Immunity
Article

CellPress

# Type I Interferon Protects Antiviral CD8+ T Cells from NK Cell Cytotoxicity

Haifeng C. Xu,[1,2,10] Melanie Grusdat,[1,10] Aleksandra A. Pandyra,[2] Robin Polz,[1] Jun Huang,[1] Piyush Sharma,[2] René Deenen,[3] Karl Köhrer,[3] Ramtin Rahbar,[4] Andreas Diefenbach,[5] Kathrin Gibbert,[6] Max Löhning,[7,8] Lena Höcker,[9] Zoe Waibler,[9] Dieter Häussinger,[1] Tak W. Mak,[4] Pamela S. Ohashi,[4] Karl S. Lang,[2,11] and Philipp A. Lang[1,11,*]
[1]Department of Gastroenterology, Hepatology, and Infectious Diseases, Heinrich-Heine-University Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany
[2]Institute of Immunology, Medical Faculty, University of Duisburg-Essen, Hufelandstrasse 55, Essen 45147, Germany
[3]Biological and Medical Research Center (BMFZ), Cluster of Excellence on Plant Sciences (CEPLAS), Heinrich-Heine-University, Moorenstrasse 5, Düsseldorf D-40225, Germany
[4]Campell Family Institute for Breast Cancer Research, Princess Margaret Cancer Center, University Health Network (UHN), 620 University Avenue, Toronto, Ontario M5G 2C1, Canada
[5]Institute of Medical Microbiology and Hygiene, University of Mainz Medical Center, Obere Zahlbacher Strasse 67, 55131 Mainz, Germany
[6]Institute of Virology, University Hospital of Essen, University of Duisburg-Essen, Essen 45147, Germany
[7]Experimental Immunology, Department of Rheumatology and Clinical Immunology, Charité University Medicine Berlin, 10117 Berlin, Germany
[8]German Rheumatism Research Center (DRFZ), a Leibniz Institute, 10117 Berlin, Germany
[9]Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, 63225 Langen, Germany
[10]Co-first author
[11]Co-senior author
*Correspondence: philipp.lang@med.uni-duesseldorf.de
http://dx.doi.org/10.1016/j.immuni.2014.05.004


## SUMMARY

**Despite development of new antiviral drugs, viral infections are still a major health problem. The most potent antiviral defense mechanism is the innate production of type I interferon (IFN-I), which not only limits virus replication but also promotes antiviral T cell immunity through mechanisms, which remain insufficiently studied. Using the murine lymphocytic choriomeningitis virus model system, we show here that IFN-I signaling on T cells prevented their rapid elimination in vivo. Microarray analyses uncovered that IFN-I triggered the expression of selected inhibitory NK-cell-receptor ligands. Consequently, T cell immunity of IFN-I receptor (IFNAR)-deficient T cells could be restored by NK cell depletion or in NK-cell-deficient hosts (*Nfil3*$^{-/-}$). The elimination of *Ifnar1*$^{-/-}$ T cells was dependent on NK-cell-mediated perforin expression. In summary, we identified IFN-I as a key player regulating the protection of T cells against regulatory NK cell function.**


## INTRODUCTION

More than 500 million people worldwide suffer from hepatitis B virus (HBV) or hepatitis C virus (HCV) infections (Rehermann, 2013; Rehermann and Nascimbeni, 2005). Both virus infections can lead to chronic disease, resulting in chronic liver inflammation, tissue damage, end-stage liver failure, and hepatocellular carcinoma (Rehermann and Nascimbeni, 2005). A critical defense mechanism to effectively eradicate viral infections is type I interferon (IFN-I) production by innate immune cells such as plasmacytoid dendritic cells (pDCs) (Gilliet et al., 2008). IFN-I binds to the IFN-I receptor (IFNAR) and not only limits viral replication (Müller et al., 1994) but also exhibits regulatory functions during immunity by activating other immune cells (González-Navajas et al., 2012; Sadler and Williams, 2008). Earlier reports indicate that IFN-I exhibits inhibitory effects on T cells (Petricoin et al., 1997). This can be explained by IFNAR signaling through the signal transducer and activator of transcription 1 (STAT-1), which blunts T cell effector function, while the competing STAT4 signaling is essential to promote cytokine expression of antiviral T cells following IFNAR activation (Nguyen et al., 2002). Conversely, IFNAR can activate antigen-presenting cells (APCs), thus promoting T cell activation during viral infection (Gautier et al., 2005; Santini et al., 2000). Furthermore, studies using IFNAR-deficient T cells show direct effects of IFN-I on T cell survival (Aichele et al., 2006; Kolumam et al., 2005; Le Bon et al., 2006). Consistently, IFN-I is a critical signal for effector and memory T cell differentiation (Agarwal et al., 2009; Marshall et al., 2010). However, the underlying mechanisms by which IFN-I affects T cell immunity are not completely understood.

Natural killer (NK) cells can be activated by IFN-I during viral infection (Biron et al., 2002) and exhibit cytotoxicity triggered by the molecule perforin (Kägi et al., 1994). While there is ample evidence demonstrating that NK cells are important for immunity against a variety of viral infections, more recent studies indicate that NK cells can produce immunoregulatory molecules such as interleukin-10 (IL-10) following activation (Lee et al., 2009; Su et al., 2001). Consequently, recent reports indicate that NK cells contribute to antiviral T cell dysfunction during chronic viral infection (Cook and Whitmire, 2013; Lang et al., 2012; Waggoner

CrossMark

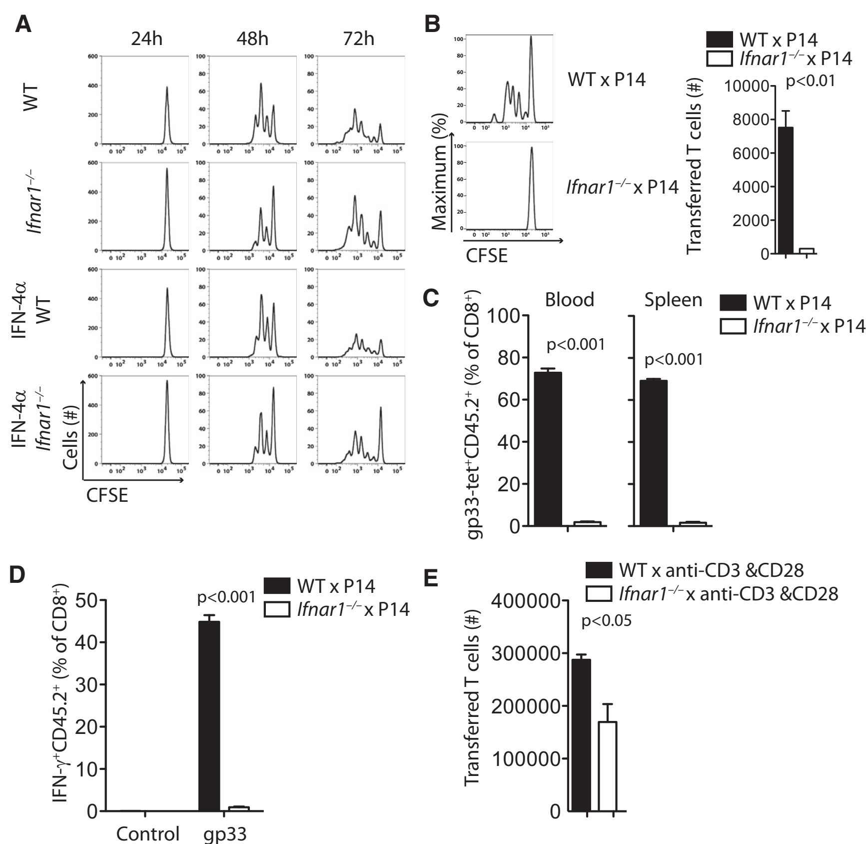



**Figure 1. IFN-I Affects T Cell Immunity In Vivo**

(A) Negatively sorted CFSE labeled $CD8^+$ T cells from WT and IFNAR1-deficient animals were stimulated for 24 hr (left panels), 48 hr (middle panels), and 72 hr (right panels) with anti-CD3 antibody in presence or absence of IFN-4α (50U/mL, one representative of n = 6 is shown).

(B) $CD45.1^+$ animals were infected with 200 pfu LCMV WE. $10^6$ negatively sorted CFSE labeled T cells from $P14^+$ WT and *Ifnar1*$^{-/-}$ mice were transferred into infected $CD45.1^+$ mice 2 days postinfection (p.i.). At day 4 p.i., CFSE expression on T cells (left panel) and cell number of transferred cells was analyzed (right panel, error bars show SEM; n = 3, one of two independent experiments is shown).

(C and D) Prior to infection, $10^5$ negatively sorted T cells from $P14^+$ or $P14^+$*Ifnar1*$^{-/-}$ animals were transferred into $CD45.1^+$ mice. (C) Gp33-$tetramer^+CD8^+CD45.2^+$ T cells were determined in the blood (left panel) and in spleen tissue (right panel) 8 days p.i. (percentage of $CD8^+$ cells, error bars show SEM; n = 5) (D) IFN-$\gamma^+CD8^+CD45.2^+$ cells were measured after restimulation with the LCMV epitope gp33 8 days p.i. (percentage of $CD8^+$ cells, error bars show SEM; n = 5).

(E) We stimulated 2 × $10^6$ negatively sorted $CD8^+$ T cells from WT and *Ifnar1*$^{-/-}$ mice in vitro with anti-CD3 and anti-CD28 antibodies for 72 hr followed by injection into $CD45.1^+$ animals. Two days following infection with 200 pfu of LCMV WE, transferred T cells were measured in spleen tissue (error bars show SEM; n = 3–4, one of two independent experiments is shown).

et al., 2012). These data are accompanied by clinical findings showing that in cohorts of HCV patients, expression of NK cell inhibitory receptors correlates with virus control (Khakoo et al., 2004; Knapp et al., 2010; Paladino et al., 2007). Consistently, NK cell cytotoxicity can be detected in human HCV patients when compared to healthy controls (Ahlenstiel et al., 2010). Moreover, after treatment of HCV infected individuals with IFN-I, NK cell cytotoxicity increases (Edlich et al., 2012). However, the effectiveness of IFN-I therapy correlates positively with NK cell cytotoxicity after IFN-I treatment (Edlich et al., 2012). Furthermore, patients with enhanced NK cell cytotoxicity show a sustained suppression of HCV during IFN-I treatment (Oliviero et al., 2013). These data argue against an influence of regulatory NK cell functions during HCV infection and rather indicate a beneficial role of NK cells during HCV infection. However, the role of regulatory factors, which can influence the interaction between NK cells and T cells, remains still unclear.

