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Type I Interferons Protect T Cells against NK Cell Attack Mediated by the Activating Receptor NCR1

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SUMMARY

Direct type I interferon (IFN) signaling on T cells is necessary for the proper expansion, differentiation, and survival of responding T cells following infection with viruses prominently inducing type I IFN. The reasons for the abortive response of T cells lacking the type I IFN receptor (*Ifnar1*$^{-/-}$) remain unclear. We report here that *Ifnar1*$^{-/-}$ T cells were highly susceptible to natural killer (NK) cell-mediated killing in a perforin-dependent manner. Depletion of NK cells prior to lymphocytic choriomeningitis virus (LCMV) infection completely restored the early expansion of *Ifnar1*$^{-/-}$ T cells. *Ifnar1*$^{-/-}$ T cells had elevated expression of natural cytotoxicity triggering receptor 1 (NCR1) ligands upon infection, rendering them targets for NCR1 mediated NK cell attack. Thus, direct sensing of type I IFNs by T cells protects them from NK cell killing by regulating the expression of NCR1 ligands, thereby revealing a mechanism by which T cells can evade the potent cytotoxic activity of NK cells.

INTRODUCTION

Proper activation, expansion, and differentiation of T cells is critical for the clearance of viral infections and is dependent on three key signals: antigen presentation, costimulation, and cytokine signaling. The nature of the infecting pathogen determines which cytokines serve as signal 3 cytokines, with the two most studied being interleukin-12 (IL-12) and type I interferons (IFNs) (Aichele et al., 2006; Keppler et al., 2009; Kolumam et al., 2005). T cell responses are dependent on type I IFNs during lymphocytic choriomeningitis virus (LCMV) infection, where the inability to directly sense type I IFNs leads to curtailed expansion (Aichele et al., 2006; Kolumam et al., 2005) and altered differentiation of antiviral T cells (Wiesel et al., 2012). The cause(s) for the abortive expansion of T cells lacking the type I IFN receptor (*Ifnar1*$^{-/-}$) are unknown and their identification is critical to understand the regulation of T cell dynamics during acute viral infections.

In addition to their role as signal 3 cytokines for T cell activation, type I IFNs act on numerous cell types leading to the establishment of an antiviral state (Stark et al., 1998). Natural killer (NK) cells can be activated directly by type I IFNs (Biron et al., 1984; Gidlund et al., 1978). NK cells play a central role in the innate immune response and contribute to the clearance of certain viral infections and cancerous cells (French and Yokoyama, 2003; Wu and Lanier, 2003), either via direct cytotoxic functions, via provision of inflammatory cytokines such as IFN-γ, or via crosstalk to antigen-presenting cells (APCs) and subsequent regulation of T cells (Cook and Whitmire, 2013). NK cells recognize target cells through an array of activating and inhibitory receptors (Cerwenka and Lanier, 2001). When signals from activating ligands outweigh those of inhibitory signals, NK cell effector functions are triggered. Inhibitory ligands, such as major histocompatibility complex (MHC) class I, are important for maintaining NK cell self-tolerance and preventing autoimmunity (Yokoyama, 1995), whereas activating ligands, such as the mouse cytomegalovirus (MCMV) viral protein m157 recognized by Ly49H on NK cells are important for protection against viral infection (Arase et al., 2002). In addition to their direct role in viral clearance and control of "altered" self cells, NK cells can regulate T cell responses in a positive and negative manner. Such regulation can occur in an indirect manner, for instance via the elimination of antigen-presenting dendritic cells, having a negative impact on the induction of T cell responses and the success of vaccination (Andrews et al., 2010; Hayakawa et al., 2004). In a direct manner, NK cells were shown to regulate T cell responses through cytokine secretion or direct cytolysis, whereby NK cells can directly kill CD8$^+$ and CD4$^+$ T cells (Lang et al., 2012; Lu et al., 2007; Soderquest et al., 2011; Waggoner et al., 2010, 2012, 2014), thereby affecting the size of the antigen-specific T cell pool and the control of an infection. Multiple NK cell ligands are proposed to play a role in this killing process; blockade of the activating receptor

NKG2D leads to enhanced CD8$^+$ T cell responses in the context of peptide vaccination (Soderquest et al., 2011) and high dose LCMV infection (Lang et al., 2012), whereas expression of the inhibitory ligands Qa1 and CD48 on T cells can confer protection against NK cell mediated lysis (Lu et al., 2007; Waggoner et al., 2010). What remains to be explained is whether and how T cells can protect themselves from NK-cell-mediated killing.

In this study we sought to address these questions by identifying the mechanisms responsible for the impaired expansion of *Ifnar1*$^{-/-}$ LCMV-specific CD8$^+$ and CD4$^+$ T cells during acute LCMV infection. By performing a whole-genome gene-expression analysis, we found many molecules involved in cell death being differentially regulated in *Ifnar1*$^{-/-}$ LCMV-specific CD8$^+$ T cells compared to their WT counterparts, among which were multiple NK cell activating and inhibitory ligands. In vivo depletion of NK cells revealed a key role for NK cells in the negative regulation of *Ifnar1*$^{-/-}$ T cells, with NK cell depletion during priming leading to a complete recovery of the early *Ifnar1*$^{-/-}$ T cell expansion. We further found that NK cells selectively killed activated *Ifnar1*$^{-/-}$ T cells in a perforin-dependent manner via engagement of natural cytotoxicity triggering receptor 1 (NCR1) ligands being specifically upregulated on *Ifnar1*$^{-/-}$ T cells. Our data establish a mechanism whereby type I IFN signaling on activated T cells is pivotal to protect them from NCR1-mediated NK cell attack.

RESULTS

Microarray Analysis Reveals Distinct Gene-Expression Profiles in *Ifnar1*$^{-/-}$ and WT P14 Cells

To investigate the reason(s) for the compromised expansion of CD8$^+$ T cells lacking the ability to sense type I IFN during LCMV infection, we performed a whole-genome microarray comparing type I IFN receptor sufficient (WT) and deficient (*Ifnar1*$^{-/-}$) LCMV-specific CD8$^+$ T cells. WT and *Ifnar1*$^{-/-}$ T cell receptor (TCR) transgenic CD8$^+$ T cells specific for the LCMV glycoprotein gp_{33-41} (P14) were cotransferred into naive C57BL/6 (Bl6) mice followed by LCMV infection. To increase the low *Ifnar1*$^{-/-}$ P14 T cell numbers recovered following LCMV infection, we utilized the previously described LCMV8.7 and vaccinia virus (VVG2) coinfection (Wiesel et al., 2011), where the inflammatory environment is provided by the LCMV8.7 mutant, which is not recognized by P14 cells, and antigen is provided by the LCMV-GP recombinant VVG2. This setup leads to greater expansion of both *Ifnar1*$^{-/-}$ and WT P14 T cells compared to LCMV, allowing for analysis of *Ifnar1*$^{-/-}$ P14 cells at early time points. Importantly, the ratio between *Ifnar1*$^{-/-}$ and WT cells is comparable in the coinfection and single infection models (Figures 1A and 1B) and it has been previously shown that there is no difference in the phenotype or differentiation of P14 cells in the two infection models (Wiesel et al., 2012). For microarray analysis, WT and *Ifnar1*$^{-/-}$ P14 cells were sorted to high purity based on expression of the congenic markers Ly5.1 (WT) and Thy1.1 (*Ifnar1*$^{-/-}$) at day 3 post coinfection, the time point corresponding to the peak of *Ifnar1*$^{-/-}$ P14 expansion (data not shown) (Figure 1C). Analysis of genes more than 2-fold differentially regulated revealed 631 genes that were differentially expressed in activated *Ifnar1*$^{-/-}$ and WT P14 cells. Of these genes, 374 were upregulated in *Ifnar1*$^{-/-}$ compared to WT P14 cells and 257 were downregulated. Pathway analysis of the 631 genes revealed that many genes grouped together into distinct functional pathways (Figure 1D). The pathways with the greatest number of differentially expressed genes were those related to signaling and signal transduction, metabolic processes, cell death and apoptosis, and IFN signaling. Of particular interest were those genes associated with cell death regulation, as these could be of direct relevance for the abortive expansion of *Ifnar1*$^{-/-}$ P14 cells. Further analysis revealed differential expression of many genes encoding ligands for NK cell activating or inhibitory receptors (Figure 1E). In combination with recent reports demonstrating NK cell regulation of T cell responses, we decided to further examine a potential role of NK cells in the negative regulation of *Ifnar1*$^{-/-}$ P14 cell expansion.

NK Cell Depletion Restores *Ifnar1*$^{-/-}$ P14 Cell Expansion

To test for a regulatory role of NK cells, we cotransferred WT and *Ifnar1*$^{-/-}$ P14 cells into NK cell depleted (aNK1.1) or undepleted (Bl6) mice followed by acute LCMV strain WE (LCMV-WE) infection. Depletion of NK cells led to the complete recovery of *Ifnar1*$^{-/-}$ P14 cell expansion at day 4 postinfection (p.i.), both in percentage and total cell numbers (Figures 2A and 2B). This ~20-fold increase in expansion was observed in spleen and lymph nodes (data not shown). Consistent with previous reports (Lang et al., 2012; Lu et al., 2007; Soderquest et al., 2011; Waggoner et al., 2012; Waggoner et al., 2010), we observed a moderate increase (~1.5-fold) in expansion of WT P14 cells in NK-cell-depleted mice. Furthermore, previous reports found that *Ifnar1*$^{-/-}$ CD4$^+$ T cells also show a strong reduction in expansion following LCMV infection (Havenar-Daughton et al., 2006). Therefore, we generated *Ifnar1*$^{-/-}$ Smarta (SM) cells, being transgenic CD4$^+$ T cells specific for the LCMV glycoprotein gp_{61-80} epitope. Cotransfer of WT and *Ifnar1*$^{-/-}$ SM cells into NK-cell-depleted mice followed by LCMV infection revealed that *Ifnar1*$^{-/-}$ SM cell expansion could also be completely recovered to WT amounts by NK cell depletion (Figures 2A and 2B). In addition, *Ifnar1*$^{-/-}$ P14 cell expansion was also recovered with the anti-Asialo GM1 NK-cell-depleting antibody, which targets an epitope not expressed on NKT or T cells (see Figure S1 available online). Analysis of P14 cell function at day 4 p.i. revealed that NK cell depletion had no significant effect on the activation (CD44) or effector functions of WT and *Ifnar1*$^{-/-}$ P14 cells, as shown by percentage of IFN-γ, perforin, and granzyme B-positive T cells (Figure 2C). Furthermore, comparing the global gene-expression profiles of P14 cells isolated from Bl6 and NK-cell-depleted mice by cluster analysis revealed that NK cell depletion had little effect on the overall expression profile of WT or *Ifnar1*$^{-/-}$ P14 cells (Figure 2D).

