Type I IFN promotes NK cell expansion during viral infection by protecting NK cells against fratricide

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Type I interferon (IFN) is crucial in host antiviral defense. Previous studies have described the pleiotropic role of type I IFNs on innate and adaptive immune cells during viral infection. Here, we demonstrate that natural killer (NK) cells from mice lacking the type I IFN-α receptor (Ifnar−/−) or STAT1 (which signals downstream of IFNAR) are defective in expansion and memory cell formation after mouse cytomegalovirus (MCMV) infection. Despite comparable proliferation, Ifnar−/− NK cells showed diminished protection against MCMV infection and exhibited more apoptosis compared with wild-type NK cells. Furthermore, we show that ifnar−/− NK cells express increased levels of NK group 2 member D (NKG2D) ligands during viral infection and are susceptible to NK cell–mediated fratricide in a perforin- and NKG2D-dependent manner. Adoptive transfer of ifnar−/− NK cells into NK cell–deficient mice reverses the defect in survival and expansion. Our study reveals a novel type I IFN–dependent mechanism by which NK cells evade mechanisms of cell death after viral infection.

Type I IFNs provide a potent line of antiviral defense through direct and indirect effects on cells of the immune system, leading to their activation and effector function (Biron, 2001; Gonzalez–Navajas et al., 2012) and resulting in the attenuation of viral replication (Müller et al., 1994). IFN-α and IFN-β are members of the type I IFN family. All members of the type I IFN family signal through a ubiquitously expressed heterodimeric receptor that is composed of the IFN-α receptor 1 (IFNAR1) and IFNAR2 chains. Type I IFNs act directly on NK cells to promote their activation, cell cycle entry, and cytotoxic function during viral infection (Biron et al., 1984; Orange and Biron, 1996; Biron, 2001; Nguyen et al., 2002; Martinez et al., 2008; Baranek et al., 2012; Fortin et al., 2013). However, the experimental systems used in previous studies—direct infection of IFN receptor–deficient mice or WT mice with IFN neutralization—are complicated by potential differences in the degree of inflammation, effects on many cell types, and viral load. Thus, the direct influence of type I IFN on effector and long-lived antiviral NK cell responses, while eliminating pleotropic effects on other cells, has not been investigated previously.

Although substantial amounts of type I IFN are produced during viral infection, this cytokine is constitutively present at basal levels and affects the development and homeostasis of various hematopoietic lineages (Honda et al., 2004; Sato et al., 2009; Gough et al., 2012). An indirect effect of type I IFN on NK cell development and maturation has been described recently (Mizutani et al., 2012; Guan et al., 2014). Because the prolific expansion and generation of memory NK cells during mouse cytomegalovirus (MCMV) infection are dependent predominantly on the proinflammatory cytokines IL-12 and IL-18 (Andoniou et al., 2005; Sun et al., 2012; Madera and Sun, 2015), it was of interest to determine whether type I IFNs play a role in these processes. Here, we use NK cells deficient in the IFNAR1 chain (Ifnar−/−) in an adoptive cotransfer system and mixed bone marrow chimeric mice to investigate the direct influence of type I IFN signaling on NK cells responding against MCMV infection.

RESULTS

Type I IFN and STAT1 are required for optimal NK cell responses after MCMV infection

Given the pleiotropic effects of type I IFNs (Uzé et al., 2007), we assessed the ability of ifnar−/− and WT Ly49H+ NK cells to expand in response to MCMV infection using an adoptive cotransfer system (Sun et al., 2012) where both transferred NK cell populations respond against virus and experience similar inflammatory cues within the same host environment. WT and ifnar−/− NK cells were cotransferred into Ly49H-deficient mice, whose NK cells are unable to recognize the virus-encoded glycoprotein m157 during MCMV infection and undergo clonal expansion (Sun et al., 2009). In contrast to the WT NK cells that robustly expanded after MCMV infection, ifnar−/− NK cells failed to expand robustly (Fig. 1 A). Although they exhibited an expansion defect, ifnar−/− NK cells...
cells were able to mature nearly as well as WT NK cells in response to MCMV infection, as indicated by the down-regulation of CD27 and up-regulation of CD11b and KLRG1 (Fig. 1, B and C). We also investigated the contribution of type I IFN signaling in NK cells for protection against lethal MCMV challenge. Equal numbers of naive WT or Ifnar−/− NK cells were transferred into separate neonatal mice and then challenged with MCMV. In contrast to mice receiving WT NK cells, which protected ∼50% of recipients, all mice receiving Ifnar−/− NK cells succumbed to infection by day 15 postinfection (Fig. 1 D), highlighting the importance of type I IFN signaling, specifically in NK cells, for protective immunity against viral challenge.

