., 1994), Stat1−/−/C0 (Meraz et al., 1996), Stat2−/−/C0 (Park et al., 2000) (JAX stock #023309), Irf9−/−/C0 (Kimura et al., 1996), Klra8−/−/C0 (Ly49H deficient; (Fodil-Cornu et al., 2008)), Nkp46iCre (referred to as NKp46Cre, (Narni-Mancinelli et al., 2011)), Rag2−/−/C0 (Taconic), and Rag2−/−/C0 x R26DTA (The Jackson Laboratory). Nkp46Cre x R26DTA mice were generated at MSKCC. Experiments were conducted using age- and gender-matched mice in accordance with approved institutional protocols. Animals were typically 6-10 weeks old at the time of use and consisted of males and females.

Virus
MCMV (Smith strain) was serially passaged through BALB/c hosts twice, then viral stocks were prepared by dissociating salivary glands 3 weeks after infection with a dounce homogenizer.

Cell Lines
Ba/F3 cells were maintained at 37°C in RPMI medium 1640 containing 10% FBS, 1mM sodium pyruvate, 2 mM glutamine, 1 mM HEPES, and 100 µg/mL penicillin/streptomycin. Ba/F3-m157 (Voigt et al., 2003) were maintained in the same conditions with the addition of 1 μM puromycin.

METHOD DETAILS

Mixed Bone Marrow Chimeras
Mixed bone marrow chimeric mice were generated by lethally irradiating (900 cGy) host CD45.1xCD45.2 mice, which were reconstituted 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. Host CD45.1+ CD45.2+ NK cells were excluded from all analyses.

In vivo Virus Infection
Adoptive transfer studies were performed by mixing splenocytes from WT (CD45.1) and genetic-deficient (CD45.2) mice to achieve equal numbers of Ly49H+KLRG1β NK cells, and injecting intravenously into adult Ly49H-deficient mice 1 day prior to MCMV infection. Recipient mice in adoptive transfer studies were infected by i.p. injection of 7.5 × 10² PFU of MCMV. Experimental mixed bone marrow chimera mice were infected by i.p. injection of 7.5 × 10³ PFU of MCMV. For survival studies, mice were infected by i.p. injection of 4 × 10⁴ PFU of MCMV.
Ex vivo were cultured in media alone as a negative control. 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

Irf9

To test the cytotoxic capacity of NK cells, Ba/F3 target cells and Ba/F3-m157 control cells were labeled differentially with Cell Trace Violet (CTV, Thermofisher) before transfer, and CTV labeling was performed according to manufacturer protocol.

Apoptosis was evaluated by caspase activity staining using the carboxyfluorescein FLICA poly caspase assay kit (BioRad) or Annexin V (BioLegend). NK cell proliferation was analyzed by labeling cells with 5 μM Cell Trace Violet (CTV, Thermofisher) before transfer, and CTV labeling was performed according to manufacturer protocol.

Ex vivo stimulation of Lymphocytes

Approximately 10^6 spleen lymphocytes were stimulated for 4 hr 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.

Ex vivo killing assay

To test the cytotoxic capacity of Irf9−/− NK cells, Ba/F3 target cells and Ba/F3-m157 control cells were labeled differentially with Cell Trace Violet (CTV, Invitrogen), according to manufacturer protocol (Ba/F3, CTV; Ba/F3-m157, CTV). 5 x 10^3 of each cell line were mixed with 5 x 10^4 purified NK cells from WT or Irf9−/− mice, 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. Percentages of target cell killing were determined using the following formula (adapted from Viant et al., 2017): 100 - {[(% Ba/F3-m157 cells/% Ba/F3 cells)]/[(% Ba/F3-m157 cells/% Ba/F3 cells) control] x 100}. For this formula, only CTV+ cells within live cells were considered. Percentage of Pi+ target and control cells was determined from total CTVhi and CTVlo cells respectively.

To test the ability of activated WT NK cells to directly kill Irf9−/− NK cells, NK cells were first enriched from spleens of pooled WT or Irf9−/− mice by negative selection over BioMag goat anti-rat IgG beads (QIAGEN) coated with rat anti-mouse CD8α, CD4, CD19, and Ter-119 antibodies (Bio X Cell, clones 2.43, GK1.5, 1D3, and TER-199 respectively). Enriched CD45.1+CD45.2+ WT NK cells were used as effectors cells against a 1:1 mix of CD45.1+ WT and CD45.2+ Irf9−/− NK cell targets. Effector and target cells were co-cultured for 16 hours at 37°C in RPMI-1640 containing 10% FBS and IL-2 (10 ng/mL), IL-12 (100 ng/mL), IL-18 (10 ng/mL), and IFN-α (100U/mL). After 16 hours, cells were stained with Annexin V and Fixable Viability Dye (eBioscience) prior to flow cytometry; double positive cells were considered dead.
RNA Sequencing
To determine the transcriptome of NK cells following stimulation with IFN-α, NK cells (TCRβ+ CD19+ CD3ε- F4/80- NK1.1-CD49b+) were sorted from C57BL/6 mice, incubated overnight with 100 U/mL IFN-α or no cytokine (unstimulated), then 5-8 × 10⁶ NK cells were sorted again for purity. To determine the STAT1-, IFNAR1- and IRF9-dependent transcriptional events in NK cells during MCMV infection, 1.5-10 × 10⁵ Ly49H⁺ NK cells (TCRβ+ CD19+ CD3ε- F4/80- NK1.1-CD49b+) were sorted from spleens of individual mBMC mice harboring WT (CD45.1) and IFN signaling-deficient (CD45.2) cells at d0, d2 or d4 PI with MCMV. RNA was isolated from sorted cell populations using TRIzol (Invitrogen) 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). STAT1-ChIP was performed twice, while IRF9-ChIP was performed 3 times.