Next, we addressed the question of whether naive *Ifnar1*$^{-/-}$ P14 cells are also sensitive to NK-cell-mediated killing. To this end, we infected mice with LCMV8.7, following cotransfer with WT and *Ifnar1*$^{-/-}$ P14 cells. In this setting, P14 cells remain antigen inexperienced but the inflammatory environment and NK cell activation remains the same as in LCMV-WE infection. WT and *Ifnar1*$^{-/-}$ P14 cell numbers were comparable during LCMV8.7 infection with or without NK cell depletion (Figure 2E), indicating that T cells need to be activated for negative regulation by NK cells. Finally, NK cell depletion could also recover *Ifnar1*$^{-/-}$ P14 cell expansion to the level of WT P14 cells during infection with vesicular stomatitis virus expressing the LCMV glycoprotein

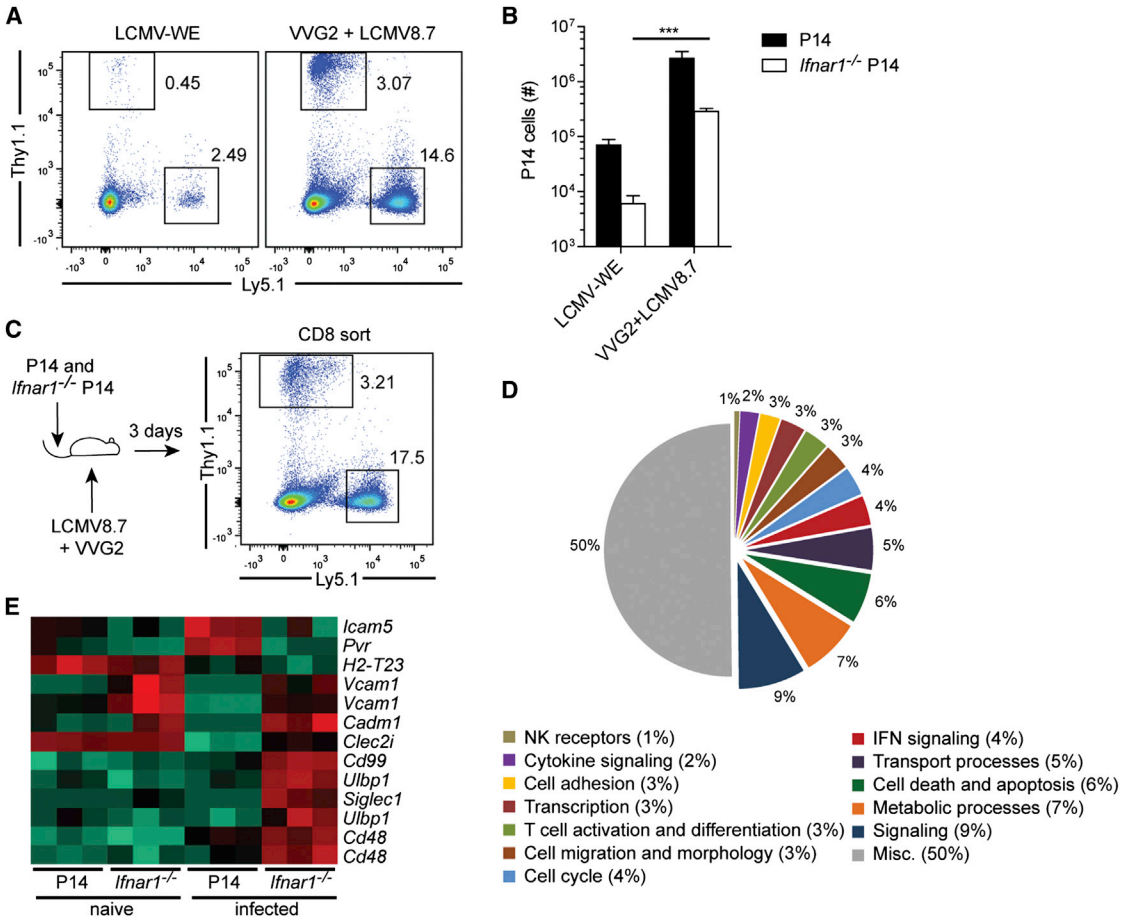

Figure 1. Microarray Analysis Reveals Distinct Gene-Expression Profiles in *Ifnar1*$^{-/-}$ and WT P14 Cells

(A) and (B) LCMV-WE infection and LCMV8.7 + VVG2 coinfection following cotransfer of 10^6 WT and *Ifnar1*$^{-/-}$ P14 cells. (A) Representative flow cytometry dot plots of WT (Ly5.1$^+$) and *Ifnar1*$^{-/-}$ (Thy1.1$^+$) P14 cells 3 days p.i. in the spleen, numbers indicate the percentage of total CD8$^+$ T cells. (B) Total P14 cell numbers in the spleen 3 days p.i. Data are shown as mean ± SEM of n = 12 mice pooled from three independent experiments. ***$p < 0.001$ (unpaired two-tailed t test).

(C) Experimental setup for microarray analysis; WT and *Ifnar1*$^{-/-}$ P14 cells were cotransferred into naive Bl6 mice followed by LCMV8.7 + VVG2 coinfection. 3 days p.i. WT (Ly5.1$^+$) and *Ifnar1*$^{-/-}$ (Thy1.1$^+$) P14 cells were sorted to purity and used for microarray analysis.

(D) Pie chart representing functional pathways enriched among genes more than 2-fold differentially expressed between WT and *Ifnar1*$^{-/-}$ P14 cells. Numbers represent the percentage of genes found in the respective pathways.

(E) Heatmap expression pattern of genes involved in NK cell activation and inhibition. Expression ranges from low expression (green) to high expression (red). Each box represents a group from the microarray analysis; each group was performed in triplicates.

(VSVGP), another infection associated with high amounts of type I IFNs (Figure 2F). Taken together, we found that both CD8$^+$ and CD4$^+$ T cells lacking the ability to sense type I IFNs are highly susceptible to negative regulation by NK cells.

NK Cell Depletion Leads to Enhanced Memory *Ifnar1*$^{-/-}$ P14 Cell Formation

NK cell depletion leads to a full recovery of expansion of *Ifnar1*$^{-/-}$ P14 cells up to day 4 p.i., but at day 7 p.i., *Ifnar1*$^{-/-}$ P14 cells exhibited reduced expansion compared to WT P14 cells, which could partially be recovered by NK cell depletion (Figures 3A and 3B). Nonetheless, NK cell depletion led to a ~100-fold increase in total *Ifnar1*$^{-/-}$ P14 cell numbers compared to undepleted controls. Similar to *Ifnar1*$^{-/-}$ P14 cells, *Ifnar1*$^{-/-}$ SM cells also exhibited a partial recovery of expansion at day 7 p.i. when NK cells were depleted (Figures 3A and 3B). Comparable results were obtained with aNK1.1 and anti-Asialo-GM1 targeted NK cell depletion (Figure S2). To examine the effect of NK cells on the endogenous *Ifnar1*$^{-/-}$ T cell response, we utilized mice in which T cells specifically lack the type I IFN receptor (*Cd4*^{cre}*Ifnar1*$^{flox/flox}$) (Kamphuis et al., 2006). We found a substantial recovery in the expansion of total activated (CD44^{hi}) or gp33 tetramer and np396 tetramer-specific CD8$^+$ T cells in NK-cell-depleted (aNK1.1) *Cd4*^{cre}*Ifnar1*$^{flox/flox}$ mice compared to undepleted (Ø) mice at day 7 p.i. (Figure 3C). As previously reported, *Ifnar1*$^{-/-}$ P14 cells failed to differentiate into short-lived effector cells (SLECs) and were skewed toward a memory precursor effector cell (MPEC) phenotype. Depletion of NK cells had no effect on acquisition of this differential phenotype between WT or *Ifnar1*$^{-/-}$ P14 cells (Figure 3D). Next, we examined memory formation of WT and *Ifnar1*$^{-/-}$ P14 cells with and without NK cell depletion. Depletion of NK cells during priming led to a substantial increase