Type I IFNs signal through STAT1–STAT2 heterodimers and STAT1–STAT1 homodimers (Li et al., 1996). Therefore, we determined the role of STAT1 in the NK cell response to MCMV infection using STAT1-deficient mice. Equal numbers of WT and Stat1−/− Ly49H+ NK cells were cotransferred into Ly49H-deficient hosts and then infected with MCMV. Similar to Ifnar−/− NK cells, Stat1−/− NK cells exhibited a striking defect in expansion during the immune response to MCMV infection (Fig. 2 A), even though Stat1−/− NK cells were able to mature nearly as well as WT NK cells after infection (Fig. 2, B and C). These data support the importance of STAT1-mediated type I IFN signaling in NK cells for an optimal antiviral NK cell response.

Type I IFN promotes the activation and effector function in NK cells after MCMV infection

Type I IFN has both direct and indirect effects on NK cell development and maturation (Mizutani et al., 2012; Guan et al., 2014). In accordance with these prior studies, resting splenic NK cells from Ifnar−/− mice exhibited an increase in immature NK cells as indicated by CD27, CD11b, and KLRG1 expression when compared with WT (unpublished data). Thus, we generated mixed WT:Ifnar−/− bone marrow chimeras in which development of Ifnar−/− mice exhibited an increase in immature NK cells as indicated by CD27, CD11b, and KLRG1 expression when compared with WT (unpublished data). Therefore, we determined the role of STAT1 in the NK cell response to MCMV infection using STAT1-deficient mice. Equal numbers of WT and Stat1−/− Ly49H+ NK cells were cotransferred into Ly49H-deficient hosts and then infected with MCMV. Similar to Ifnar−/− NK cells, Stat1−/− NK cells exhibited a striking defect in expansion during the immune response to MCMV infection (Fig. 2 A), even though Stat1−/− NK cells were able to mature nearly as well as WT NK cells after infection (Fig. 2, B and C). These data support the importance of STAT1-mediated type I IFN signaling in NK cells for an optimal antiviral NK cell response.
also failed to up-regulate granzyme B compared with their WT counterparts at day 1.5 PI (Fig. 3 B), consistent with a previous study (Baranek et al., 2012). Interestingly, Ifnar−/− NK cells produced more IFN-γ than WT NK cells at day 1.5 PI (Fig. 3 B). STAT1 phosphorylation was completely ablated in NK cells that cannot sense type I IFNs (Fig. 3 C). These findings demonstrate the ability of type I IFNs to directly impact the expression of key effector molecules in NK cells, likely via robust STAT1 phosphorylation.

Given the rescue of the maturation defect of Ifnar−/− NK cells in mixed bone marrow chimeric mice (Mizutani et al., 2012), we assessed the ability of Ly49H+ Ifnar−/− and Stat1−/− NK cells from mixed chimeras to undergo clonal expansion in response to MCMV infection. After adoptive transfer and infection, Ifnar−/− NK cells were unable to expand as robustly as their WT counterparts (Fig. 3 D). Similarly, mixed chime-ra-derived Stat1−/− NK cells also exhibited a marked defect in expansion after MCMV infection compared with WT NK cells (Fig. 3 E). Long-lived NK cells were also diminished in NK cell populations that lack IFNAR or STAT1 (Fig. 3, D and E), highlighting the critical role of type I IFN signaling in NK cells for a robust viral-specific response.