Here we show that IFN-I can improve T cell immunity by protecting antiviral T cells from regulatory NK cell functions. IFN-I prevented rapid elimination of antiviral T cells during viral infection in vivo. Microarray analyses uncovered that IFN-I triggered expression of selective NK cell inhibiting ligands on T cells. Consequently, NK-cell-deficient animals were able to mount a functional antiviral T cell response—even in absence of IFN-I signaling in these T cells. The regulatory effects of NK cells were dependent on perforin expression in vitro and in vivo.

## RESULTS

### IFN-I Deficiency Results in Elimination of Proliferating T Cells In Vivo

IFN-I is a critical factor that triggers T cell immunity in vivo (Aichele et al., 2006; Kolumam et al., 2005). However, when negatively sorted T cells from wild-type (WT) and IFN-I receptor-deficient (*Ifnar1*$^{-/-}$) mice (Müller et al., 1994) were stimulated with anti-CD3 in presence or absence of IFN-I, no difference was detected in the proliferation rate of T cells (Figure 1A; see also Figure S1A available online). When we transferred negatively sorted CFSE-labeled T cells from a mouse carrying the transgenic TCR (P14) recognizing the lymphocytic choriomeningitis virus (LCMV) peptide gp33 (Pircher et al., 1989) into WT animals following infection with LCMV, we observed as expected, proliferation of the transferred WT T cells (Figure 1B). In sharp contrast to WT T cells, IFNAR-deficient virus-specific T cells were rapidly eliminated after transfer into WT animals followed by infection with LCMV WE (Figure 1B). Transfer of IFNAR-deficient cells resulted in the absence of functional antiviral T cells during the course of infection, whereas WT T cells were readily detectable in infected animals (Figure 1C). Consequently, restimulation with the virus-specific epitope gp33 induced cytokine production in WT T cells, whereas there was almost no IFN-γ production detectable in *Ifnar1*$^{-/-}$ T cells (Figure 1D). The transferred T cells were eliminated from their host after activation, but naive T cells from both WT and IFNAR deficient animals were

detectable in naive animals throughout the course of the experiment (Figures S1B and S1C). Furthermore, nonspecific viral infection such as with the cytolytic vesicular stomatitis virus did not result in diminished quantity of *Ifnar1*$^{-/-}$ T cells, when they were not activated (Figure S1D). In addition, when we transferred WT and *Ifnar1*$^{-/-}$ P14 cells expressing the congenical markers CD45.1 and CD90.1, respectively, we observed similar numbers in the bloodstream (Figure S1E). Moreover, when we transferred bone marrow harvested from WT and *Ifnar1*$^{-/-}$ animals at a ratio of 1:1 into lethally irradiated mice, we observed no difference in CD8$^{+}$ T cell numbers (Figure S1F). Next, we transferred in vitro proliferated T cells into mice followed by infection with LCMV. Consistently, we observed removal of proliferated *Ifnar1*$^{-/-}$ T cells when compared to WT controls (Figure 1E). These data indicate that absence of IFN-I signaling might protect T cells from negative regulators of T cell immunity in vivo.

### IFN-I Triggers Expression of Genes Encoding for Major Histocompatibility Complex Class I and MHC Ib Molecules on T Cells

To further investigate the mechanism by which T cells show sustained effector function through IFN-I signaling, we performed microarray analyses on negatively sorted primary CD8$^{+}$ T cells. T cells were activated with an anti-CD3 antibody in the presence or absence of IFN-I (500 U/mL IFN-4α). IFN-I regulated 1162 genes in naive T cells, while 270 genes were differentially expressed in anti-CD3 stimulated T cells after IFNAR activation (Figure 2A; Figure S2A; Table S1). As expected, expression of interferon-stimulated genes (ISGs) was significantly higher after treatment with IFN-I ($p_{(corr)} < 0.05$) so that expression changes in IFN-treated cells were clustered independently of anti-CD3 stimulation (Figure S2B). Further on, differentially expressed IFN-I responsive genes were globally categorized into functional GeneOntology (GO) classes. Among others, several IFN-related GO classes showing significant enrichment of differentially expressed genes could be identified (Figure 2B). Becuase no major differences were observed during T cell proliferation after IFN-4α treatment in vitro, we speculated that plasma membrane proteins might play a critical role during the protection of virus specific T cells in vivo. Among genes encoding for plasma membrane proteins, genes encoding for MHC I or MHC Ib molecules were upregulated following IFN-I treatment (Figures 2C and 2D). Taken together, these data indicate that among genes regulated by IFN-I, expression of genes encoding for MHC I and MHC Ib molecules was increased.

### NK-Cell-Receptor Ligand Expression Is Triggered by IFN-I

H2 proteins are part of MHC I and MHC Ib molecules, which can act as ligands of NK cell inhibitory receptors (Vivier et al., 2011; Vivier et al., 2008). Expression patterns of samples obtained from IFN-I-treated cells clustered when H2 and other NK-cell-receptor ligands were analyzed, indicating that IFN-I might trigger the expression of NK-cell-receptor ligands (Figure 2D). Validation of the genes encoding for NK-cell-receptor ligands uncovered that expression of several genes were significantly elevated in response to IFN-I treatment on T cells (Figure 3A). Although NKG2D ligands were significantly increased after CD3 stimulation, their expression was not affected by IFN-I in these experiments (Figures S3A and S3B). We also tested other NK-cell-receptor ligands, but did not identify statistically different expression in T cells in this setting, neither during anti-CD3 stimulation nor during incubation with IFN-4α (Figures S3C–S3G). Next, we analyzed whether MHC I and Qa-1b protein expression was affected following IFN-I treatment. Consistent with our gene-expression analyses, Qa-1b expression was induced on the cell surface of CD8$^{+}$ T cells after stimulation with anti-CD3 (Figure 3B). However, coincubation with IFN-4α led to further increase of Qa-1b expression in a concentration-dependent manner (Figure 3B). Moreover, MHC I expression was significantly increased on WT CD8$^{+}$ T cells when compared to *Ifnar1*$^{-/-}$ cells (Figures 3C and 3D). These data indicate that NK-cell-receptor ligands are regulated by IFN-I and that absence of IFNAR results in limited expression of the inhibitory NK-cell-receptor ligands MHC I and Qa-1b in vitro.

### NK Cells Target IFNAR1-Deficient T Cells

Our data indicate that the expression of NK-cell-receptor ligands are affected by IFN-I. We next hypothesized that regulatory NK cell functions might target antiviral T cells in the absence of IFN-I. Moreover, when we transferred in vitro stimulated T cells from WT and *Ifnar1*$^{-/-}$ animals into LCMV-infected WT hosts, we observed that absence of IFNAR1 resulted in decreased expression of Qa-1b (Figure 4A). Consistently, LCMV-specific T cells from WT mice expressed Qa-1b on the cell surface when transferred into infected C57BL/6 mice. In the absence of IFN-I signaling however, Qa-1b expression was reduced when compared to WT P14 (Figure 4B; Figure S4A). Similar results were obtained when MHC I expression was determined (Figure 4C; Figure S4B). When we transferred T cells into naive animals, we did not observe any expression differences between WT and IFNAR1-deficient T cells (Figures 4B and 4C). Intermediate doses of LCMV infection triggers T cell dysfunction of antiviral T cells (Lang et al., 2012). As shown by others and us, NK cell depletion can rescue T cell function with higher doses of LCMV (Figure S4C) (Cook and Whitmire, 2013; Lang et al., 2012; Waggoner et al., 2012). After NK cell depletion, LCMV-specific T cells showed reduced expression of Qa-1b, suggesting that low Qa-1b correlates with elimination of anti-viral T cells (Figure S4D). Following their transfer, negatively sorted *Ifnar1*$^{-/-}$ P14 were rapidly deleted from infected animals. However, after depletion of NK cells (Koo and Peppard, 1984), the numbers of IFNAR1-deficient T cells were increased (Figure 4D). Next we asked whether NK cell depletion might affect prolonged antiviral T cell immunity of *Ifnar1*$^{-/-}$ T cells. After NK cell depletion, *Ifnar1*$^{-/-}$ P14$^{+}$ T cells were detectable in increased numbers 8 days after infection when compared to control animals (Figure 4E). Furthermore, the T cell response was restored in *Nfil3*$^{-/-}$ mice. *Nfil3*$^{-/-}$ mice lack NK cell function but exhibit normal NKT cell development when compared to WT controls (Gascoyne et al., 2009; Kamizono et al., 2009). Consequently, cytokine production by IFNAR1-deficient T cells was highly increased when compared to their corresponding controls, in the absence of NK cells, either eliminated by NK cell depletion or reduced in number and function in *Nfil3*$^{-/-}$ animals (Figure 4F). These data indicate that NK cells target antiviral T cells in absence of IFN-I signaling. Furthermore, these effects were