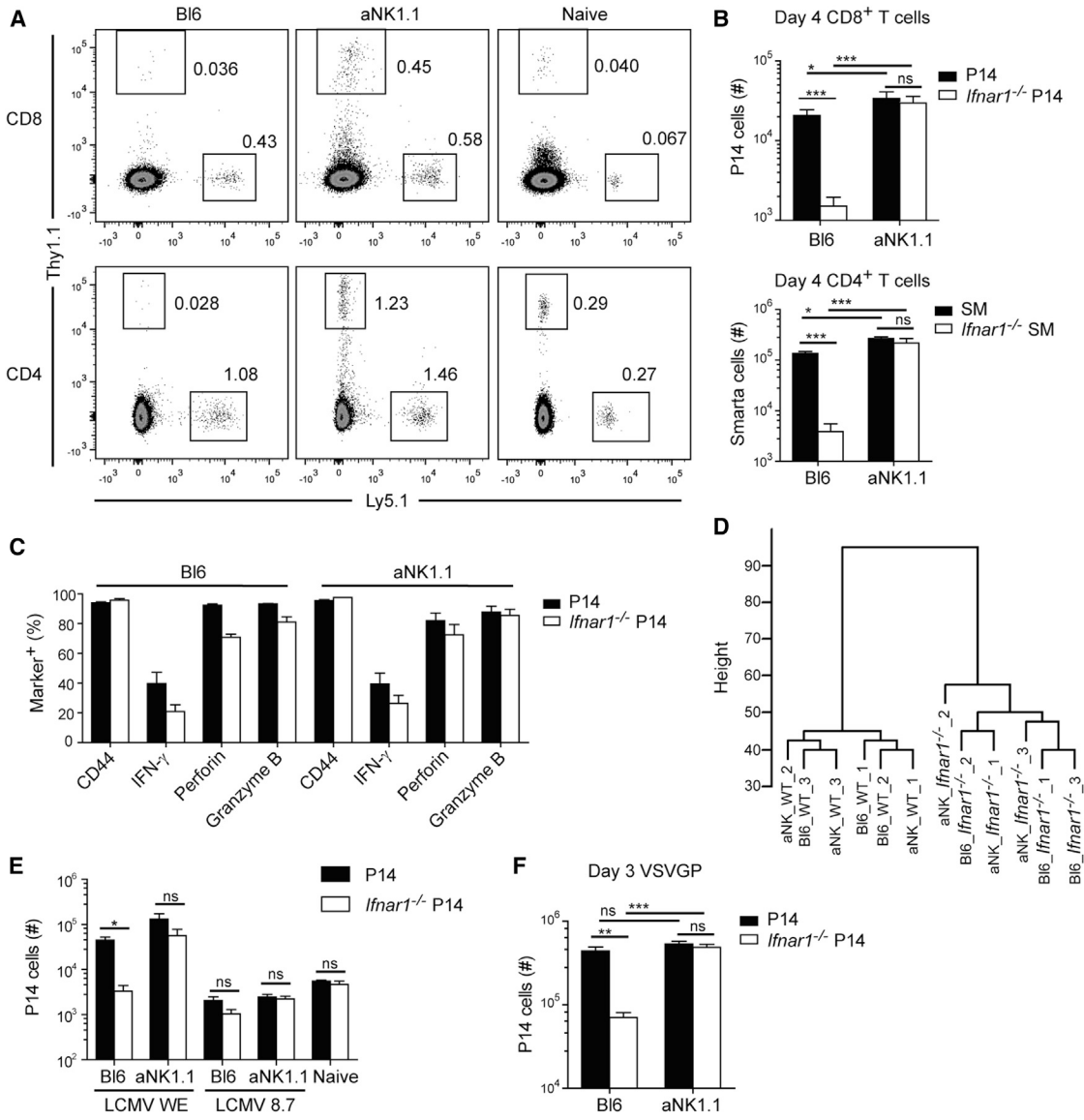

Figure 2. NK Cell Depletion Restores *Ifnar1*$^{-/-}$ P14 Cell Expansion

(A and B) Percentage and total T cell numbers of WT and *Ifnar1*$^{-/-}$ P14 or SM cells in LCMV infected NK cell depleted (aNK1.1) or undepleted (Bl6) mice at day 4 p.i. Naive mice received cell transfers but were left uninfected. (A) Representative flow cytometry dot plots pregated on CD8$^+$ (top row) or CD4$^+$ (bottom row) T cells. Numbers represent the percentage of total CD8$^+$ or CD4$^+$ T cells in the spleen. (B) Total cell numbers of WT and *Ifnar1*$^{-/-}$ P14 and SM cells 4 days p.i. (C) Functionality of T cells from NK cell depleted (aNK1.1) and undepleted (Bl6) mice 4 days p.i. Shown is the percentage of cells positive for the indicated marker. No significant differences were found when comparing activation markers of *Ifnar1*$^{-/-}$ P14 cells from Bl6 and NK-cell-depleted mice.
(D) Hierarchical clustering of overall gene-expression patterns of WT and *Ifnar1*$^{-/-}$ P14 cells from undepleted (Bl6) and depleted (aNK) mice.
(E) Day 4 p.i. cell numbers of WT and *Ifnar1*$^{-/-}$ P14 cells in the spleen as in (A) from mice infected with LCMV-WE or LCMV8.7.
(F) Total number of P14 cells 3 days following VSVGP infection. (B, D–F) Data shown are mean ± SEM of n = 4 mice representative of at least three experiments. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001 (unpaired two-tailed t test). See also Figure S1.

in the percentage and number (Figures 3E and 3F) of *Ifnar1*$^{-/-}$ memory P14 cells compared to the undepleted situation. Phenotypically, NK cell depletion during priming had no effect on the differentiation of memory cells into central and effector memory cells at day 80 p.i. and VVG2 challenge revealed that WT and *Ifnar1*$^{-/-}$ P14 cells were equally functional with respect to recall potential and exertion of effector functions, regardless of whether they had been primed in the presence or absence of NK cells (Figure S3). Taken together, NK cell depletion leads to a partial recovery of *Ifnar1*$^{-/-}$ T cell expansion at day 7 p.i. and substantially increased formation of functional memory cells.

Memory *Ifnar1*$^{-/-}$ P14 Cell Expansion Is Curtailed by NK Cells during LCMV Infection

Similar to primary T cell responses, memory recall of CD8$^+$ T cells is also dependent on signal 3 cytokines as memory *Ifnar1*$^{-/-}$ CD8$^+$

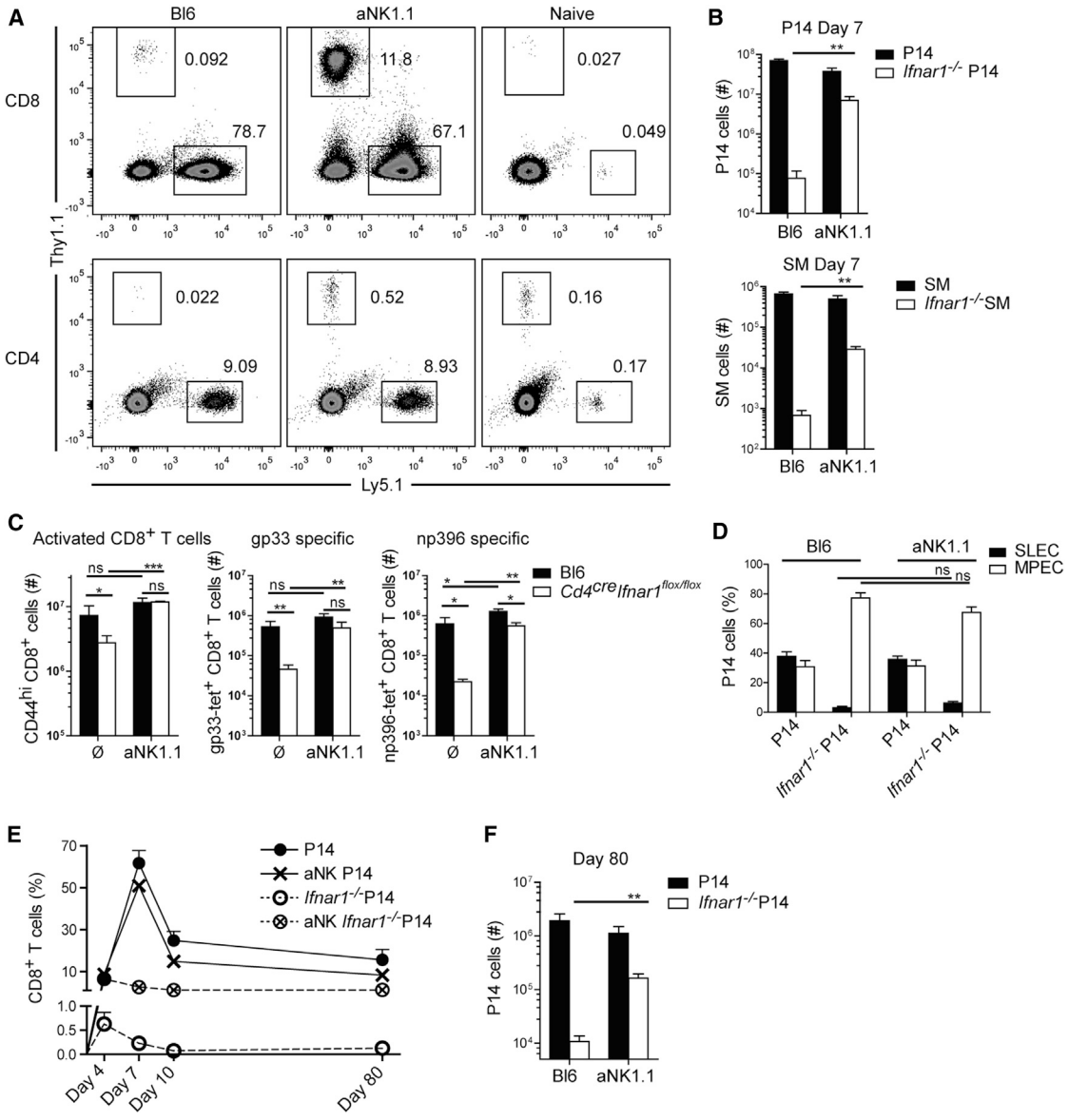

Figure 3. NK Cell Depletion Leads to Enhanced Memory *Ifnar1*$^{-/-}$ P14 Cell Formation

(A and B) Percentage and total T cell numbers of WT and *Ifnar1*$^{-/-}$ P14 or SM cells in NK cell depleted (aNK1.1) or undepleted (Bl6) mice at day 7 p.i. Naive mice received cell cotransfers but were left uninfected. (A) Representative flow cytometry dot plots pregated on CD8$^+$ (top row) or CD4$^+$ (bottom row) T cells are shown. Numbers represent the percentage of total CD8$^+$ or CD4$^+$ T cells in the spleen. (B) Total numbers of WT and *Ifnar1*$^{-/-}$ P14 and SM cells 7 days post LCMV infection.