Type I IFN is dispensable for NK cell proliferation but shields NK cells against apoptosis after MCMV infection

We explored two possibilities for the expansion defect of Ifnar−/− NK cells in response to MCMV infection: proliferation and apoptosis. To investigate whether a proliferative defect exists in NK cells that are unable to sense type I IFNs, cell trace violet (CTV)–labeled Ly49H+ NK cells from WT:Ifnar−/− chimeras were transferred into Ly49H− deficient hosts and infected with MCMV. Ifnar−/− NK cells exhibited a modest increase in proliferation when compared with WT NK cells at day 4 PI (Fig. 4 A), suggesting that type I IFN signals actually restrain NK cell proliferation after activation. We next compared the amount of apoptosis in Ifnar−/− and WT NK cells that are activated during MCMV infection. After adoptive transfer and MCMV infection, Ifnar−/− NK cells incorporated significantly more fluorescent-labeled inhibitor of caspases (FLICA), a measurement of activated caspases, than WT NK cells at day 4 PI (Fig. 4 B; 2.1 ± 0.5% vs. 12.0 ± 1.8%, respectively; P = 0.006). Together, these findings suggest that type I IFNs shield activated NK cells from cell death during viral infection.

Type I IFN protects NK cells from fratricide via a perforin- and NKG2D-dependent mechanism

To evaluate whether host NK cells were killing Ifnar−/− NK cells, we transferred equal numbers of WT and Ifnar−/− NK cells into WT or perforin-deficient (Prf1−/−) hosts and infected them with lymphocytic choriomeningitis virus (LCMV). LCMV infection elicits a strong type I IFN response (Biron et al., 1999) but does not drive the antigen-specific proliferation of Ly49H+ NK cells observed during MCMV infection, thus ruling out the influence of the m157–Ly49H interaction in determining relative NK cell numbers. After adoptive transfer and LCMV infection in WT hosts, WT NK cells persisted, whereas the percentage of Ifnar−/− NK cells diminished at day 7 PI (Fig. 5 A). The decrease in Ifnar−/− NK cells was seen as early as day 3 PI (not depicted). However, Ifnar−/− NK cells

Figure 3. Type I IFN promotes NK cell activation and expression of effector molecules after MCMV infection. (A and B) WT:Ifnar−/− chimeric mice were infected with MCMV. CD69, IFN-γ, and granzyme B are shown for splenic WT and Ifnar−/− NK cells (compared with uninfected mice) at day 1.5 PI. (C) STAT1 phosphorylation of NK cells at day 1.5 PI is shown, and bar graph plots mean fluorescent intensity (MFI). (D and E) Ly49H+ NK cells from WT:Ifnar−/− chimeric mice (D) or WT:Stat1−/− chimeric mice (E) were transferred into Ly49H− deficient hosts and infected with MCMV. Percentages of Ly49H+ NK cells are shown. Data are mean ± SEM and representative of three independent experiments with at least n = 3 biological replicates per condition. *, P < 0.05 using paired Student’s t test.

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transferred into Prf1−/− hosts were able to persist, and larger percentages were observed compared with the cotransferred WT NK cell population (Fig. 5 A), uncovering a novel role for type I IFNs in protecting NK cells against perforin-mediated elimination. Similar results were observed when we infected mice with an MCMV strain lacking m157 (not depicted). To confirm that the elimination of Ifnar−/− infected mice with an MCMV strain lacking m157 (not de- 
ediated elimination. Similar results were observed when we cotransferred into WT and Ncr1gfp/gfp hosts that were then infected with LCMV. Similar to WT hosts, Ncr1gfp/gfp hosts exhibited a loss of NK cells unable to sense type I IFN during viral infection (Fig. 5 B), indicating that NKp46 does not play a role in NK cell–mediated killing of Ifnar−/− NK cells.