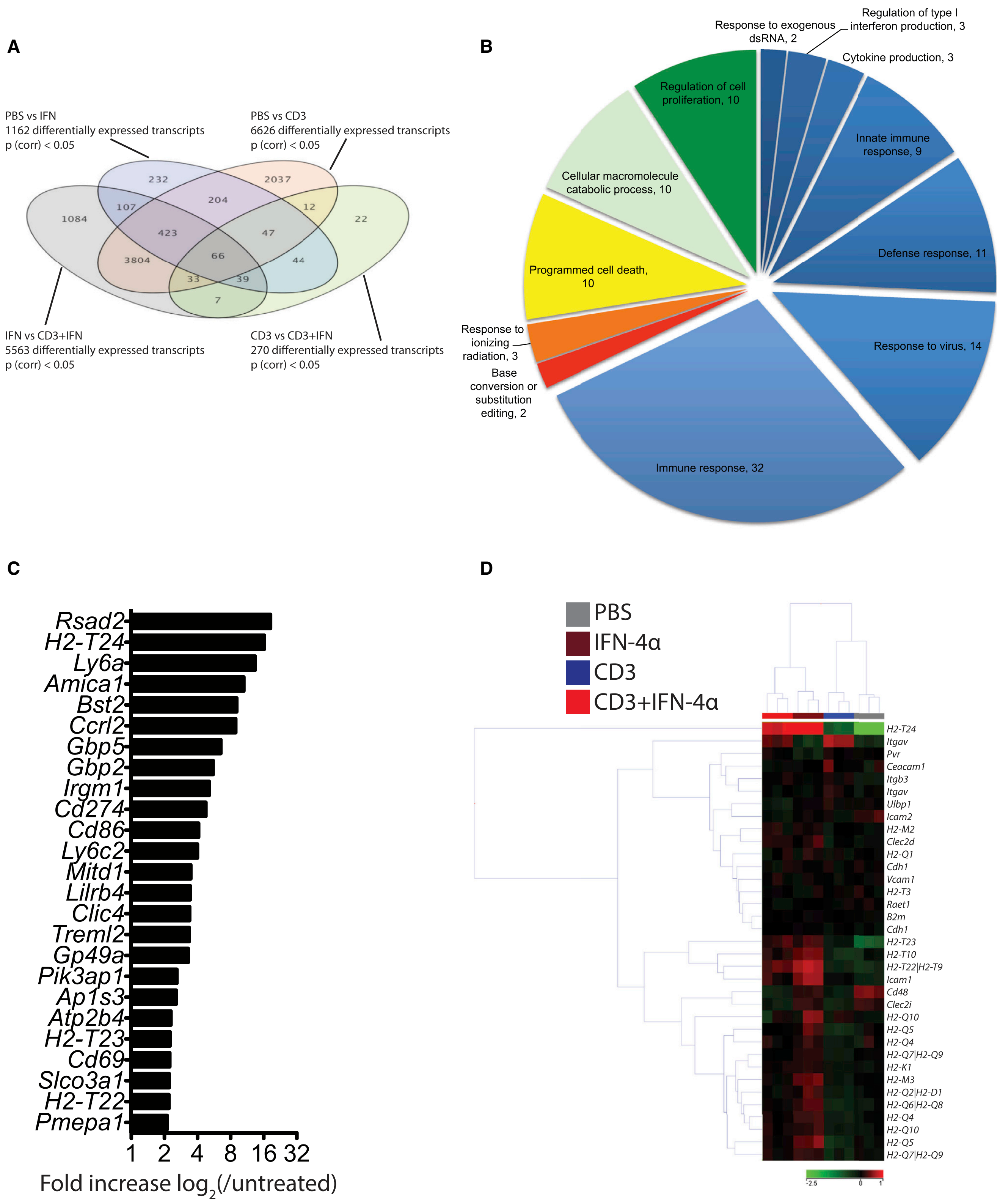



**Figure 2. IFN-I Triggers Expression of Genes Encoding for Major Histocompatibility Complex Class I and MHC Ib Molecules on T Cells**

(A–C) We stimulated $10^6$ negatively sorted $CD8^+$ T cells with anti-CD3 antibodies in the presence or absence of IFN-4α (500 U/mL). After 6 hr, total RNA was extracted from cell suspensions and microarray analyses were performed (n = 3). (A) Venn diagram of genes regulated by IFN-4α treatment, CD3 stimulation, and

(legend continued on next page)

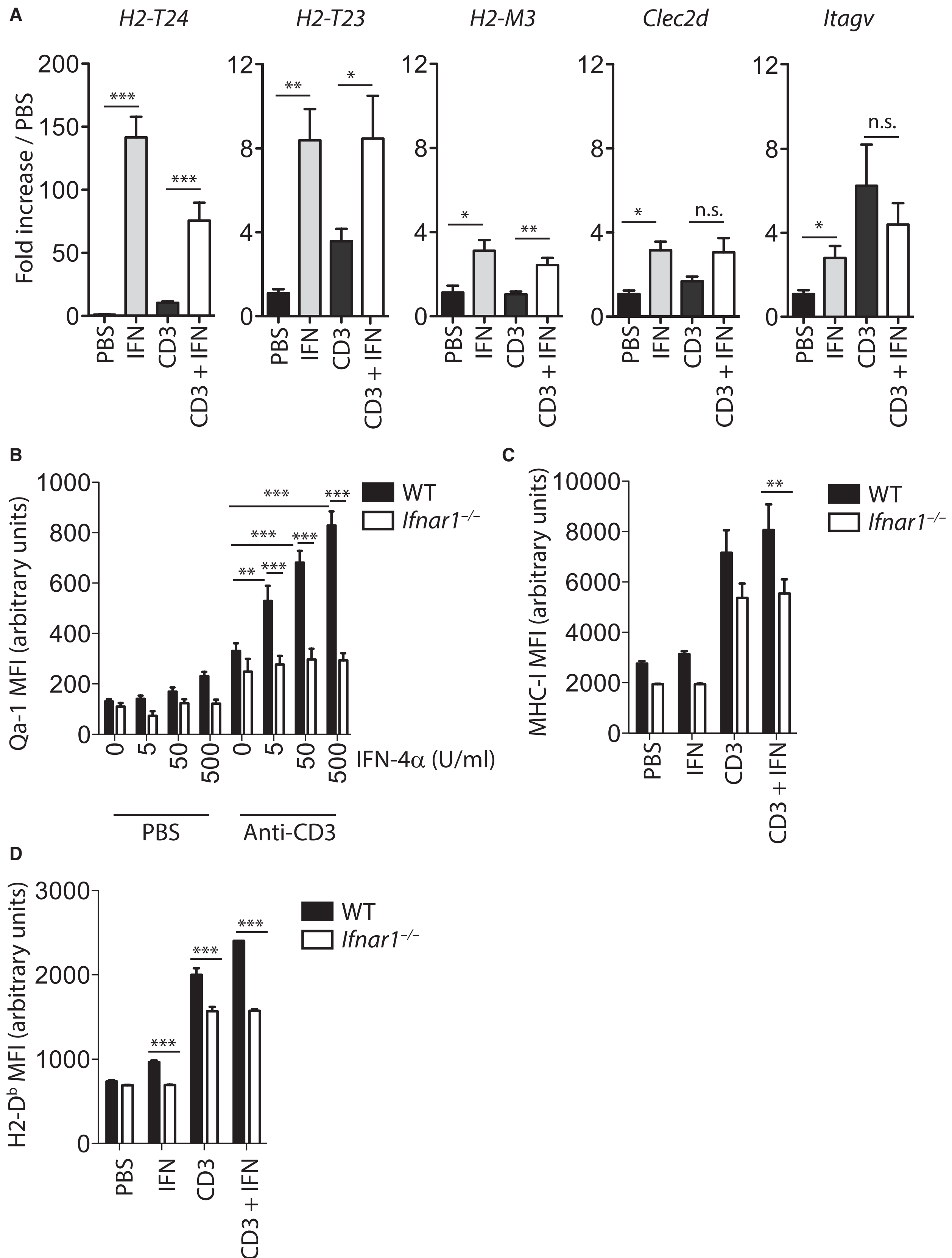
rather dependent on NK cell function, because in animals with competent NKT cell responses but deficient for NK cells, *Ifnar1*$^{-/-}$ T cells were recovered. We wondered whether similar effects could be observed with CD4$^{+}$ T cells. Consistent with the described data, *Ifnar1*$^{-/-}$Smarta$^{+}$ T cells, which express a TCR specific for the LCMV MHC II epitope gp61 as a transgene (Oxenius et al., 1998), were eliminated by NK cells following infection with LCMV (Figures S4E and S4F). Moreover, cytokine production of IFNAR1-deficient antiviral CD4$^{+}$ T cells could be increased in absence of NK cells (Figure S4G).