(C) WT (Bl6) or *Cd4*^{cre}*Ifnar1*$^{flox/flox}$ mice were depleted of NK cells (aNK1.1) or left un-depleted (Ø) and infected with LCMV-WE. At day 7 p.i., total activated CD8$^+$ T cells were analyzed by gating on CD44^{hi} CD8$^+$ cells. Gp33 and np396-specific responses were analyzed by tetramer staining. Shown are the total numbers of the indicated cells in the spleen. Data shown are representative of n = 4 mice. ns, not significant; *p < 0.05; **p < 0.01 (unpaired two-tailed t test).

(D) Differentiation phenotype of WT and *Ifnar1*$^{-/-}$ P14 cells as in (A) in NK cell depleted (aNK1.1) and undepleted (Bl6) mice, shown are the percentage of short-lived effector cells (CD44^{hi}, KLRG1^{hi}, CD127^{lo}) and memory precursor effector cells (CD44^{hi}, KLRG1^{lo}, CD127^{hi}) among WT or *Ifnar1*$^{-/-}$ P14 cells 7 days post LCMV infection. Data shown are mean ± SEM of n = 12 mice pooled from three independent experiments. ns, not significant (unpaired two-tailed t test).

(E and F) Time course analysis of WT and *Ifnar1*$^{-/-}$ P14 cells in NK cell depleted (aNK) and undepleted (Bl6) mice following LCMV infection. (E) Percentage of P14 cells among total CD8$^+$ T cells in blood. (F) Total P14 cell number day 80 p.i. in the spleen of undepleted (Bl6) and NK-cell-depleted (aNK1.1) mice. (B, D–F) Data shown are mean ± SEM of n = 4 mice representative of three independent experiments. ns, not significant; **p < 0.01 (unpaired two-tailed t test). See also Figure S2.

T cells show greatly reduced recall expansion during LCMV infection (Keppler and Aichele, 2011). Therefore, we examined whether the memory response to LCMV is also negatively regulated by NK cells in the absence of type I IFN signaling on CD8$^+$ T cells. To generate memory P14 cells, we cotransfected WT and *Ifnar1*$^{-/-}$ P14 cells into naive mice followed by VVG2 infection (Figure 4A).

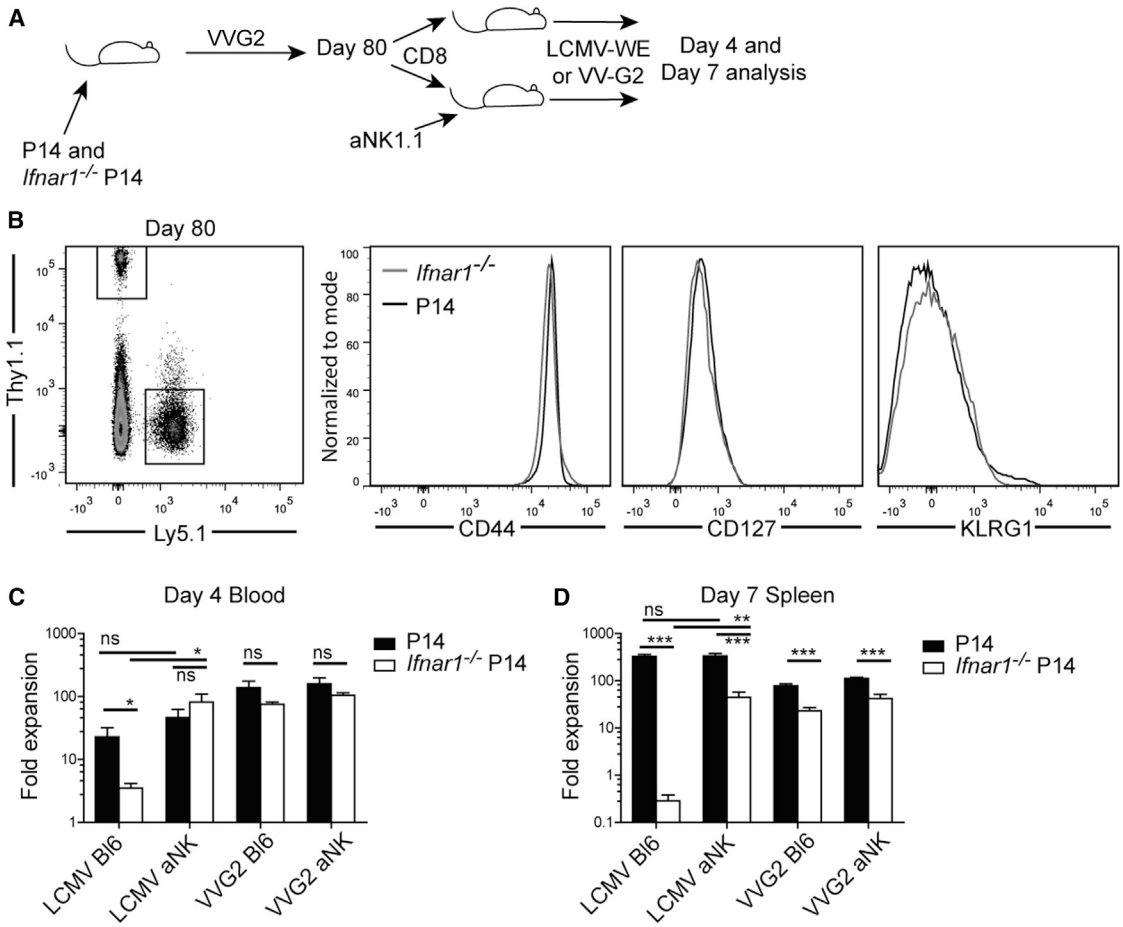

Figure 4. Memory *Ifnar1*$^{-/-}$ P14 Cell Expansion Is Curtailed by NK Cells during LCMV Infection

(A) WT and *Ifnar1*$^{-/-}$ memory P14 cells were generated after infection with VVG2. At day 80 p.i., CD8$^+$ T cells were MACS purified and transferred into naive NK-cell-depleted (aNK1.1) or undepleted (Bl6) hosts followed by challenge with LCMV or VVG2.

(B) Phenotypic analysis of P14 memory T cells at day 80 post VVG2 infection. Flow cytometry dot plots and histograms of the indicated markers are shown and are representative of three independent experiments with n = 4 mice.

(C) Fold expansion of WT and *Ifnar1*$^{-/-}$ P14 cells at day 4 and (D) day 7 post challenge. (C and D) Data shown are mean ± SEM of n = 12 mice pooled from three independent experiments. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001 (unpaired two-tailed t test). See also Figure S3.

At day 80 p.i., WT and *Ifnar1*$^{-/-}$ P14 cells had differentiated into phenotypically equivalent memory cells (Figure 4B). At this time point, memory CD8$^+$ T cells were purified and transferred into either NK cell depleted or undepleted mice, followed by challenge with LCMV-WE or VVG2. At day 4 postchallenge, analysis of T cell expansion in the blood revealed reduced expansion of *Ifnar1*$^{-/-}$ P14 cells during LCMV challenge, which could be completely restored by NK cell depletion during the challenge phase (Figure 4C). At day 7 post LCMV challenge, as during the primary response, NK cell depletion led to a partial recovery of *Ifnar1*$^{-/-}$ P14 cell expansion (Figure 4D). In summary, both primary and secondary CD8$^+$ T cell responses to LCMV are dependent on type I IFN signaling, and depletion of NK cells leads to enhanced expansion of both naive and memory *Ifnar1*$^{-/-}$ P14 cells.

