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European Journal of Immunology

Adaptive immunity

Basic

Research Article

NKG2D stimulation of CD8$^+$ T cells during priming promotes their capacity to produce cytokines in response to viral infection in mice

Inga Kavazović[1], Maja Lenartić[1], Vedrana Jelenčić[1], Slaven Jurković[2], Niels A.W. Lemmermann[3], Stipan Jonjić[1], Bojan Polić[1] and Felix M. Wensveen[1,4]

[1] Department of Histology and Embryology, Faculty of Medicine, University of Rijeka, Rijeka, Croatia
[2] Department of Medical Physics, University Hospital Rijeka, Rijeka, Croatia
[3] Institute for Virology,University Medical Center of the Johannes Gutenberg–University Mainz, Mainz, Germany
[4] Department of Experimental Immunology, Amsterdam Medical Center, Amsterdam, The Netherlands

Natural killer group 2 member D (NKG2D) is an activating receptor that is expressed on most cytotoxic cells of the immune system, including NK cells, γδ, and CD8$^+$ T cells. It is still a matter of debate whether and how NKG2D mediates priming of CD8$^+$ T cells in vivo, due to a lack of studies where NKG2D is eliminated exclusively in these cells. Here, we studied the impact of NKG2D on effector CD8$^+$ T-cell formation. NKG2D deficiency that is restricted to murine CD8$^+$ T cells did not impair antigen-specific T-cell expansion following mouse CMV and lymphocytic choriomeningitis virus infection, but reduced their capacity to produce cytokines. Upon infection, conventional dendritic cells induce NKG2D ligands, which drive cytokine production on CD8$^+$ T cells via the Dap10 signaling pathway. T-cell development, homing, and proliferation were not affected by NKG2D deficiency and cytotoxicity was only impaired when strong T-cell receptor (TCR) stimuli were used. Transfer of antigen-specific CD8$^+$ T cells demonstrated that NKG2D deficiency attenuated their capacity to reduce viral loads. The inability of NKG2D-deficient cells to produce cytokines could be overcome with injection of IL-15 superagonist during priming. In summary, our data show that NKG2D has a nonredundant role in priming of CD8$^+$ T cells to produce antiviral cytokines.

Keywords: Cytokines · Priming · CD8$^+$ T cells · Costimulation · mCMV · NKG2D

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Correspondence: Dr. Felix M. Wensveen
e-mail: Felix.Wensveen@gmail.com

Introduction

$CD8^+$ T-cell activation following viral infection is a tightly controlled process that does not only require stimulation of their antigen receptor, but also costimulation by membrane-bound ligands and cytokines from antigen-presenting cells (APCs) and $CD4^+$ helper T cells [1, 2]. Costimulation provides activated $CD8^+$ T cells with essential signals that prevent activation-induced cell death [1]. In addition, costimulation unlocks the full potential of $CD8^+$ T cells, promoting proliferation, cytokine production, and cytotoxicity [1]. A large number costimulatory receptors have been identified, most notably CD28 and members of the TNF receptor superfamily [3, 4].

A molecule of particular interest for T-cell activation is the c-type lectin-like receptor natural killer group 2 member D (NKG2D), encoded by killer cell lectin like receptor K1 (*Klrk1*). NKG2D is an activating receptor that is expressed on cytotoxic cells such as NK cells, NKT cells, and γδ T cells [5]. NKG2D is not present on naïve murine $CD8^+$ T cells, but is induced after antigen encounter [6]. Multiple ligands for NKG2D have been identified, including MICA, MICB, and ULBP1-6 in humans and Rae1α-ε, H60a-c, and MULT1 in mice [7]. NKG2D ligands are typically induced in response to stress factors such as infection or oncogenic transformation [5]. NKG2D ligands are also observed in absence of overt infection such as on bone marrow (BM) stroma [5]. NKG2D signals through the adaptor molecules DAP10 and DAP12, which mediate direct cytotoxic killing of target cells upon recognition of ligands by NK cells [8]. $CD8^+$ T cells lack expression of DAP12 and in these cells NKG2D was proposed to function as a costimulatory receptor [9]. In vitro systems trying to establish the exact costimulatory role of NKG2D on $CD8^+$ T cells have previously shown contradicting results [10, 11]. In vivo, NKG2D engagement using models systems with transgenic expression of its ligands enhances both effector and memory $CD8^+$ T-cell responses [12]. However, hyperstimulation of costimulatory molecules may lead to superphysiological responses or conversely, cause anergy [13, 14]. The impact of NKG2D on T-cell priming is therefore best studied using NKG2D-deficient cells. However, NKG2D plays a role in development of NK and B cells [15] and *Krk1*$^{-/-}$ mice have hyperresponsive NK cells. Studies in which NKG2D is specifically eliminated in $CD8^+$ T cells are so far lacking. Currently, it is unknown whether, under physiological conditions, NKG2D functions as a costimulatory factor for $CD8^+$ T-cell priming in vivo, or whether it only mediates direct effector responses to target cells expressing its ligands [15].