Next, we wondered whether lack of IFNAR1 phenotypically affected T cell immunity when compared to WT T cells, and whether NK cell depletion could completely rescue *Ifnar1*$^{-/-}$ T cell immunity. When we monitored transferred WT and *Ifnar1*$^{-/-}$ T cells over time, we found that removal of IFNAR1-deficient T cells was mainly dependent on the presence of NK cells and that *Ifnar1*$^{-/-}$ T cell immunity was similar to WT T cell immunity in absence of NK cells (Figure 5A). Consistently, NK cell depletion also improved the cytokine production of IFNAR1-deficient T cells (Figure 5B). IFN-$\gamma$, tumor necrosis factor alpha (TNF-$\alpha$), and interleukin-2 (IL-2) production by *Ifnar1*$^{-/-}$ T cells was rescued in absence of NK cells (Figure 5B). In line with this, lysosomal-associated membrane protein-1 (Lamp-1) staining, indicating degranulation, of *Ifnar*$^{-/-}$ T cells was also restored after NK cell depletion (Figure 5B). However, we still observed significant differences in cytokine production between WT T cells and *Ifnar1*$^{-/-}$ T cells in NK-cell-depleted animals (Figure 5B). Furthermore, we observed higher expression of PD-1 and lower expression of KLRG1 in *Ifnar1*$^{-/-}$ T cells when compared to WT T cells (Figure 5C; Figure S5A). This differential expression of PD-1 was independent of the presence of NK cells and could potentially contribute to the deficient T cell immunity in absence of IFN-I (Barber et al., 2006). The differential expression of KLRG1 suggests an additional role of IFN-I during antiviral T cell differentiation (Gründemann et al., 2010; Kaech and Cui, 2012; Kaech et al., 2003). Expression of other factors involved in regulating antiviral CD8$^{+}$ T cell immunity such as IL-7R or TIM-3 (Jin et al., 2010; Jones et al., 2008; Kaech et al., 2003; Wherry, 2011) were similar or only slightly different between *Ifnar1*$^{-/-}$ and WT T cells, when NK cells were depleted (Figure 5C; Figure S5A). Consistently, when we determined viral titers in spleen tissue, we observed higher titers in animals that received *Ifnar1*$^{-/-}$ T cells (Figure 5D). However, after NK cell depletion, viral titers were reduced, indicating that in absence of NK cells, IFNAR1-deficient T cells were capable of eliminating virus-infected cells (Figure 5D). At later time points, the virus was cleared from the animals (Figure S5B). When we measured the IFN-$\alpha$ concentration, we did not observe any differences shortly after infection (Figure S5C). At day 4 however, serum IFN-$\alpha$ concentration in animals receiving *Ifnar1*$^{-/-}$ T cells were significantly higher than in other mice (Figure S5C), and this is likely attributable to the higher viral titers due to dysfunctional T cell immunity (Figures 5A–5D). To investigate whether these effects affected the interaction of NK cells with T cells in an IFN-I-dependent manner, we transferred negatively sorted T cells from WT and *Ifnar1*$^{-/-}$ mice into the same hosts. Consistent with our previous findings, we observed higher expression of PD-1 and lower expression of KLRG1 in *Ifnar1*$^{-/-}$ T cells, independent of the presence of NK cells (Figure S5D). However, when we measured the number of transferred T cells, we detected higher T cell numbers of *Ifnar1*$^{-/-}$ T cells in the absence of NK cells when compared to animals with NK cells (Figure S5E). Furthermore, in line with the differential PD-1 and KLRG1 expression, we detected a significant difference between WT and *Ifnar1*$^{-/-}$ T cells even in absence of NK cells (Figure S5E). However, NK cells seemed to be a critical regulator of T cell immunity in the absence of IFN-I receptor signaling. Notably, NK cells can also affect T cell immunity by regulating antigen-presenting cells (Andrews et al., 2010). In our setting, however, selective increase in *Ifnar1*$^{-/-}$ T cells rather points to a direct interaction between IFNAR1-deficient T cells and NK cells. To further evaluate whether the observed effects could have been cofounded by other factors, we used *Ifnar1*$^{\text{loxp/loxp}}$ × *Cd4-cre*$^{+}$ animals (Kamphuis et al., 2006). Similarly, T cell immunity was impaired in animals, which lack IFNAR1 signaling in T cells (Figure 5E). However, in the absence of NK cells, antiviral T cells could be recovered in *Ifnar1*$^{\text{loxp/loxp}}$ × *Cd4-cre*$^{+}$ animals (Figure 5E). Taken together, these data indicate that NK cells are critical regulators of T cells deficient in IFN-I signaling in vivo.

## NK Cells Target *Ifnar1*$^{-/-}$ T Cells through Perforin-Mediated Cytotoxicity

To evaluate whether NK cells target T cells directly, we coincubated sorted WT and *Ifnar1*$^{-/-}$ T cells from infected animals with cultured NK cells. NK cells selectively eliminated *Ifnar1*$^{-/-}$ T cells while WT T cells were protected from NK-cell-mediated killing (Figure 6A). Furthermore, NK cells selectively induced apoptosis in T cells deficient for IFNAR1 when compared to WT T cells (Figure 6A). During LCMV infection, IFN-I can activate NK cell cytotoxicity, which is mediated by perforin (Kägi et al., 1994). While perforin expression of CD8$^{+}$ T cells is critical to eliminate LCMV from the organism, NK-cell-mediated cytotoxicity does not influence early virus distribution or elimination during LCMV infection (Lang et al., 2012). To investigate whether perforin expression by NK cells affected *Ifnar1*$^{-/-}$ T cells, we coincubated WT and *Ifnar1*$^{-/-}$ T cells with perforin-deficient NK cells. Cell number and cell viability of WT T cells was similar when the cells were incubated with WT NK cells or perforin-deficient NK cells. However, *Ifnar1*$^{-/-}$ T cell numbers and cell viability were significantly reduced when incubated with WT NK cells in contrast to perforin-deficient NK cells (Figure 6A), indicating that the interaction between NK cells and T cells was dependent on perforin expression in vitro. Next, we performed experiments with RMA-s and RMA cell lines, which exhibit differential MHC I expression (Figure S6A). As expected, RMA-s cells, which have lower MHC I expression, were highly susceptible toward NK-cell-mediated killing (Figure S6B). Furthermore, the killing of RMA-s cells was dependent on perforin expression (Figure S6B).

CD3 stimulation in combination with IFN-4$\alpha$ treatment. (B) Pie diagram of significantly enriched gene ontology clusters ($p < 0.1$) after treatment of CD8$^{+}$ T cells with IFN-I (amount of genes regulated within the cluster regulated is shown). (C) Change of gene expression (IFN-4$\alpha$ against PBS) of loci encoding for plasma membrane proteins, which were significantly regulated by IFN-I treatment ($p_{(corr)} < 0.05$).
(D) Cluster of gene expression of genes encoding for MHC I and MHC Ib molecules and encoding for proteins involved in NK cell regulation are shown.




(legend on next page)

CellPress

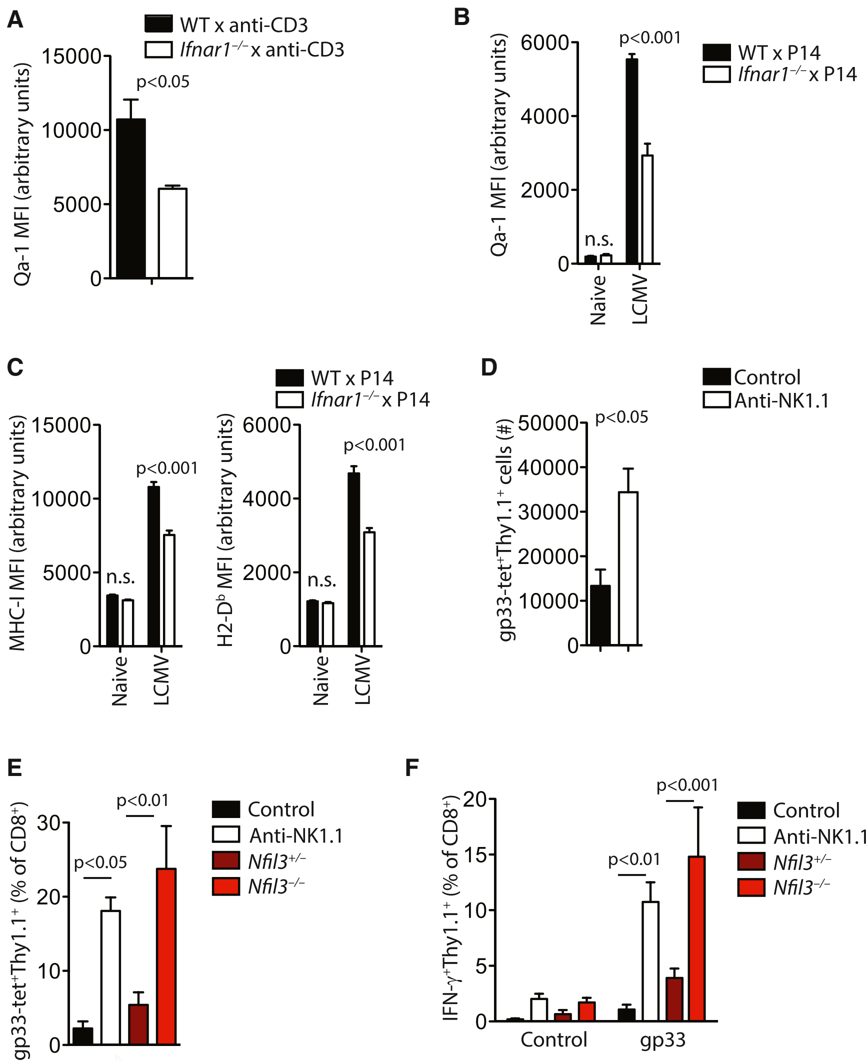



**Figure 4. NK Cells Target Antiviral T Cells in the Absence of IFNAR Signaling**

(A) Negatively sorted CFSE labeled CD8$^+$ T cells from WT and IFNAR1-deficient animals were stimulated for 72 hr with anti-CD3 and anti-CD28 antibodies followed by transfer of $2 \times 10^6$ cells into CD45.1$^+$ animals. At 2 days p.i. with 200 pfu LCMV WE, Qa-1b expression on transferred T cells was measured in spleen tissue (error bars show SEM; n = 3–4).

(B and C) We transferred $10^6$ negatively sorted CD8$^+$ T cells from P14$^+$ WT and *Ifnar1*$^{-/-}$ animals into CD45.1$^+$ hosts followed by no infection or infection with $2 \times 10^6$ pfu of LCMV WE. (B) Qa-1b, (C) MHC I (left panel), and H2-D$^b$ (right panel) expression was determined on transferred T cells 20 hr p.i. (error bars show SEM; n = 4, one of two independent experiments is shown).

(D) Negatively sorted CFSE labeled T cells from Thy1.1$^+$P14$^+$*Ifnar1*$^{-/-}$ were transferred into NK cell depleted and control animals 2 days p.i. with 200 pfu LCMV WE. At 4 days p.i., cell number was determined (error bars show SEM; n = 4, one of two independent experiments is shown).

(E and F) We transferred $10^5$ negatively sorted T cells from Thy1.1$^+$P14$^+$*Ifnar1*$^{-/-}$ were transferred into *Nfil3*$^{-/-}$, *Nfil3*$^{+/-}$, and NK-cell-depleted and control animals prior to infection with 200 pfu of LCMV WE. (E) Gp33-tetramer$^+$Thy1.1$^+$ T cells are shown 8 days p.i. (error bars show SEM; n = 5–7). (F) IFN-γ production of Thy1.1$^+$ T cells is shown 8 days p.i. (error bars show SEM; n = 5–7).

Consistently, when we transferred in vitro activated *B2m*$^{-/-}$ T cells into LCMV infected hosts, we observed their rapid removal, whereas WT T cells persisted in the infected hosts (Figure S6C). This rapid depletion was also dependent on NK cells, since *B2m*$^{-/-}$ T cells were not eliminated in the absence of NK cells (Figure S6C).