Negative Regulation of *Ifnar1*$^{-/-}$ T Cell Expansion by NK Cells Is Perforin Dependent

To investigate the mechanisms of how this negative regulation occurs, we focused on a potential role of perforin-mediated cytotoxicity. To this end, we cotransferred WT and *Ifnar1*$^{-/-}$ P14 cells into perforin-deficient (*Prf1*$^{-/-}$) mice followed by LCMV infection. Analysis at day 4 p.i. revealed that *Ifnar1*$^{-/-}$ P14 cell priming in *Prf1*$^{-/-}$ mice led to the complete recovery of early expansion (Figures 5A and 5B). The expansion of *Ifnar1*$^{-/-}$ P14 cells in *Prf1*$^{-/-}$ mice was comparable to NK cell depletion in Bl6 mice, and NK cell depletion in *Prf1*$^{-/-}$ mice had no additional effect (Figure S4). Furthermore, at day 7 p.i., expansion of *Ifnar1*$^{-/-}$ P14 cells in *Prf1*$^{-/-}$ mice was also partially recovered (Figures 5A and 5B), in analogy to NK-cell-depleted Bl6 hosts. Similar results were obtained when *Ifnar1*$^{-/-}$ SM cells were transferred into *Prf1*$^{-/-}$ mice, with complete recovery of *Ifnar1*$^{-/-}$ SM cell expansion by day 4 p.i. and partial recovery by day 7 in NK-cell-depleted hosts, and a full recovery in *Prf1*$^{-/-}$ mice (Figures 5C and 5D). The reduced expansion of WT SM cells at day 7 in *Prf1*$^{-/-}$ compared to Bl6 mice is due to increased viral burden in the former, as LCMV control is dependent on perforin-mediated CD8$^+$ T cell effector function. The total number of *Ifnar1*$^{-/-}$ SM cells was comparable between

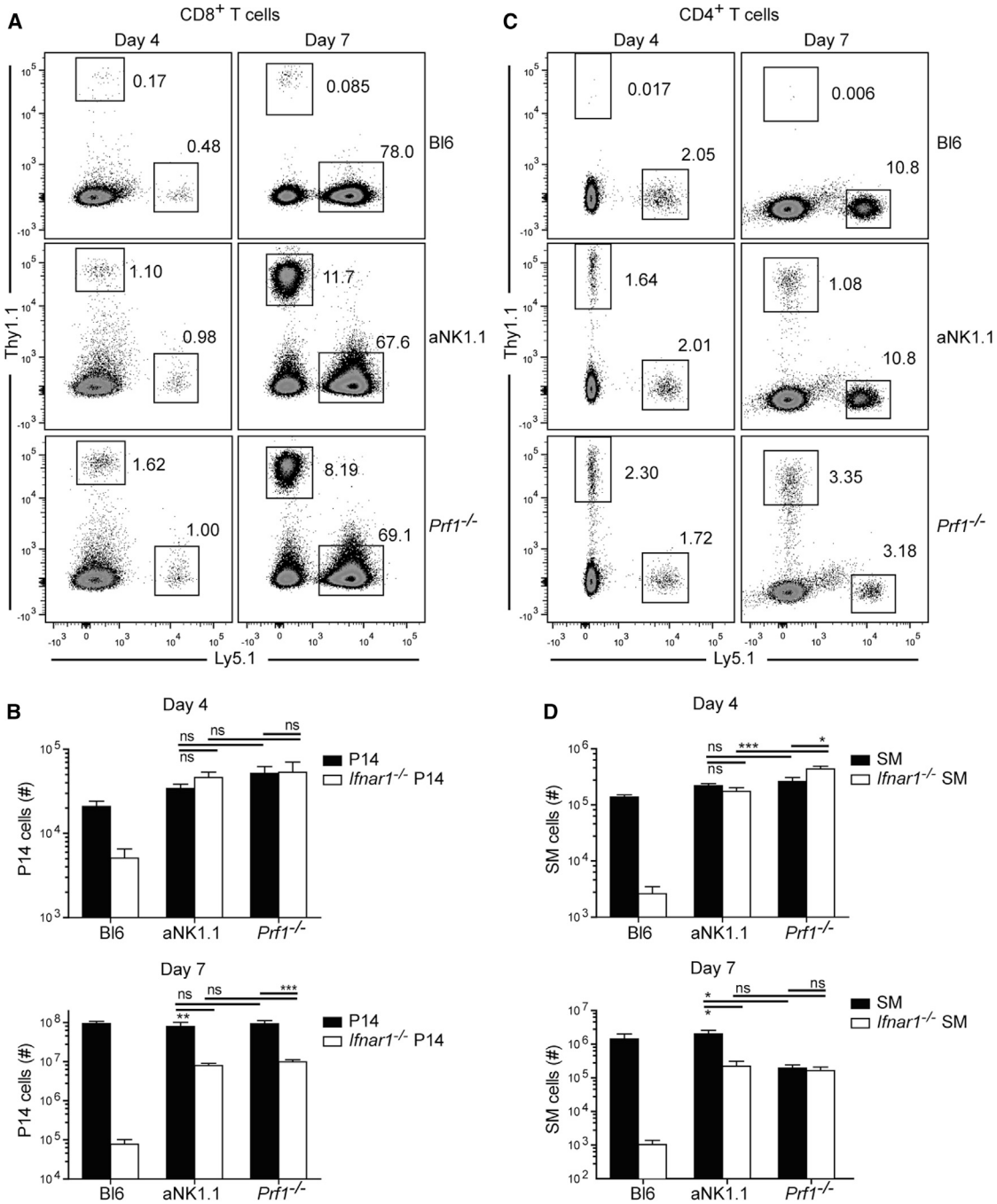

Figure 5. Negative Regulation of *Ifnar1*$^{-/-}$ T Cell Expansion by NK Cells Is Perforin Dependent

(A–D) Cotransfer of WT and *Ifnar1*$^{-/-}$ P14 or SM cells into NK-cell-depleted (aNK1.1), perforin-deficient (*Prf1*$^{-/-}$), or undepleted (Bl6) mice followed by LCMV infection. On day 4 and day 7 p.i., the percentage and total cell numbers were determined. (A and C) Representative flow cytometry dot plots pregated on CD8$^+$ (A) or CD4$^+$ (C) T cells, numbers represent the percentage of total CD8$^+$ (A) or CD4$^+$ (C) T cells in the spleen. (B and D) Total number of WT and *Ifnar1*$^{-/-}$ P14 or SM cells at day 4 (top) and day 7 (bottom) post LCMV infection. Data shown are mean ± SEM of n = 12 mice pooled from three experiments. ns, not significant; **p < 0.01; ***p < 0.001 (unpaired two-tailed t test). See also Figure S4.

Prf1$^{-/-}$ mice and NK-cell-depleted Bl6 mice. Thus, our results demonstrate that the early negative regulation of *Ifnar1*$^{-/-}$ T cells by NK cells is perforin dependent, implicating a direct killing mechanism.

NK Cells Preferentially Kill *Ifnar1*$^{-/-}$ T Cells

Next, we investigated whether NK cells were preferentially killing *Ifnar1*$^{-/-}$ P14 cells. To demonstrate direct killing of CD8$^+$ T cells by NK cells, we established an in vivo killer assay. WT and

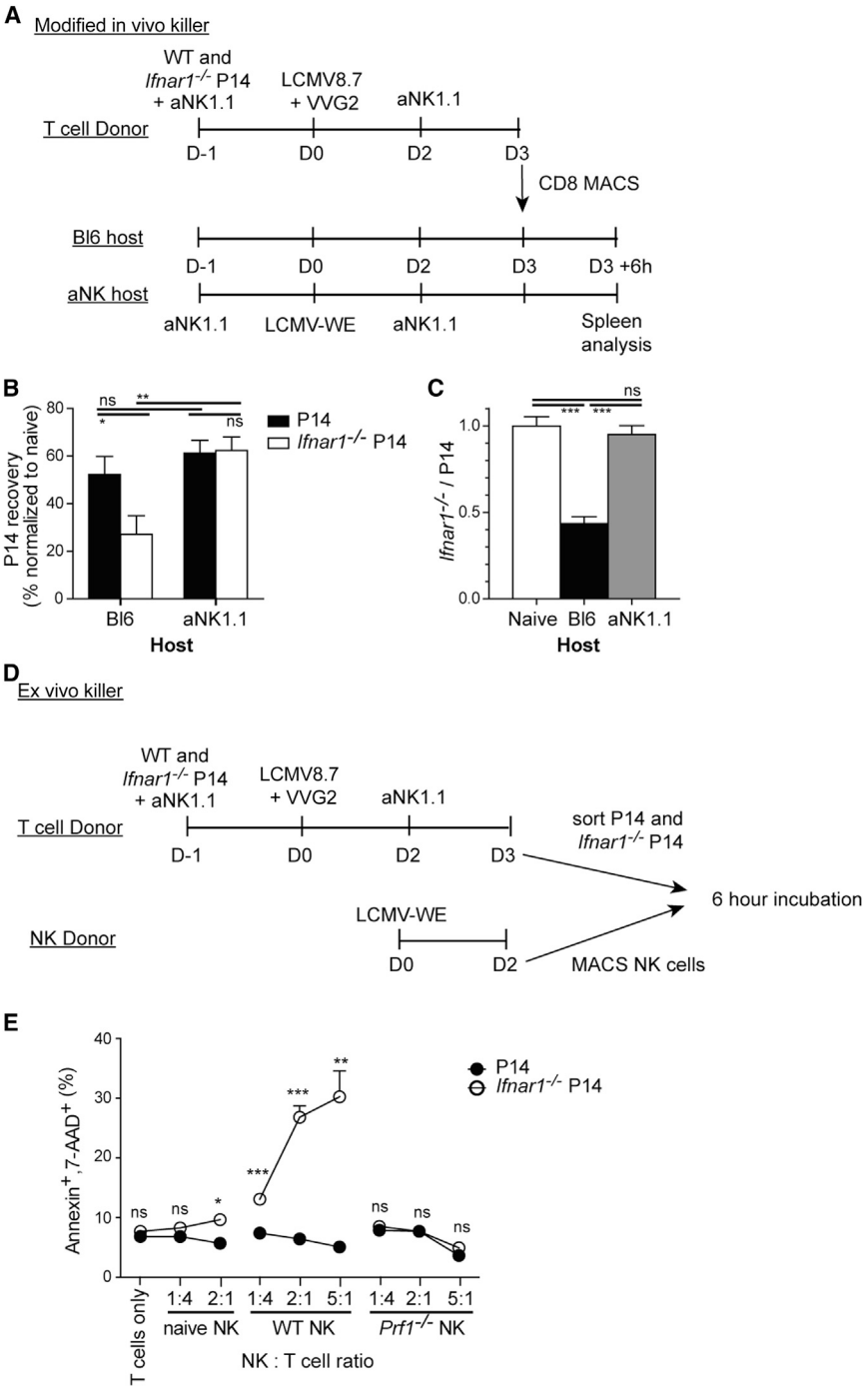

Figure 6. NK Cells Preferentially Kill *Ifnar1*$^{-/-}$ T Cells

(A) Experimental setup of the in vivo killer assay used in (B) and (C). T cell donors were NK cell depleted and cotransferred WT and *Ifnar1*$^{-/-}$ P14 cells followed by LCMV8.7 + VVG2 coinfection. Day 3 p.i. P14 cells were isolated, MACS purified, and transferred into LCMV-WE infection matched undepleted (Bl6) or NK-cell-depleted (aNK1.1) hosts. P14 cell recovery was determined 6 hr later.