To evaluate the contribution of NKG2D triggering in NK cell fratricide, equal numbers of WT and Ifnar−/− NK cells were cotransferred into WT hosts that received a nondepleting neutralizing anti-NKG2D–blocking antibody (or PBS as a negative control) and then were infected with LCMV. As expected, WT NK cells preferentially survived compared with Ifnar−/− NK cells in the PBS-treated control mice (Fig. 5 C). However, in mice receiving anti-NKG2D, a similar persistence between WT and Ifnar−/− NK cells was observed (Fig. 5 C), suggesting that the NKG2D pathway may represent at least one mechanism that activates fratricide. Similar results were observed in mice infected with MCMV lacking m157 (not depicted). Furthermore, we confirmed the expression of NKG2D ligands on the NK cells soon after infection. After LCMV infection of WT:Ifnar−/− bone marrow chimeras, Ifnar−/− NK cells expressed significantly higher amounts of NKG2D ligand (Fig. 5 D). This finding suggests that type I IFN may have a direct role in suppressing NKG2D ligand induction during viral infection. Collectively, these data indicate that type I IFN acts to combat NK cell–mediated fratricide that is dependent on NKG2D triggering and perforin release.

**Type I IFN is dispensable for NK cell survival and memory formation during MCMV infection in mice lacking WT NK cells**

If host NK cells are indeed mediating fratricide of transferred Ifnar−/− NK cells during viral infection, we hypothesized that Ifnar−/− NK cells should expand normally and generate long-lived memory after MCMV infection in a system devoid of NK cells. Equal numbers of Ly49H+ WT (CD45.1) and Ifnar−/− (CD45.2) NK cells from mixed chimeric mice were transferred into Rag2−/− × Il2rg−/− hosts (which lack T, B, and NK cells), and the recipient mice were then infected with MCMV. After MCMV infection, Ifnar−/− NK cells expanded robustly and even exhibited an increase in percentage compared with WT NK cells (Fig. 6 A). Activation and maturation of virus-specific Ifnar−/− NK cells were nearly identical to WT NK cells, as indicated by CD27, CD11b, and KLRG1 expression at day 7 PI (Fig. 6, B–D). Furthermore, Ifnar−/− NK cells of a comparable phenotype to WT NK cells could be recovered at >4 wk PI (Fig. 6, E–G), revealing the ability of Ifnar−/− NK cells to generate a long-lived memory pool during infection in hosts lacking WT NK cells.

**Type I IFN promotes NK cell expansion**

![Image](https://example.com/image.png)

**Figure 4.** *Ifnar−/−* NK cells proliferate normally but undergo greater apoptosis during MCMV infection. (A) Ly49H+ NK cells from WT:*Ifnar−/−* chimeric mice were labeled with CTV, transferred into a Ly49H-deficient host, and infected with MCMV. Proliferating cells were analyzed at day 4 PI. Bar graph shows percentages of divided and nondivided NK cells at day 4 PI for each group. (B) FLICA incorporation is shown in plots, and bar graph shows percentages of FLICA+ NK cells in each group. Data are mean ± SEM and representative of three independent experiments with at least n = 3 biological replicates per condition. *, P < 0.05 using paired Student’s t test.
DISCUSSION

Traditionally classified as a member of the innate immune system, NK cells are known to provide a crucial line of early defense against viral infections in both humans and mice (Bukowski et al., 1985; Rager-Zisman et al., 1987; Biron et al., 1989). In more recent years, NK cells have been described to possess many features of adaptive immunity (Sun and Lanier, 2011; Vivier et al., 2011). However, the molecular mechanisms behind these adaptive responses are not well understood. Our current findings demonstrate that direct type I IFN signaling in NK cells promotes their optimal activation and function during MCMV infection. In certain viral systems, such as during vaccinia virus infection, NK cell–dependent and –independent effects of type I IFN have been reported where NK cells were directly harvested from WT and Ifnar−/− or Stat1−/− mice (Martinez et al., 2008; Fortin et al., 2013). Other viral models, using mouse hepatitis virus or dengue virus infection, underscore the importance of type I IFN signaling in innate cells for viral control, with the primary focus being macrophages and dendritic cells (Cervantes-Barragán et al., 2009; Züst et al., 2014). In the context of MCMV infection, type I IFN has long been known to mediate antiviral effects, partly through its activity on NK cells (Nguyen et al., 2002; Baranek et al., 2012), our current study demonstrates that direct and indirect effects of type I IFNs on NK cells during the early course of MCMV infection. Extending a recent study reporting both direct and indirect effects of type I IFN on the early NK cell and dendritic cell response after MCMV infection (Baranek et al., 2012), our current study demonstrates that type I IFN acts directly on NK cells to promote their long-term survival by protecting them from elimination via NK cell–mediated fratricide.