To evaluate whether NK cells target IFNAR1-deficient T cells via perforin in vivo, we transferred WT and *Ifnar1*$^{-/-}$ P14$^+$ cells into WT and *Prf1*$^{-/-}$ animals and infected them with LCMV. All these groups exhibited IFN-I production shortly after infection (Figure S6D). Consistent with the in vitro data, perforin deficiency restored the presence of *Ifnar1*$^{-/-}$ T cells (Figure 6B). Furthermore, IFNAR1 deficient T cells were able to produce cytokines after restimulation with virus-specific peptides (Figure 6C), indicating that the T cells were functional. LCMV cannot be eliminated in the absence of perforin (Kägi et al., 1994). However, after transfer of *Ifnar1*$^{-/-}$ T cells, only low or no virus titers were detected in the spleen and other tissues (Figure S6E; data not shown), which indicates that *Ifnar1*$^{-/-}$ T cells were capable of eliminating virus-infected cells. Consistent with the previous data, perforin deficiency did not affect the increased expression of PD-1 and the lower expression of KLRG1 on *Ifnar1*$^{-/-}$ T cells (Figure 6D; Figure S6F), likely resulting in still significant differences between WT and *Ifnar1*$^{-/-}$ T cell immunity in absence of NK cells (Figures 6B and 6C).

Next, we depleted NK cells in one group to assess whether NK cells might have additional effects on the IFNAR1-deficient virus-specific T cells. *Ifnar1*$^{-/-}$ P14$^+$ T cell immunity was restored in *Prf1*$^{-/-}$ animals to the same extent as in NK-cell-depleted mice (Figure 6E). Moreover, there was no further restoration in *Ifnar1*$^{-/-}$ P14$^+$ T cell immunity in *Prf1*$^{-/-}$ mice after NK cell depletion,

**Figure 3. IFN-I Regulates the Expression of NK-Cell-Receptor Ligands**

(A) We stimulated $10^6$ negatively sorted CD8$^+$ T cells with anti-CD3 antibodies in the presence or absence of IFN-4α (500 U/mL). After 6 hr, total RNA was extracted from cell suspensions and *H2-T24*, *H2-T23*, *H2-M3*, *Clec2d*, and *Itagv* (from left to right) expression was analyzed by RT-PCR (error bars show SEM; n = 4–6).

(B–D) Negatively sorted T cells were treated with anti-CD3 antibodies in absence or presence of IFN-4α. (B) Qa-1b expression was determined on CD8$^+$ T cells after 20 hr in absence or presence of different indicated IFN-4α concentrations (error bars show SEM; n = 6). (C) MHC I expression was determined on CD8$^+$ T cells after 20 hr of IFN-4α treatment (500 U/mL) (error bars show SEM; n = 4). (D) H2-D$^b$ expression was determined on CD8$^+$ T cells after 20 hr exposure to IFN-4α (500 U/mL) (error bars show SEM; n = 4, one of two independent experiments is shown). *p < 0.05, **p < 0.01, ***p < 0.001 between the indicated groups.

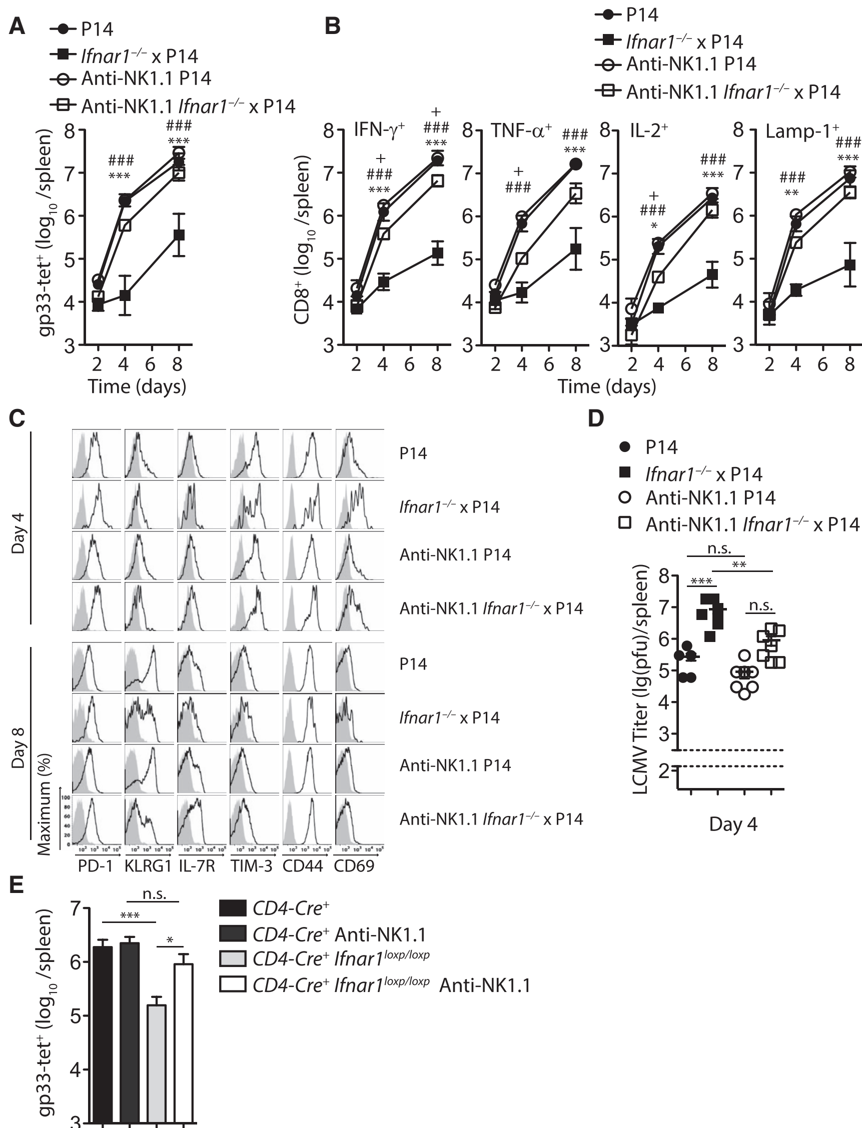



**Figure 5. NK Cells Regulate *Ifnar1*$^{-/-}$ T Cell Number and Function**

(A–D) We transferred $10^5$ negatively sorted T cells from P14$^+$ and P14$^+$ *Ifnar1*$^{-/-}$into NK-cell-depleted and control animals prior to infection with 200 pfu of LCMV WE. (A) Transferred Gp33-tetramer$^+$ T cells were measured at day 2, 4, and 8 p.i. in spleen tissue (error bars show SEM; n = 5–8, ***$p < 0.001$ between P14$^+$*Ifnar1*$^{-/-}$ transferred control and NK-cell-depleted animals, ###$p < 0.001$ between control animals transferred with P14$^+$ *Ifnar1*$^{-/-}$ and P14$^+$ cells). (B) IFN-γ, TNF-α, IL-2, and Lamp-1-positive transferred cells are shown at day 2, 4, and 8 p.i. (error bars show SEM; n = 4–6,*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between P14$^+$*Ifnar1*$^{-/-}$ transferred controls and NK-cell-depleted animals, ### $p < 0.001$ between control animals transferred with P14$^+$*Ifnar1*$^{-/-}$ and P14$^+$WT cells, + indicates $p < 0.05$ between anti-NK1.1 P14$^+$ and anti-NK1.1 *Ifnar1*$^{-/-}$P14$^+$). (C) Surface molecules were monitored on transferred cells on day 4 and 8 p.i. (n = 4–6, one representative is shown). (D) Virus titers of spleen tissue 4 days p.i. are shown (error bars show SEM; n = 7).

(E) *Ifnar1*$^{loxp/loxp}$ × *CD4-Cre*$^+$ and *CD4-Cre*$^+$ animals with and without NK cell depletion were infected with 200 pfu of LCMV WE followed by analyses of gp33-tetramer$^+$CD8$^+$ T cells in spleen tissue (error bars show SEM; n = 3–5). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, n.s. indicates not significant.

indicating that NK cells target IFNAR1-deficient T cells through perforin (Figures 6E and 6F). In conclusion, these data indicate that NK cells target *Ifnar1*$^{-/-}$ T cells directly and via perforin expression in vitro and in vivo.

## DISCUSSION

In this study we analyzed the effects of IFN-I on antiviral T cells. In our in vivo setting, IFNAR signaling protected antiviral T cells from NK-cell-mediated elimination. Considering these data, we identified NK cell-receptor ligands on T cells to be regulated by IFN-I in vitro and in vivo. NK cell deficiency increased *Ifnar1*$^{-/-}$ P14$^+$ T cell function after viral infection. These effects were triggered by NK-cell-mediated cytotoxicity because IFN-I signaling-deficient antiviral T cells were functional in perforin-deficient mice.