(B) WT and *Ifnar1*$^{-/-}$ P14 cell recovery relative to the amount recovered in naive mice. Data shown are mean ± SEM of n = 12 mice pooled from three experiments. ns, not significant; *p < 0.05; **p < 0.01 (unpaired two-tailed t test).

(C) Ratio of *Ifnar1*$^{-/-}$ to WT P14 cells from the indicated hosts, data shown are mean ± SEM of n = 4 mice representative of three independent experiments. ns, not significant; ***p < 0.001 (unpaired two-tailed t test).

(D) Experimental setup of the ex vivo killer assay used in (E). T cell donors were NK cell depleted and cotransferred WT and *Ifnar1*$^{-/-}$ P14 cells followed by LCMV8.7 + VVG2 coinfection. At day 3 p.i. WT (Ly5.1^{+}) and *Ifnar1*$^{-/-}$ (Thy1.1^{+}) cells were sorted to high purity. NK cells were isolated from LCMV-WE infected mice at day 2 p.i. and MACS purified. We placed 2 × 10^{4} WT or *Ifnar1*$^{-/-}$ P14 cells in triplicates together with the indicated amounts of NK cells for 6 hr followed by flow cytometric analysis.

(E) The percentage of apoptotic Annexin V and 7-AAD double positive cells is depicted as percentage of total WT or *Ifnar1*$^{-/-}$ P14 cells. Data shown are mean ± SEM of n = 3 representative from at least three experiments ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001 (unpaired two-tailed t test).

Ifnar1$^{-/-}$ P14 cells were activated in vivo by VVG2 and LCMV8.7 coinfection, to increase T cell recovery. Activated CD8^{+} T cells were isolated at day 3 p.i. and transferred into naive or infection-matched hosts, which had been depleted of NK cells or left untreated (Figure 6A). After 6 hr, spleens from host mice were isolated and P14 cell recovery was analyzed. The number of recovered P14 cells in infected hosts was normalized to naive hosts and the percentage of recovery was determined (Figure 6B). We found a significant reduction in the recovery of *Ifnar1*$^{-/-}$ P14 cells in undepleted hosts compared to WT P14 cells, and this reduced recovery was abolished by NK cell depletion. Also, by examining the ratio of *Ifnar1*$^{-/-}$ to WT P14 cells in the different hosts, we found a specific reduction in *Ifnar1*$^{-/-}$ P14 cells in undepleted hosts, which was abolished by NK cell depletion (Figure 6C), indicating preferential in vivo killing of *Ifnar1*$^{-/-}$ compared to WT P14 cells. Next, we developed an ex vivo killer assay to directly examine the killing of P14 cells by NK cells. WT and *Ifnar1*$^{-/-}$ P14 cells were activated in NK cell depleted mice in vivo by coinfection with VVG2 and LCMV8.7. At day 3 p.i., WT and *Ifnar1*$^{-/-}$ P14 cells were sorted to high purity and placed together with day 2 in vivo LCMV activated NK cells (Figure 6D). Following a 6 hr incubation period, T cell killing was visualized by staining with 7-AAD and Annexin V, where double-positive cells represent late apoptotic cells. We observed a strong increase in the percentage of double-positive *Ifnar1*$^{-/-}$ P14 cells with increasing effector NK to CD8^{+} T cell target ratios (Figure 6E). This increase in apoptotic *Ifnar1*$^{-/-}$ P14 cells was reduced to background amounts in the presence of naive NK cells or

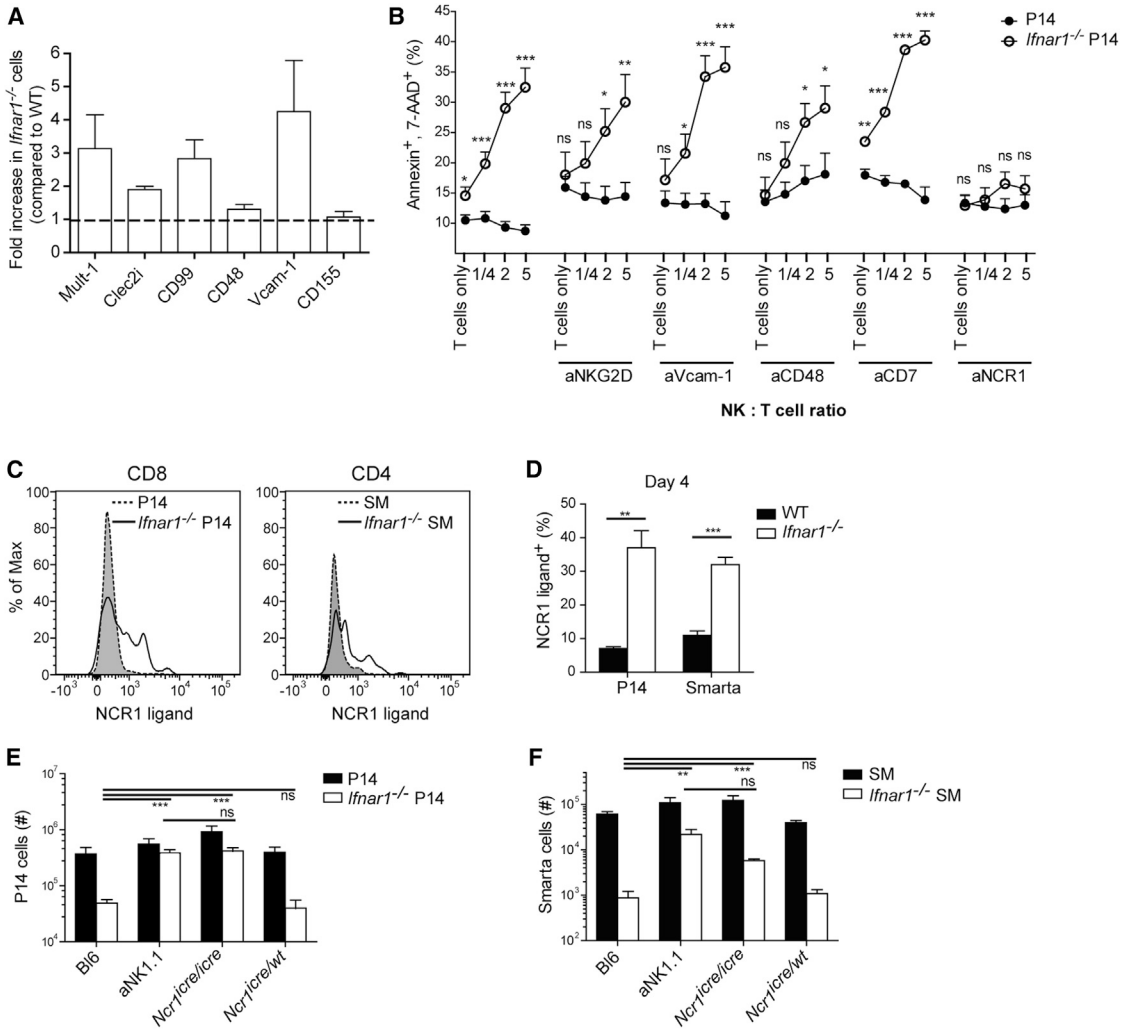

Figure 7. Activated *Ifnar1*$^{-/-}$ CD8^{+} T Cells Are Killed by NK Cells in a NCR1-Dependent Manner

(A) Quantitative real-time PCR of the indicated NK cell activating or inhibitory ligands. RNA was isolated from WT and Ifnar1$^{-/-}$ P14 cells on day 4 post LCMV infection. Shown is the fold increase in expression in *Ifnar1*$^{-/-}$ P14 cells compared to WT P14 cells.

(B) Ex vivo killer assay as described in Figure 6D in combination with blocking reagents for the indicated molecules. Shown is the percentage of Annexin V and 7-AAD double-positive cells among the total P14 or *Ifnar1*$^{-/-}$ P14 cells. Data shown are mean ± SEM of n = 12 pooled from at least three experiments.

(C and D) NCR1 ligand expression on *Ifnar1*$^{-/-}$ and WT P14 and SM cells, stained by NCR1-IgG fusion protein on day 4 post LCMV infection. (C) Overlay histogram of WT (dashed, shaded line) and *Ifnar1*$^{-/-}$ (black line) P14 cells (left) and WT (dashed, shaded line) and *Ifnar1*$^{-/-}$ (black) SM cells (right) stained with 1 μg NCR1-IgG fusion protein. (D) Summary graph of the percentage NCR1 ligand positive P14 and SM cells (C and D) Data shown are mean ± SEM of n = 4 representative from at least three experiments.

(E and F) Cotransfer of WT and *Ifnar1*$^{-/-}$ P14 or SM cells into NCR1 deficient (*Ncr1*$^{iCre/iCre}$), littermate heterozygous controls (*Ncr1*$^{iCre/WT}$), NK1.1-depleted (aNK1.1), or undepleted (Bl6) mice followed by LCMV-WE infection. On day 4 p.i., total cell numbers were determined. (E) Total numbers of P14 cells. Data shown are mean ± SEM of n = 12 mice from three pooled independent experiments. (F) Total numbers of SM cells. Data shown are mean ± SEM of n = 8 mice from two pooled independent experiments. ns, not significant; **p < 0.01; ***p < 0.001 (unpaired two-tailed t test). See also Figure S5.

activated NK cells from *Prf1*$^{-/-}$ mice. As we observed an increase of apoptotic *Ifnar1*$^{-/-}$ P14 cells but no increase in apoptotic WT P14 cells, we concluded that *Ifnar1*$^{-/-}$ P14 cells are specifically rendered susceptible to NK cell mediated killing, whereas T cells that can sense type I IFNs are protected.