Chromatin immunoprecipitation and DNA sequencing
5-10 × 10⁶ NK cells (TCRβ+ CD19+ CD3ε- F4/80- NK1.1-CD49b+) were sorted from spleens of pooled C57BL/6 mice and incubated overnight (16-18 hours) with cytokines. NK cells were stimulated with 100 U/mL IFN-α with or without 10 ng/mL IL-2. DNA and proteins were cross-linked for 8 minutes using 1% formaldehyde. ChIP was performed as previously described (Beaulieu et al., 2014; Zheng et al., 2007), using 10 μg of rabbit polyclonal anti-STAT1 antibody (Santa Cruz, sc-592, clone M-22), or 10 μg of rabbit polyclonal anti-IRF9 antibody (Santa Cruz, sc-496, clone C-20) followed by Illumina next-generation sequencing. Fragments between 100 and 600 bp were size selected and Illumina HiSeq libraries were prepared using the Kapa DNA library preparation chemistry (Kapa Biosystems) and 12-15 cycles of PCR. Adaptors were diluted 1/10 or 1/50 depending on the starting amount of material available. Barcoded libraries were run on Hiseq 2500 1T in a 50bp/50bp paired-end run, using the TruSeq SBS Kit v3 (Illumina). STAT1-ChIP was performed twice, while IRF9-ChIP was performed 3 times.

QUANTIFICATION AND STATISTICAL ANALYSIS
RNA-Seq Analysis
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). Read counts overlapping exons according to the UCSC Known Gene model were generated using the summizarizeOverlaps 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). Genes were considered differentially expressed if they showed an FDR adjusted p value < 0.05, adjusted for multiple hypothesis correction as calculated by the DESeq2 software. For gene set enrichment analysis, differentially expressed genes were passed into goseq (v1.32.0) using the PANTHER and REACTOME databases. Top pathways were ranked by p-value, selected only from those passing a threshold of FDR-corrected p < 0.05.

ChIP-Seq analysis and peak annotation
For STAT1-ChIP, 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). Concordantly aligned paired mates were used for individual peak calling and pooled replicate peak calling by MACS2 (v2.1.2.160309)(Zhang et al., 2008) with the arguments “-p 1e-2 -m 2 50 –to-large.” Irreproducible discovery rate (IDR)(Li et al., 2011) calculations using scripts provided by the ENCODE project (https://www.encodeproject.org/software/idr/; v2.0.2 and v2.0.3) were performed on all pairs of individual replicate peak lists against the oracle peak list of pooled replicates. Reproducible peaks were thresholded for those showing an IDR value of 0.05 or less and used for further analysis. Peak assignment to genes and gene features was performed using ChipPeakAnno(Zhu et al., 2010). Promoter peaks were defined as peaks that overlapped a region that was +2kb to −0.5kb from the transcriptional start site (TSS). Intragenic (intronic and exonic) peaks were defined as any peak that overlapped with annotated intronic and exonic regions, respectively, based on the UCSC Known Gene annotation database(Speir et al., 2016). Intergenic peaks were defined as any non-promoter or non-intragenic peaks, and were assigned to the gene of the nearest TSS based on the distance from the start of the peak. Priority was given to transcripts that were canonical, based on the UCSC Known Canonical database.

For IRF9-ChIP, paired-end reads were mapped to the Mus musculus genome (mm10 assembly) using Bowtie2 (v2.3.2) with parameters “–no-unal -X 500 –no-mixed –no-discount.” Concordantly aligned paired mates were used for individual peak calling and pooled replicate peak calling by MACS2 with the arguments “–nomodel –shift 100 –extsize 200 -p 0.2 -B –mfold 3 50 –SPMR –keep-dup ‘auto’.” IDR was calculated using custom bash scripts on all pairs of individual replicate peak lists against the oracle peak list of pooled replicates. Reproducible peaks were thresholded for those showing IDR < 0.01 and used for further analysis.

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).
Statistical Analyses
For graphs, data are shown as mean ± SEM, and unless otherwise indicated, statistical differences were evaluated using a two-tailed unpaired Student’s t test, and Welch’s correction. Statistical differences in survival were determined by Gehan-Breslow-Wilcoxon Test analysis. For RNA-seq data, significance cutoff was determined using multiple hypothesis corrected p values as calculated by DESeq2. p < 0.05 was considered significant and is indicated in figures by an “*.” Graphs were produced and statistical analyses were performed using GraphPad Prism.

DATA AND SOFTWARE AVAILABILITY
The accession number for all RNA-seq and ChIP-seq data reported in this paper is GEO: GSE106139.