NK cells might target virus-specific T cells during chronic viral infection. Human chronic viral infections such as infections with hepatitis B or hepatitis C virus lead to enhanced cytotoxicity of NK cells (Ahlenstiel et al., 2010; Oliviero et al., 2009; Rehermann, 2013). This NK cell activation might be triggered by IFN-I (Ahlenstiel et al., 2010), which can be produced by plasmacytoid dendritic cells (pDCs) or Kupffer cells after sensing HCV RNA (Lau et al., 2013; Takahashi et al., 2010). Also during LCMV infection in mice, pDCs contribute to IFN-I production (Lang et al., 2010), which is a critical component of NK cell activation and cytotoxicity (Biron et al., 2002). In humans, the expression of inhibitory NK cell receptors correlates with elimination of HCV and decreased liver cell damage in human cohorts (Khakoo et al., 2004; Knapp et al., 2010; Paladino et al., 2007). However, recent studies show that following IFN-I treatment, immediate increase in serum ALT concentration correlated with an increase in NK cell cytotoxicity and virus elimination, suggesting a protective role of NK cells during viral induced hepatitis (Ahlenstiel et al., 2011). Furthermore, the reaction of NK cells to interferon treatment can serve as an indicator of effectiveness in patients suffering from hepatitis C virus infection (Oliviero et al., 2013) and predictor of treatment outcome (He et al., 2006; Sarasin-Filipowicz et al., 2008). Considering the data obtained in the LCMV model system, NK cell depletion results in enhanced antiviral T cell immunity, which triggers virus elimination and prevents chronic viral infection in mice (Cook and Whitmire, 2013; Lang et al., 2012; Waggoner et al., 2012). Furthermore, NK cell depletion can improve viral infections also at later time points during chronic infection (Waggoner et al., 2014). These seemingly contradictory findings

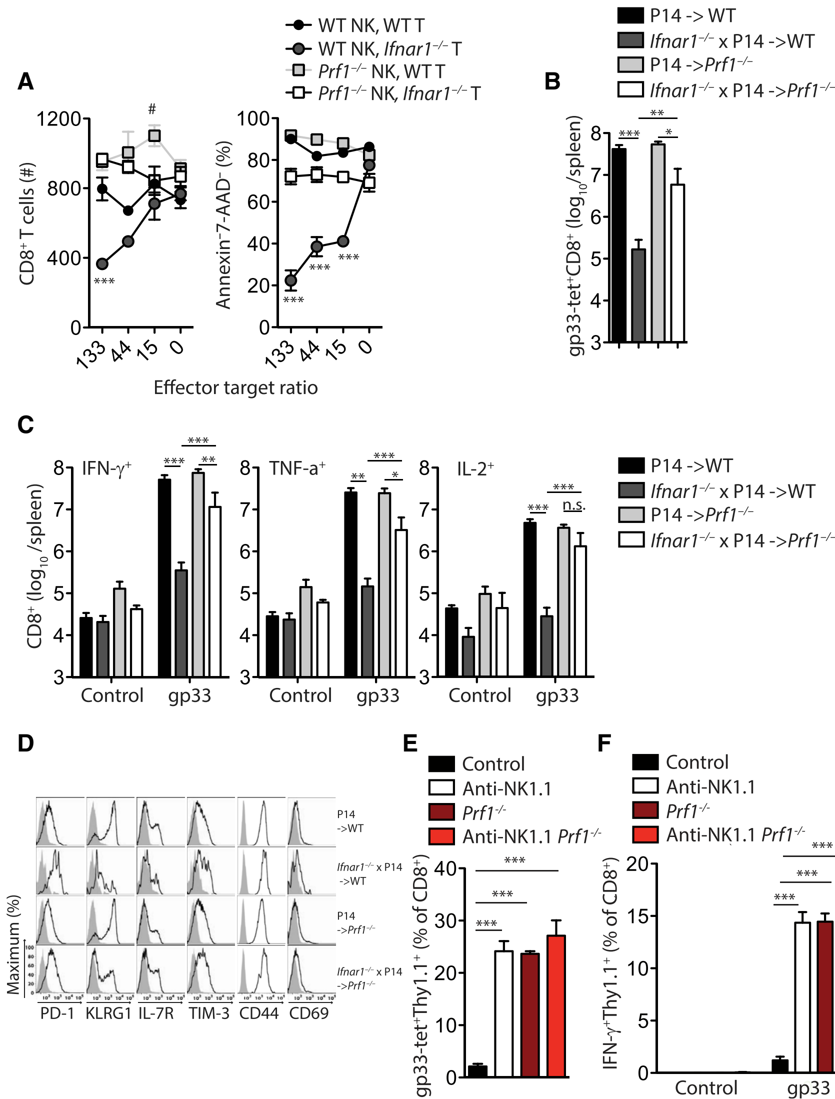


**Figure 6. NK Cells Target *Ifnar1*$^{-/-}$ T Cells through Perforin-Mediated Cytotoxicity**

(A) We cotransferred $2 \times 10^6$ negatively sorted T cells from P14$^+$ and P14$^+$*Ifnar1*$^{-/-}$ into NK cell depleted WT animals, followed by administration of $2 \times 10^6$ pfu of LCMV WE and 5 μg peptide gp33. P14$^+$ cells were isolated by FACS on day 2 and coincubated with IL-2-derived NK cells at the indicated effector target ratios. Following a 6 hr incubation, T cells numbers (left panel) and Annexin V$^-$ 7AAD$^-$ cells (right panel) were measured (error bars show SEM; n = 3, one of two independent experiments is shown, ***p < 0.001 between P14$^+$*Ifnar1*$^{-/-}$ T cells cocultured with WT and *Prf1*$^{-/-}$ NK cells, #p < 0.05 between P14$^+$ T cells cocultured with WT and *Prf1*$^{-/-}$ NK cells.

(B–D) We transferred $10^5$ negatively sorted T cells from P14$^+$ and P14$^+$*Ifnar1*$^{-/-}$into *prf1*$^{-/-}$ and control animals prior to infection with 200 pfu of LCMV WE. (B) Transferred Gp33-tetramer$^+$ T cells were measured at day 8 p.i. in spleen tissue (error bars show SEM; n = 4). (C) IFN-γ, TNF-α, and IL-2-positive transferred cells are shown at day 8 p.i. (error bars show SEM; n = 4). (D) Surface molecules were monitored on transferred cells on day 8 p.i. (n = 4, one representative is shown).

(E and F) We transferred $10^5$ negatively sorted T cells from Thy1.1$^+$P14$^+$*Ifnar1*$^{-/-}$into NK-cell-depleted control and *Prf1*$^{-/-}$ animals prior to infection with 200 pfu of LCMV WE. (E) Gp33-tetramer$^+$Thy1.1$^+$ T cells are shown at day 8 p.i. (error bars show SEM; n = 4). (F) IFN-γ production of Thy1.1$^+$ T cells is shown 8 days p.i. (error bars show SEM; n = 4, one of two independent experiments is shown). *p < 0.05, **p < 0.01 and ***p < 0.001, n.s. indicates not significant.

indicating that both NK cell activation and inhibition might have positive clinical benefits could be explained by our data, which suggest that IFN-I might protect T cells from NK cell cytotoxicity in addition to their potential antiviral effects during HCV infection. In patients with strong IFN-I effects, not only might the NK cell cytotoxicity be higher but also might the protection of T cells against NK-cell-mediated elimination. These data could also explain the immediate slight increase of ALT activity following IFN-I treatment in serum of HCV patients, which could be mediated not only by NK cells but also by increased cytotoxic T cell activity.

NK cell receptors might orchestrate the regulatory effects of NK cells. NK cell cytotoxicity can be mediated through a variety of activating and inhibitory receptors. During HCV and HBV infection, the activating NK cell receptors NKp30, NKp46, NKG2C, NKG2D, CD122, and the inhibitory receptor NKG2A are upregulated (Rehermann, 2013). Considering data from murine infection models, activating NK-cell-receptor ligands are upregulated on virus-specific T cells such as NKG2D ligands (Lang et al., 2012; Rabinovich et al., 2003). Furthermore, CD48 triggers inhibitory signals on virus-specific T cells, which is sensed by the NK cell receptor 2B4 (Waggoner et al., 2010). In our study, we found no significant suppression of NKG2D ligands by IFN-I, arguing against a role of NKG2D ligands in our experimental settings (Figures S3A and S3B). However, we identified that the expression of inhibitory NK-cell-receptor ligands on T cells are increased after IFN-I treatment. Involvement of signaling through other receptors, especially activating NK cell receptors, is likely, as described in more detail in an accompanying manuscript by Crouse et al. (2014). Therefore, during a viral infection, IFN-I not only activates NK cells but also triggers protection against regulatory NK cell functions on antiviral T cells. Furthermore, we identified other factors, which are regulated by IFN-I on antiviral T cells. *Ifnar1*$^{-/-}$ T cells exhibited an increased expression of PD-1, a well-known suppressor of T cell immunity (Barber et al., 2006). Moreover, IFN-I might phenotypically affect T cell immunity independently of NK cells, as evident by differential expression of KLRG1 (Kaech and Cui, 2012; Kaech et al., 2003). These and potentially other mechanisms might contribute to the efficiency of antiviral treatment by IFN-I, and they remain to be further explored. In conclusion, IFN-I is a critical regulator for antiviral T cell immunity to protect against regulatory NK cell functions.

## EXPERIMENTAL PROCEDURES

### Mice, Viruses, Virus Titration, and Cell Depletion

*Nfil3*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice were previously described (Kamizono et al., 2009; Müller et al., 1994). P14$^{+}$ and Smarta$^{+}$ mice were previously described (Oxenius et al., 1998; Pircher et al., 1989). *CD45.1*$^{+}$ and *B2m*$^{-/-}$ mice were purchased from Jackson Laboratory (Koller et al., 1990). All mice were on a C57BL/6 genetic background. All mice were maintained under specific pathogen-free conditions and experiments have been approved by commission of the Ontario Cancer Institute Animal Resource Centre following institutional guidelines or under the authorization of the LANUV in accordance with German laws for animal protection. NK cells were depleted with i.v. injection of anti-NK1.1 (clone PK136) as previously described (Koo and Peppard, 1984; Lang et al., 2012). LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute) and was propagated in L929 cells as described. Virus titers were measured with a plaque-forming assay as previously described (Lang et al., 2013). Briefly, organs were harvested into HBSS and homogenized with a Tissue Lyser (QIAGEN). MC57 cells were added to diluted virus samples on 24 well plates. After 3 hr, 1% methylcellulose containing medium was added. Forty-eight hr later, plates were fixed (4% formalin), permeabilized (1% Triton X HBSS), and stained with anti-LCMV-NP (clone: VL-4) antibody, Peroxidase anti-rat secondary antibody. Mice were infected i.v. with 200 or $2 \times 10^{6}$ pfu of LCMV WE as indicated. We administrated $2 \times 10^{6}$ pfu of VSV (Indiana strain) i.v. as indicated.

### Purification of T Cells

For T cell purification, single cell suspended splenocytes were enriched following the manufacturer's instructions with the pan T cell MACS kit and the CD8 purification kit (Miltenyi). Purified T cells were labeled with CFSE as previously described (Invitrogen) (Lang et al., 2012). For NK cell in vitro killer assay, WT and *Ifnar1*$^{-/-}$ P14$^{+}$ T cells were sorted with a FACS Aria III (BD) using congenicmarkers (CD45.1 for WT and CD90.1 for *Ifnar1*$^{-/-}$).