Activated *Ifnar1*$^{-/-}$ CD8^{+} T Cells Are Killed by NK Cells in an NCR1-Dependent Manner

Because *Ifnar1*$^{-/-}$ P14 cells were more susceptible to NK-cell-mediated killing, we examined the mechanisms of how *Ifnar1*$^{-/-}$ P14 cells are specifically recognized by NK cells. From the microarray analysis, we found differential regulation of various molecules involved in NK cell activation or inhibition. To reconfirm differential expression of activating or inhibiting ligands for NK cells, we analyzed expression of a selection of ligands by quantitative real-time PCR (Figure 7A). Among the reconfirmed ligands we found a 3-fold upregulation of the NKG2D activating ligand Mult-1 (Carayannopoulos et al., 2002) on *Ifnar1*$^{-/-}$ compared to WT P14 cells. *Ifnar1*$^{-/-}$ P14 cells had a 2-fold upregulation of Clec2i (Clr-g), the activating ligand for NKR-P1F

(Iizuka et al., 2003), and a 3-fold upregulation of CD99, which has been shown to have both activating and inhibitory functions, depending on the receptor it binds to (Shiratori et al., 2004). The inhibitory ligand CD48, which binds 2B4 (Waggoner et al., 2010), was expressed at similar amounts on *Ifnar1*$^{-/-}$ and WT P14 cells. Strongly upregulated on *Ifnar1*$^{-/-}$ compared to WT P14 cells was the adhesion molecule VCAM-1, but no difference was seen in the expression of the activating ligand CD155 (Bottino et al., 2003). Of note, the classical inhibitory ligand, MHC class I, was not differentially expressed on WT or *Ifnar1*$^{-/-}$ P14 cells (data not shown). To investigate a possible role of those activating ligands, we utilized the ex vivo killer assay in combination with various blocking antibodies. Blocking of NKG2D, VCAM-1, CD48, or CD7, a ligand involved in T cell apoptosis (Pace et al., 2000), had no impact on preferential NK-cell-mediated killing of *Ifnar1*$^{-/-}$ compared to WT P14 cells (Figure 7B). Confirming the ex vivo killer analysis, we found no role for NKG2D in vivo when cotransferring WT and *Ifnar1*$^{-/-}$ P14 cells into *Klrk1*$^{-/-}$ mice followed by LCMV infection (data not shown). We found that in vitro blocking of the NK-cell-activating receptor NCR1 led to a complete abrogation of apoptosis induction in *Ifnar1*$^{-/-}$ P14 cells. Consequently, we examined the expression of NCR1 ligands on WT and *Ifnar1*$^{-/-}$ P14 cells at day 4 p.i. by using an NCR1-immunoglobulin G (IgG) fusion protein, recognizing the unknown cellular NCR1 ligands (Mandelboim et al., 2001). NCR1-IgG fusion protein staining revealed increased expression of NCR1 ligands on *Ifnar1*$^{-/-}$ compared to WT P14 cells following LCMV infection, where around 40% of *Ifnar1*$^{-/-}$ cells expressed NCR1 ligands (Figures 7C and 7D). Following infection with VVG2, *Ifnar1*$^{-/-}$ P14 cells expressed NCR1 ligands to comparable amounts as WT P14 cells during LCMV infection (data not shown), indicating that the inflammatory environment associated with VV could possibly compensate for the lack of type I IFN signaling to protect the T cells from expressing NCR1 ligands. Because *Ifnar1*$^{-/-}$ CD4^{+} Smarta cells were also killed in an NK cell-dependent manner, we examined the NCR1 ligand expression on WT and *Ifnar1*$^{-/-}$ SM cells 4 days after LCMV infection. Similar to *Ifnar1*$^{-/-}$ P14 cells, we found an increased expression of NCR1 ligands on *Ifnar1*$^{-/-}$ compared to WT SM cells (Figures 7C and 7D). Additionally, by using *Cd4*^{cre}*Ifnar1*$^{flox/flox}$ mice, which lack *Ifnar1* expression specifically on T cells, we found that endogenous T cells specific for gp33 and np396 also showed an increased expression of NCR1 ligands on their surface at day 7 p.i. (Figure S5A). Furthermore, infection with LCMV8.7 following cotransfer of WT and *Ifnar1*$^{-/-}$ P14 cells revealed that the upregulation of NCR1 ligands on *Ifnar1*$^{-/-}$ P14 cells is antigen dependent, because no increase in the expression of NCR1 ligands in *Ifnar1*$^{-/-}$ P14 cells was seen upon LCMV8.7 infection (Figure S5B).

To functionally test the role of NCR1 in vivo, we cotransferred WT and *Ifnar1*$^{-/-}$ P14 cells into *Ncr1*$^{icre/icre}$ mice, which exhibit impairment of NCR1 expression, followed by LCMV infection. At four days p.i., we found a significant recovery of *Ifnar1*$^{-/-}$ P14 cell expansion compared to the expansion in WT hosts or heterozygous *Ncr1*$^{icre/WT}$ mice, despite the reported hyperactivity of NK cells in *Ncr1*$^{icre/icre}$ mice (Narni-Mancinelli et al., 2012) (Figure 7E). This recovery in expansion of *Ifnar1*$^{-/-}$ P14 cells was comparable to NK-cell-depleted Bl6 hosts, indicating a strong role for NCR1-dependent killing of *Ifnar1*$^{-/-}$ T cells in vivo. Similarly, following transfer of WT and *Ifnar1*$^{-/-}$ SM cells into *Ncr1*$^{icre/icre}$ mice, followed by LCMV infection, we found that the expansion of *Ifnar1*$^{-/-}$ SM cells could also be recovered, similar to NK-cell-depleted Bl6 mice (Figure 7F). Taken together, the recovery of *Ifnar1*$^{-/-}$ P14 and SM cell expansion in NCR1-deficient mice, together with the results from ex vivo NCR1 blocking in NK-cell-mediated T cell killing assays, led us to conclude that activated NK cells recognize *Ifnar1*$^{-/-}$ T cells via increased NCR1 ligand expression, culminating in perforin-dependent killing of T cells that lack the ability to sense type I interferons.

DISCUSSION

This study demonstrates that T cells lacking the ability to sense type I IFNs were highly susceptible to elimination by NK cells during the early phase of LCMV infection. Previous findings have demonstrated that T cells can be recognized and killed by NK cells in a perforin-dependent manner, but the conclusions and mechanisms are controversial. It has been proposed that NK cells can act as rheostats by killing CD4^{+} T cells and thereby indirectly affecting the CD8^{+} T cell response (Waggoner et al., 2012). Other groups have found that CD8^{+} T cells themselves can be direct targets for NK cell mediated killing (Lang et al., 2012; Soderquest et al., 2011). Our results complement these previous findings and indicate that both CD8^{+} and CD4^{+} T cells could be directly killed by NK cells following LCMV infection. Importantly, our results add to these previous observations by revealing a mechanism by which T cells can protect themselves from negative regulation by NK cells via direct sensing of type I IFNs. In line with previous reports, we found that WT T cells were modestly increased following NK cell depletion, whereas the massively curtailed *Ifnar1*$^{-/-}$ T cell expansion was greatly increased (~20 fold) following NK cell depletion.

Previous reports revealed several different mechanisms by which regulation of T cell responses by NK cells can occur. It has been shown that NK cells can have an indirect effect on the T cell response by limiting the availability of APCs, such as during MCMV infection when infected APCs can be directly killed by NK cells (Andrews et al., 2010). However, we found no differences in dendritic cell (DC) numbers or functionality comparing DCs from NK-cell-depleted or undepleted mice (Figure S6). Acting in a direct manner, NK cells have been found to directly recognize T cells via various activating and inhibitory ligands. While some reports indicate a role for NKG2D-dependent recognition and killing of CD8^{+} T cells (Lang et al., 2012; Soderquest et al., 2011), others found no involvement for NKG2D-dependent activation of NK cells (Waggoner et al., 2012). Our findings also indicated no role for NKG2D, despite the detected upregulation of Mult-1 on *Ifnar1*$^{-/-}$ P14 cells. In line with the previous finding that Qa1 expression on T cells can protect them from NK cell mediated killing (Lu et al., 2007), we found a modest increase in expression of Qa1 on WT compared to *Ifnar1*$^{-/-}$ P14 cells by microarray analysis. Whether or not this differential regulation of Qa1 expression on WT and *Ifnar1*$^{-/-}$ T cells has any physiological consequence remains to be addressed. We have found that *Ifnar1*$^{-/-}$ T cells could be specifically recognized and eliminated by NK cells in an NCR1-dependent manner, demonstrating a prominent involvement of NCR1 ligand expression

on T cells as a mechanism by which NK cells could recognize improperly activated T cells and eliminate them. Our data clearly indicate that type I IFN signaling on T cells protects them from NK-cell-mediated killing by regulating the balance of activating and inhibitory signals to NK cells with a dominant contribution of NCR1 ligands.