Type I IFN has long been observed to promote NK cell–mediated cytotoxicity (Biron et al., 1999). In line with previous studies (Nguyen et al., 2002; Baranek et al., 2012), we were able to detect a defect in granzyme B up-regulation by Ifnar−/− NK cells after MCMV infection (Fig. 3 B). This diminished cytotoxicity of Ifnar−/− NK cells likely explains the reduced protection during lethal MCMV infection (Fig. 1 D). In accordance with previous findings showing that type I IFN inhibits IFN-γ production by NK cells in a STAT1-dependent manner (Nguyen et al., 2000), we observed a greater production of IFN-γ by Ifnar−/− NK cells when compared with WT NK cells after MCMV infection (Fig. 3 B). Thus, the protection mediated by Ifnar−/− NK cells over the control group is likely a consequence of heightened IFN-γ production during viral challenge.

Previous studies have found conflicting roles for type I IFN in the induction of NK cell proliferation after MCMV infection (Orange and Biron, 1996; Geurs et al., 2009). These studies relied on the direct infection of IFN-γ-deficient mice (Geurs et al., 2009), which fails to consider the effects of higher viral loads or administration of IFN-neutralizing antibodies during viral infection (Orange and Biron, 1996), which could directly affect production of IL-15, a cytokine known to influence NK cell survival and proliferation. We demonstrate that NK cells unable to sense type I IFNs retain a functional proliferative program and even exhibit a modest increase in proliferation after infection when compared with WT NK cells (Figs. 4 A and 6 A); however, Ifnar−/− NK cells were found to undergo more apoptosis than WT NK cells (Fig. 4 B). Although our findings suggest that a perforin- and NK-G2D-dependent fratricide mechanism plays a major role in the increased cell death observed in Ifnar−/− NK cells.
during viral infection, additional survival mechanisms may be lacking in these cells. Type I IFN previously has been described to promote the survival of activated CD8+ T cells in a manner that is independent of Bcl-2 (Marrack et al., 1999), perhaps by enhancing the responsiveness of antigen-specific CD8+ T cells to IL-2 and IL-15 (Le Bon et al., 2006) and by regulating the expression of IL-7 and IL-15 cytokine receptors (Schluns and Lefrançois, 2003). Consistent with this hypothesis, we found no difference in the expression of Bcl-2 between WT and Ifnar−/− NK cells after MCMV infection (unpublished data). Thus, a Bcl-2-independent mechanism is promoting survival of antigen-specific NK cells that can sense type I IFNs, shielding these cells from apoptosis. Future studies investigating gene targets of STAT1 may reveal additional pro-survival mechanisms in NK cells exposed to type I IFNs during viral infection.

Recently, type I IFN has been shown to protect antiviral CD8+ T cells from NK cell–mediated killing, as two independent studies observed the selective elimination of Ifnar−/− CD8+ T cells after LCMV infection (Crouse et al., 2014; Xu et al., 2014). This preferential elimination of Ifnar−/− CD8+ T cells was a consequence of the differential expression of inhibitory (MHC class I; Xu et al., 2014) and activating (NKp46; Crouse et al., 2014) NK cell signals. We explored the contribution of these distinct pathways in NK–NK cell fratricide and found that Ifnar−/− NK cells exhibited a modest reduction (15–30%) in MHC class I expression after MCMV infection when compared with WT NK cells (unpublished data). However, NK cells are known to tolerate target cells that express a wide range of MHC class I surface levels (Jonsson et al., 2010; unpublished data), with rejection only occurring when target cell MHC class I expression falls below 20% of the host levels (i.e., an 80% reduction in MHC class I expression; Brodin et al., 2010). Thus, a 30% decrease in MHC class I expression will unlikely result in missing self-recognition and killing. However, because the triggering of NK cell activation and cytotoxicity stems from the net balance of activating and inhibitory input signals (Joncker et al., 2009), a modest decrease in MHC class I expression compounded by a strong activating signal, like that provided by NKG2D ligands, could possibly contribute to the selective killing of Ifnar−/− NK cells observed in our study. Expression of NKp46 ligands was another mechanism suggested to trigger NK cell–mediated killing of Ifnar−/− CD8+ T cells (Crouse et al., 2014); however, we found no role for NKp46 in NK cell–mediated elimination of Ifnar−/− NK cells. Thus, unlike with NK cell–mediated killing of CD8+ T cells, our data do not support a role for MHC class I molecules or NKp46 ligands in NK–NK cell fratricide during viral infection.