### Purification and Culture NK Cells

For NK cell purification, single cell suspended splenocytes were enriched following the manufacturer's instructions with the DX5 NK MACS kit (Miltenyi). For NK culture, sorted NK cells were stimulated with 1,000 U/ml IL-2 (Miltenyi) for 4 days.

### Flow Cytometry Analysis

Flow cytometry was performed as previously described (Lang et al., 2013). For intracellular cytokine, stain-single suspended splenocytes were incubated with the LCMV specific peptides gp33. After 1 hr Brefeldin A (eBiosciences) was added, followed by additional 5 hr incubation at 37°C. For Lamp-1 staining, anti-CD107a antibody (eBiosciences) was added for the 5 hr incubation and measured after additional staining with anti-CD8 (eBioscience) antibody. For cytokine production, cells were fixed with 2% formalin after surface stain with anti-CD8 (eBiosciences), permeabilized with 0.1% Saponin, and stained with anti-IFN-$\gamma$, anti-TNF-$\alpha$, and anti-IL-2 (eBiosciences) for 30 min at 4°C. LCMV specific T cells and NKG2D ligand stainings were performed as previously described (Lang et al., 2013; Lang et al., 2012). Cells were incubated with gp33-tetramer or NKG2D tetramer for 15 min at 37°C followed by surface molecule stainings for 30 min at 4°C (with anti-CD8, anti-PD-1, anti-KLRG1, anti-CD127, anti-TIM-3, anti-CD44, anti-CD69, anti-CD45.1, anti-CD45.2, anti-CD90.1, eBioscience).

### RT-PCR Analyses

RNA purification and RT-PCR analyses were performed as previously described according to manufacturer's instructions (QIAGEN) (Lang et al., 2013). Gene expression of *H2-T24*, *H2-T23*, *Clec2d*, *Itagv*, *Itgb3*, *B2m*, *Cd48*, *Cdh1*, *Pvr*, *Ulbp1*, and *Gapdh* was performed with kits from Applied Biosystems. For analysis, the expression of all target genes was normalized to *Gapdh* expression ($\Delta$Ct). Gene-expression values were then calculated based on the $\Delta\Delta$Ct method, with the mean naive MEFs as a control to which all other samples were compared. Relative quantities (RQ) were determined with the equation: RQ = $2^{\wedge}$-$\Delta\Delta$Ct.

### Microarray Analyses

RNA preparations were checked for RNA integrity by Agilent 2100 Bioanalyzer quality control. All samples in this study showed high quality RNA Integrity Numbers (RIN; median = 8). RNA was quantified by photometric Nanodrop measurement. Synthesis of cDNA and subsequent biotin labeling of cRNA was performed according to the manufacturer's protocol (WT Plus Kit; Affymetrix). Briefly, 100 ng of total RNA were converted to cDNA, followed by in vitro transcription and biotin labeling of cDNA. After fragmentation labeled cDNA was hybridized to Affymetrix Mouse Gene 2.0 ST Gene Expression Microarrays for 16 hr at 45°C, stained by strepatavidin/phycoerythrin conjugate and scanned as described in the manufacturer's protocol.

Data analyses on Affymetrix CEL files were conducted with GeneSpring GX software (Vers. 12.5; Agilent Technologies). Probes within each probeset were summarized by GeneSprings' ExonRMA16 algorithm after quantile normalization of probe-level signal intensities across all samples to reduce interarray variability (Bolstad et al., 2003). Input data preprocessing was concluded by baseline transformation to the median of all samples.

After grouping of samples (three biological replicates each) according to their respective experimental condition, a given probeset had to be expressed above background (i.e., fluorescence signal of a that probeset was detected within the 20$^{th}$ and 100$^{th}$ percentiles of the raw signal distribution of a given array) in all three replicates in at least one of two, or both conditions to be further analyzed in pairwise comparisons. Differential gene expression was statistically determined by Welch's unpaired t test. Resulting p values were corrected for multiple testing (Benjamini-Hochberg FDR). The significance threshold was set to $p_{(corr)} = 0.05$. Hierarchical cluster analysis was performed with Euclidian similarity measures and Ward's linkage. GeneOntology (GO) analyses were done with the DAVID Functional Annotation Tool (http://david.abcc.ncifcrf.gov) (Huang et al., 2009a, 2009b) testing for enrichment of differentially expressed transcripts in distinct functional GO categories. Significant GO term enrichment was determined at an EASE score of $p < 0.1$.

### Statistical Analysis

Data except microarray, which is described above, are expressed as mean ± SEM. Statistical significance between two groups was analyzed with Student's t test. For experiments involving analysis of multiple time points, two-way ANOVA with an additional Bonferroni posttest was used. Mantel-Cox test was used for analysis of survival curves. p values < 0.05 were considered as statistically significant.

## ACCESSION NUMBERS

The microarray data set has been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO Series accession number GSE57421.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.05.004.

## AUTHOR CONTRIBUTIONS

H.C.X. performed experiments and wrote the paper. M.G. performed experiments. A.A.P. performed experiments and wrote the paper. R.P. J.H., and P.S. performed experiments. R.D. performed experiments and wrote the paper. K.K., R.R., A.D., K.G., M.L., L.H., Z.W., D.H., T.W.M., and P.S.O. provided reagents, discussed the data, and wrote the paper. K.S.L. initiated the study and wrote the paper. P.A.L. designed the study, performed experiments, and wrote the paper.

## ACKNOWLEDGMENTS

The authors are grateful for the technical assistance of Eugen Bäcker and Anne Trenkel. This study was supported by the Alexander von Humboldt Foundation (SKA2008 and SKA2010), the German Research Council (SFB974, LA2558/3-1, LA2558/5-1, TRR60, LA1419/5-1, RTG1949), and the NIH Tetramer Facility. Further support came from the Strategic Research Fund and the Forschungskomission of the Heinrich Heine University.

Received: December 4, 2013
Accepted: April 18, 2014
Published: June 5, 2014

## REFERENCES

Agarwal, P., Raghavan, A., Nandiwada, S.L., Curtsinger, J.M., Bohjanen, P.R., Mueller, D.L., and Mescher, M.F. (2009). Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory. J. Immunol. *183*, 1695–1704.

Ahlenstiel, G., Titerence, R.H., Koh, C., Edlich, B., Feld, J.J., Rotman, Y., Ghany, M.G., Hoofnagle, J.H., Liang, T.J., Heller, T., and Rehermann, B. (2010). Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. Gastroenterology *138*, 325–335, e321–322.

Ahlenstiel, G., Edlich, B., Hogdal, L.J., Rotman, Y., Noureddin, M., Feld, J.J., Holz, L.E., Titerence, R.H., Liang, T.J., and Rehermann, B. (2011). Early changes in natural killer cell function indicate virologic response to interferon therapy for hepatitis C. Gastroenterology *141*, 1231–1239, e1231–e1232.

Aichele, P., Unsoeld, H., Koschella, M., Schweier, O., Kalinke, U., and Vucikuja, S. (2006). CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. J. Immunol. *176*, 4525–4529.

Andrews, D.M., Estcourt, M.J., Andoniou, C.E., Wikstrom, M.E., Khong, A., Voigt, V., Fleming, P., Tabarias, H., Hill, G.R., van der Most, R.G., et al. (2010). Innate immunity defines the capacity of antiviral T cells to limit persistent infection. J. Exp. Med. *207*, 1333–1343.

Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G.J., and Ahmed, R. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. Nature *439*, 682–687.

Biron, C.A., Nguyen, K.B., and Pien, G.C. (2002). Innate immune responses to LCMV infections: natural killer cells and cytokines. Curr. Top. Microbiol. Immunol. *263*, 7–27.

Bolstad, B.M., Irizarry, R.A., Astrand, M., and Speed, T.P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics *19*, 185–193.

Cook, K.D., and Whitmire, J.K. (2013). The depletion of NK cells prevents T cell exhaustion to efficiently control disseminating virus infection. J. Immunol. *190*, 641–649.

Crouse, J., Bedenikovic, G., Wiesel, M., Ibberson, M., Xenarios, I., Von Laer, D., Kalinke, U., Vivier, E., Jonjic, S., and Oxenius, A. (2014). Type I Interferons Protect T Cells against NK Cell Attack Mediated by the Activating Receptor NCR1. Immunity *40*. Published online June 5, 2014. http://dx.doi.org/10.1016/j.immuni.2014.05.003.

Edlich, B., Ahlenstiel, G., Zabaleta Azpiroz, A., Stoltzfus, J., Noureddin, M., Serti, E., Feld, J.J., Liang, T.J., Rotman, Y., and Rehermann, B. (2012). Early changes in interferon signaling define natural killer cell response and refractoriness to interferon-based therapy of hepatitis C patients. Hepatology *55*, 39–48.

Gascoyne, D.M., Long, E., Veiga-Fernandes, H., de Boer, J., Williams, O., Seddon, B., Coles, M., Kioussis, D., and Brady, H.J. (2009). The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. Nat. Immunol. *10*, 1118–1124.

Gautier, G., Humbert, M., Deauvieau, F., Scuiller, M., Hiscott, J., Bates, E.E., Trinchieri, G., Caux, C., and Garrone, P. (2005). A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. J. Exp. Med. *201*, 1435–1446.

Gilliet, M., Cao, W., and Liu, Y.J. (2008). Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat. Rev. Immunol. *8*, 594–606.

González-Navajas, J.M., Lee, J., David, M., and Raz, E. (2012). Immunomodulatory functions of type I interferons. Nat. Rev. Immunol. *12*, 125–135.

Gründemann, C., Schwartzkopff, S., Koschella, M., Schweier, O., Peters, C., Voehringer, D., and Pircher, H. (2010). The NK receptor KLRG1 is dispensable for virus-induced NK and CD8+ T-cell differentiation and function in vivo. Eur. J. Immunol. *40*, 1303–1314.

He, X.S., Ji, X., Hale, M.B., Cheung, R., Ahmed, A., Guo, Y., Nolan, G.P., Pfeffer, L.M., Wright, T.L., Risch, N., et al. (2006). Global transcriptional response to interferon is a determinant of HCV treatment outcome and is modified by race. Hepatology *44*, 352–359.