The finding that NCR1 ligands are upregulated on *Ifnar1*$^{-/-}$ T cells following LCMV infection implies that type I IFNs somehow block the upregulation of NCR1 ligands following activation, implicating the possibility that NCR1 ligand upregulation is a default program induced after T cell activation and that additional cytokine signaling is necessary to block its upregulation. The finding that *Ifnar1*$^{-/-}$ P14 cells did not upregulate NCR1 ligands following VVG2 infection leads to the possibility that the inflammatory environment associated with a VV infection could compensate for the lack of type I IFN signaling and thereby protect T cells from upregulating NCR1 ligands and becoming targets for NK cell killing. Because some redundancy between the signal 3 cytokines IL-12 and type I IFN has been demonstrated for expansion and differentiation of T cells (Curtsinger et al., 2005), it is possible that IL-12 signaling on *Ifnar1*$^{-/-}$ P14 cells following VVG2 infection can suppress the upregulation of NCR1 ligands and thereby protect the activated T cells. However, further studies need to be performed to address this hypothesis.

NCR1 activation on NK cells was shown to play an important role in various viral infections; *Ncr1*$^{-/-}$ mice, despite normal in vitro killing of tumor cells, are more susceptible to lethal influenza infection (Gazit et al., 2006) due to the ability of NK cells to directly recognize and eliminate influenza infected cells (Mandelboim et al., 2001). Furthermore, in humans, low expression of NCR1 has been associated with the persistent infections hepatitis C (Nattermann et al., 2006) and HIV (De Maria et al., 2003), leading to the speculation that hosts might downregulate the expression of NCR1 on NK cells to protect antiviral T cells from elimination in a setting of persistent antigen exposure with low abundance of signal 3 cytokines. It will be interesting to experimentally address such a hypothesis in future studies. Our findings expand on the role of NCR1 activation of NK cells and demonstrate that in addition to its role in direct viral clearance it also has a potent regulatory role on T cell expansion.

Despite the decisive role found for NK cells in the regulation of *Ifnar1*$^{-/-}$ T cell expansion at early time points, at the peak of T cell expansion (day 7) *Ifnar1*$^{-/-}$ T cell numbers were reduced compared to WT T cells, which was independent of NK cell regulation. These findings demonstrate that type I IFN signaling has additional roles in the expansion of T cells. This could be due to type I IFN-dependent regulation of other cytokine receptors such as IL-7R and IL-15R, which both have important roles in T cell survival (Schluns and Lefrançois, 2003). In addition, the inability of *Ifnar1*$^{-/-}$ T cells to differentiate into SLECs can affect the overall clonal burst size, because this population of cells with greater ability to expand (Sarkar et al., 2008) is missing from the *Ifnar1*$^{-/-}$ T cell pool. In line with this, our microarray data indicate that many cellular pathways involved in cell survival and cell division are differentially regulated in *Ifnar1*$^{-/-}$ P14 cells, indicating that type I IFN signaling could imprint WT P14 cells for better survival and expansion. In line with this reasoning, it has been shown that type I IFN is important for T cell survival (Marrack et al., 1999), and both signal 3 cytokines IL-12 and type I IFN have been found to induce chromatin remodeling in CD8$^+$ T cells, leading to a complex gene regulation program that promotes survival and expansion of CD8$^+$ T cells (Agarwal et al., 2009).

In conclusion, we demonstrate that NK-cell-mediated killing of improperly activated T cells is responsible for the abortive expansion of *Ifnar1*$^{-/-}$ T cells early after LCMV infection and also after VSV infection, both infections being dominated by a biased type I IFN milieu. The results lend support to a scenario in which activated T cells need to efficiently safeguard themselves against NK-cell-mediated cytotoxicity, and this protection is afforded by type I IFN-driven inhibition of NCR1 ligand expression.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6, *Prf1*$^{-/-}$ mice (Kägi et al., 1994), P14 transgenic (Ly5.1$^+$) mice (Pircher et al., 1990), *Ifnar1*$^{-/-}$ P14 mice (Thy1.1$^+$), SM transgenic (Ly5.1$^+$) mice (Oxenius et al., 1998), and *Ifnar1*$^{-/-}$ SM mice (Thy1.1$^+$) were housed and bred in specific pathogen-free facilities. *Ncr1*$^{icre/icre}$ genetically targeted mice (Narni-Mancinelli et al., 2012) were generated by breeding *Ncr1*^{icre} mice to homozygosity (Narni-Mancinelli et al., 2011). T-cell-specific *Ifnar1*$^{-/-}$ mice (*Cd4*^{cre}*Ifnar1*$^{flox/flox}$) mice were a kind gift from Professor Ulrich Kalinke. All animals were used at 6 to 12 weeks of age. All animal experiments have been performed in accordance with institutional policies and have been reviewed by the cantonal veterinary office.

Viruses, Infections, and Depletion

The LCMV-WE and the mutant strain LCMV-WE8.7 (LCMV8.7) (Pircher et al., 1990) were provided by Dr. R.M. Zinkernagel (University Hospital, Zurich, Switzerland) and propagated at a low multiplicity of infection on L929 fibroblast cells. Recombinant VSV expressing the LCMV glycoprotein (VSVGP) was grown on L292 cells. Recombinant Vaccinia virus expressing the LCMV glycoprotein (VVG2) was originally obtained from Dr. D.H.L. Bishop (Oxford University) and was grown on BSC40 cells at low multiplicity of infection.

Coinfection of LCMV8.7 and VVG2 was performed by intraperitoneal (i.p.) injection of 1×10^4 ffu LCMV8.7 and 5×10^6 pfu VVG2. Infections with 200 ffu LCMV-WE or 2×10^6 pfu VSVGP were performed intravenously (i.v.). Infection with 5×10^6 pfu VVGP was performed i.p.

Depletion of NK cells with either aNK1.1 (clone PK136, Bio X cell) or anti-Asialo GM1 (wako chem) was performed by i.p injection of 300 μg aNK1.1 or 10 μl anti-Asialo GM1 1 day before and after infection.

Adoptive Transfer

CD8$^+$ and CD4$^+$ T cells were isolated from naive P14 or SM (Ly5.1$^+$) or *Ifnar1*$^{-/-}$ P14 or SM (Thy1.1$^+$) mice using anti-CD8 or anti-CD4 beads (Miltenyi Biotech) and adoptively cotransferred into naive recipient mice. To study the functionality of memory P14 cells, we isolated lymphocytes 80 days p.i. and purified a total of 10^6 CD8$^+$ T cells by anti-CD8 MACS beads and transferred them into naive recipient mice. Prior to transfer, the frequency of memory WT and *Ifnar1*$^{-/-}$ P14 cells among the total CD8$^+$ T cells was determined by flow cytometry.

Flow Cytometry

Staining was performed on whole-blood or single-cell suspensions from the indicated organs. All fluorescent antibodies used were purchased from BioLegend, eBioscience, or BD Biosciences. NCR1-IgG fusion protein was a kind gift from Professor Mandelboim and was described previously (Mandelboim et al., 2001). Data were acquired on a LSRII flow cytometer (BD Bioscience) and analyzed with Flowjo software (Treestar).

Microarray

WT and *Ifnar1*$^{-/-}$ P14 T cells were isolated from day 3 LCMV8.7 + VVG2 co-infected mice that were undepleted or depleted of NK cells. T cells were sorted to purity by flow cytometry. Microarray analysis was performed by Miltenyi Biotec and data analysis was done in collaboration with the Vital-IT

Group, SIB Swiss Institute of Bioinformatics (Supplemental Experimental Procedures).

In Vivo Killer Assay

Activated T cells were isolated from VVG2 and LCMV8.7 coinfected mice following adoptive cotransfer of 10^6 WT and *Ifnar1*$^{-/-}$ P14 cells at day 3 p.i. CD8$^+$ T cells were MACS purified and adoptively transferred into naive or infection-matched hosts. Following 6 hr incubation, spleens were isolated and analyzed by flow cytometry. The recovery of P14 cells from infected hosts was normalized to the number recovered in the naive hosts.

Ex Vivo Killer Assay

Activated NK cells were isolated from day 2 post LCMV (10^6 ffu)-infected mice by DX5 MACS bead purification. NK cells were added to round bottom 96 well plates together with the indicated blocking antibodies (Supplemental Experimental Procedures). Activated T cells were isolated from VVG2 and LCMV8.7 coinfected mice following adoptive transfer of WT and *Ifnar1*$^{-/-}$ P14 cells at day 3 p.i. Activated T cells were FACS sorted to purity based on the expression of CD8 and Ly5.1 (WT) or Thy1.1 (*Ifnar1*$^{-/-}$ P14). We added 2×10^4 purified P14 or *Ifnar1*$^{-/-}$ T cells to the 96 well plates separately. Following a 6 hr incubation, T cells were stained with 7-AAD and Annexin V according to manufactures recommendation.

Quantitative Real-Time PCR

Total RNA was isolated from sorted P14 T cells 3 days after LCMV8.7 + VVG2 coinfection of NK-cell-depleted mice. RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions, and cDNA was reverse transcribed with M-MLV reverse transcriptase RNase, H minus (Promega). Real-time PCR was performed with Rotorgene 3000 (Corbett). qPCR was performed with TaqMan probes for the indicated genes together with GAPDH as internal control. Analysis was performed by determining the Ct value for each sample normalized to GAPDH (ΔCt). Fold expression was then calculated with the ΔΔCt method, briefly ΔCt from *Ifnar1*$^{-/-}$ P14 cells was subtracted from the ΔCt of WT P14 cells giving a ΔΔCt. Fold induction was calculated with the Equation $2^{-\Delta\Delta Ct}$.

Statistical Analysis

Statistical significance was determined by a two-tailed unpaired t test with GraphPad Prism.

ACCESSION NUMBER

The microarray data discussed in this paper have been deposited in NCBI's Gene Omnibus and are accessible through the GEO accession number GSE57355 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57355).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.05.003.

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