We previously demonstrated that the proinflammatory cytokine IL-12 and downstream signaling components STAT4 and Zbtb32 are critical for the clonal-like proliferation of Ly49H+ NK cells during MCMV infection (Sun et al., 2012; Beaulieu et al., 2014). Interestingly, the expansion defect in Ifnar−/− NK cells is nearly as pronounced as that of the Il12rb−/− NK cells, demonstrating a nonredundant role for these cytokines in promoting NK cell expansion. Based on our current findings, we propose a complementary role for IL-12 and type I IFN in simultaneously driving cellular proliferation and protecting against cell death via fratricide,
respectively, during virus-specific NK cell expansion. Given the use of type I IFN in the clinic and its potential impact in vaccination strategies, it is important to elucidate the direct and indirect roles IFN has on specific cellular compartments during the immune response. Our work brings us one step closer by uncovering a novel protective role of type I IFN signaling in NK cells during viral infection.

MATERIALS AND METHODS

Mice and infections. All mice used in this study were bred and maintained 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−/− (Müller et al., 1994), Stat1−/− (Meraz et al., 2008), Kira8−/− (Ly49H deficient; Fodil-Cornu et al., 2008), Nkp46 iCre (referred to as NK-p46Cre; Narni-Mancinelli et al., 2011), B2m−/− (Taconic), Rag2−/−Il2rg−/− (Taconic), Ncr1gfp/gfp (Gazit et al., 2006), Prf1−/− (The Jackson Laboratory), and R26DTA (The Jackson Laboratory). Nkp46Cre R26DTA mice were generated at MSKCC. Adoptive transfer studies and the generation of mixed bone marrow chimeric mice were performed as previously described (Sun et al., 2009). Bone marrow chimeric mice were infected by i.p. injections of 7.5 × 10^5 PFU of salivary gland–derived Smith strain MCMV. Mice used in adoptive transfer studies were infected with 7.5 × 10^5 PFU of MCMV. Newborn Ly49H-deficient mice were infected with 2 × 10^5 PFU of MCMV. LCMV infection was performed as described previously (Sun et al., 2004). In vivo blockade of NKG2D signaling was accomplished by i.p. injection of anti-NKG2D (clone CX5; 200 µg/mouse) on day 0 of LCMV infection.

Flow cytometry and cell sorting. The blocking of Fc receptors was performed with 2.4G2 mAb before staining with the indicated surface or intracellular antibodies (BD, BioLegend, and eBioscience). Flow cytometry was performed using an LSR-II flow cytometer (BD). NKG2D ligand staining was done using mouse NKG2D–human Ig fusion protein for 30 min at 4°C, followed by PE-conjugated goat anti–human IgG Fc antibody (Luminex) for 15 min at 4°C. In proliferation assays, NK cells were labeled with 5-µM CTV (Invitrogen) before transfer, and labeling was performed according to the manufacturer’s protocol (Invitrogen). The data were analyzed using Flowjo software (Tree Star). The enrichment and adoptive transfer of NK cells were performed as previously described (Sun et al., 2012).

Statistical methods. Mean ± SEM is depicted in all graphs. A two-tailed unpaired Student’s t test was used to determine statistical differences. Statistical significance was assigned to p-values <0.05. All statistical analyses and plots were produced in Prism (GraphPad Software).

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