Huang, W., Sherman, B.T., and Lempicki, R.A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. *37*, 1–13.

Huang, W., Sherman, B.T., and Lempicki, R.A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. *4*, 44–57.

Jin, H.T., Anderson, A.C., Tan, W.G., West, E.E., Ha, S.J., Araki, K., Freeman, G.J., Kuchroo, V.K., and Ahmed, R. (2010). Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. Proc. Natl. Acad. Sci. USA *107*, 14733–14738.

Jones, R.B., Ndhlovu, L.C., Barbour, J.D., Sheth, P.M., Jha, A.R., Long, B.R., Wong, J.C., Satkunarajah, M., Schweneker, M., Chapman, J.M., et al. (2008). Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. J. Exp. Med. *205*, 2763–2779.

Kaech, S.M., and Cui, W. (2012). Transcriptional control of effector and memory CD8+ T cell differentiation. Nat. Rev. Immunol. *12*, 749–761.

Kaech, S.M., Tan, J.T., Wherry, E.J., Konieczny, B.T., Surh, C.D., and Ahmed, R. (2003). Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat. Immunol. *4*, 1191–1198.

Kägi, D., Ledermann, B., Bürki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., and Hengartner, H. (1994). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature *369*, 31–37.

Kamizono, S., Duncan, G.S., Seidel, M.G., Morimoto, A., Hamada, K., Grosveld, G., Akashi, K., Lind, E.F., Haight, J.P., Ohashi, P.S., et al. (2009). Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. J. Exp. Med. *206*, 2977–2986.

Kamphuis, E., Junt, T., Waibler, Z., Forster, R., and Kalinke, U. (2006). Type I interferons directly regulate lymphocyte recirculation and cause transient blood lymphopenia. Blood *108*, 3253–3261.

Khakoo, S.I., Thio, C.L., Martin, M.P., Brooks, C.R., Gao, X., Astemborski, J., Cheng, J., Goedert, J.J., Vlahov, D., Hilgartner, M., et al. (2004). HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science *305*, 872–874.

Knapp, S., Warshow, U., Hegazy, D., Brackenbury, L., Guha, I.N., Fowell, A., Little, A.M., Alexander, G.J., Rosenberg, W.M., Cramp, M.E., and Khakoo, S.I. (2010). Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus. Hepatology *51*, 1168–1175.

Koller, B.H., Marrack, P., Kappler, J.W., and Smithies, O. (1990). Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8+ T cells. Science *248*, 1227–1230.

Kolumam, G.A., Thomas, S., Thompson, L.J., Sprent, J., and Murali-Krishna, K. (2005). Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J. Exp. Med. *202*, 637–650.

Koo, G.C., and Peppard, J.R. (1984). Establishment of monoclonal anti-Nk-1.1 antibody. Hybridoma *3*, 301–303.

Lang, P.A., Recher, M., Honke, N., Scheu, S., Borkens, S., Gailus, N., Krings, C., Meryk, A., Kulawik, A., Cervantes-Barragan, L., et al. (2010). Tissue macrophages suppress viral replication and prevent severe immunopathology in an interferon-I-dependent manner in mice. Hepatology *52*, 25–32.

Lang, P.A., Lang, K.S., Xu, H.C., Grusdat, M., Parish, I.A., Recher, M., Elford, A.R., Dhanji, S., Shaabani, N., Tran, C.W., et al. (2012). Natural killer cell activation enhances immune pathology and promotes chronic infection by limiting CD8+ T-cell immunity. Proc. Natl. Acad. Sci. USA *109*, 1210–1215.

Lang, P.A., Xu, H.C., Grusdat, M., McIlwain, D.R., Pandyra, A.A., Harris, I.S., Shaabani, N., Honke, N., Maney, S.K., Lang, E., et al. (2013). Reactive oxygen species delay control of lymphocytic choriomeningitis virus. Cell Death Differ. *20*, 649–658.

Lau, D.T., Negash, A., Chen, J., Crochet, N., Sinha, M., Zhang, Y., Guedj, J., Holder, S., Saito, T., Lemon, S.M., et al. (2013). Innate immune tolerance and the role of kupffer cells in differential responses to interferon therapy among patients with HCV genotype 1 infection. Gastroenterology *144*, 402–413, e412.

Le Bon, A., Durand, V., Kamphuis, E., Thompson, C., Bulfone-Paus, S., Rossmann, C., Kalinke, U., and Tough, D.F. (2006). Direct stimulation of T cells by type I IFN enhances the CD8+ T cell response during cross-priming. J. Immunol. *176*, 4682–4689.

Lee, S.H., Kim, K.S., Fodil-Cornu, N., Vidal, S.M., and Biron, C.A. (2009). Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD8 T cell regulation during viral infection. J. Exp. Med. *206*, 2235–2251.

Marshall, H.D., Prince, A.L., Berg, L.J., and Welsh, R.M. (2010). IFN-alpha beta and self-MHC divert CD8 T cells into a distinct differentiation pathway characterized by rapid acquisition of effector functions. J. Immunol. *185*, 1419–1428.

Müller, U., Steinhoff, U., Reis, L.F., Hemmi, S., Pavlovic, J., Zinkernagel, R.M., and Aguet, M. (1994). Functional role of type I and type II interferons in antiviral defense. Science *264*, 1918–1921.

Nguyen, K.B., Watford, W.T., Salomon, R., Hofmann, S.R., Pien, G.C., Morinobu, A., Gadina, M., O'Shea, J.J., and Biron, C.A. (2002). Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. Science *297*, 2063–2066.

Oliviero, B., Varchetta, S., Paudice, E., Michelone, G., Zaramella, M., Mavilio, D., De Filippi, F., Bruno, S., and Mondelli, M.U. (2009). Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. Gastroenterology *137*, 1151–1160, e1151–e1157.

Oliviero, B., Mele, D., Degasperi, E., Aghemo, A., Cremonesi, E., Rumi, M.G., Tinelli, C., Varchetta, S., Mantovani, S., Colombo, M., and Mondelli, M.U. (2013). Natural killer cell dynamic profile is associated with treatment outcome in patients with chronic HCV infection. J. Hepatol. *59*, 38–44.

Oxenius, A., Bachmann, M.F., Zinkernagel, R.M., and Hengartner, H. (1998). Virus-specific MHC-class II-restricted TCR-transgenic mice: effects on humoral and cellular immune responses after viral infection. Eur. J. Immunol. *28*, 390–400.

Paladino, N., Flores, A.C., Marcos, C.Y., Fainboim, H., Theiler, G., Arruvito, L., Williams, F., Middleton, D., and Fainboim, L. (2007). Increased frequencies of activating natural killer receptors are associated with liver injury in individuals who do not eliminate hepatitis C virus. Tissue Antigens 69 (*Suppl 1*), 109–111.

Petricoin, E.F., 3rd, Ito, S., Williams, B.L., Audet, S., Stancato, L.F., Gamero, A., Clouse, K., Grimley, P., Weiss, A., Beeler, J., et al. (1997). Antiproliferative action of interferon-alpha requires components of T-cell-receptor signalling. Nature *390*, 629–632.

Pircher, H., Bürki, K., Lang, R., Hengartner, H., and Zinkernagel, R.M. (1989). Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. Nature *342*, 559–561.

Rabinovich, B.A., Li, J., Shannon, J., Hurren, R., Chalupny, J., Cosman, D., and Miller, R.G. (2003). Activated, but not resting, T cells can be recognized and killed by syngeneic NK cells. J. Immunol. *170*, 3572–3576.

Rehermann, B. (2013). Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. Nat. Med. *19*, 859–868.

Rehermann, B., and Nascimbeni, M. (2005). Immunology of hepatitis B virus and hepatitis C virus infection. Nat. Rev. Immunol. *5*, 215–229.

Sadler, A.J., and Williams, B.R. (2008). Interferon-inducible antiviral effectors. Nat. Rev. Immunol. *8*, 559–568.

Santini, S.M., Lapenta, C., Logozzi, M., Parlato, S., Spada, M., Di Pucchio, T., and Belardelli, F. (2000). Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. J. Exp. Med. *191*, 1777–1788.

Sarasin-Filipowicz, M., Oakeley, E.J., Duong, F.H., Christen, V., Terracciano, L., Filipowicz, W., and Heim, M.H. (2008). Interferon signaling and treatment outcome in chronic hepatitis C. Proc. Natl. Acad. Sci. USA *105*, 7034–7039.

Su, H.C., Nguyen, K.B., Salazar-Mather, T.P., Ruzek, M.C., Dalod, M.Y., and Biron, C.A. (2001). NK cell functions restrain T cell responses during viral infections. Eur. J. Immunol. *31*, 3048–3055.

Takahashi, K., Asabe, S., Wieland, S., Garaigorta, U., Gastaminza, P., Isogawa, M., and Chisari, F.V. (2010). Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection. Proc. Natl. Acad. Sci. USA *107*, 7431–7436.

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., and Ugolini, S. (2008). Functions of natural killer cells. Nat. Immunol. *9*, 503–510.

Vivier, E., Raulet, D.H., Moretta, A., Caligiuri, M.A., Zitvogel, L., Lanier, L.L., Yokoyama, W.M., and Ugolini, S. (2011). Innate or adaptive immunity? The example of natural killer cells. Science *331*, 44–49.

Waggoner, S.N., Taniguchi, R.T., Mathew, P.A., Kumar, V., and Welsh, R.M. (2010). Absence of mouse 2B4 promotes NK cell-mediated killing of activated CD8+ T cells, leading to prolonged viral persistence and altered pathogenesis. J. Clin. Invest. *120*, 1925–1938.

Waggoner, S.N., Cornberg, M., Selin, L.K., and Welsh, R.M. (2012). Natural killer cells act as rheostats modulating antiviral T cells. Nature *481*, 394–398.

Waggoner, S.N., Daniels, K.A., and Welsh, R.M. (2014). Therapeutic depletion of natural killer cells controls persistent infection. J. Virol. *88*, 1953–1960.

Wherry, E.J. (2011). T cell exhaustion. Nat. Immunol. *12*, 492–499.