Here, we questioned whether NKG2D plays a role in $CD8^+$ T-cell priming following viral infection, using models in which NKG2D is specifically eliminated in these cells. We show that NKG2D is not required for $CD8^+$ T-cell expansion following viral infection and has a redundant role in mediating cytotoxicity. In contrast, cytokine production as well as their capacity to lower viral loads following infection was impaired in NKG2D-deficient $CD8^+$ T cells. Reduced cytokine production by *Klrk1*$^{-/-}$ $CD8^+$ T cells could be overcome using an IL-15 superagonist. Thus, under physiological conditions, NKG2D plays a nonredundant role in priming antiviral effector $CD8^+$ T cells for the production of cytokines.

Results

NKG2D deficiency impairs antiviral $CD8^+$ T-cell responses

We questioned whether effector $CD8^+$ T-cell responses are affected by the absence of NKG2D. First, we analyzed NKG2D expression on effector, memory precursor, and naïve T cells. Wild-type (WT) and *Klrk1*$^{-/-}$ mice were infected with mouse cytomegalovirus (mCMV). On day 8 after infection, NKG2D expression was analyzed on virus-specific ($K^b m57^+$) effector cells ($CD127^-$) and memory precursor cells ($CD127^+$) in comparison with naïve T cells ($CD62L^+CD44^{Dim/-}$) from uninfected controls. NKG2D expression was very low on naïve cells, but was strongly induced on both effector and memory cells (Fig. 1A). Next, WT and *Klrk1*$^{-/-}$ mice were infected with mCMV and antigen-specific T-cell responses were analyzed 8 days later. NKG2D deficiency resulted in a two- to threefold reduction of $CD8^+$ T cells specific for two immune-dominant viral epitopes ($K^b m57$ and $K^b m139$) in spleen and lymph nodes (LNs) (Fig. 1B). In vitro restimulation with viral peptides also showed a strong reduction in IFN-γ production by *Klrk1*$^{-/-}$ $CD8^+$ T cells compared to controls (Fig. 1C). Thus, NKG2D deficiency impairs $CD8^+$ T-cell responses against mCMV infection.

NKG2D deficiency does not affect naïve T-cell development

NKG2D deficiency impacts the development of NK and B cells [15]. Differences in effector T-cell responses of *Klrk1*$^{-/-}$ mice may therefore be the result of alterations in the naïve T-cell pool. No differences were observed in the frequencies of thymic subpopulations, peripheral $CD4^+$ and $CD8^+$ T-cell populations, or in regulatory T cells of *Klrk1*$^{-/-}$ mice compared to WT controls. Also, naïve, central memory, and effector memory $CD8^+$ T-cell subpopulations were unaffected by deficiency for NKG2D (Fig. 2A).

The impact of NKG2D on T-cell development may only become apparent in a competitive setting. Therefore, WT and *Klrk1*$^{-/-}$ BM cells were mixed in a 1:1 ratio and injected in lethally irradiated hosts to generate mixed BM chimeras (mBMCs). After reconstitution, no differences were observed in the ratio between WT and *Klrk1*$^{-/-}$ cells, either in thymic subpopulations or in total $CD8^+$ T cells (Fig. 2B). In addition, the composition of $CD8^+$ T-cell subpopulations was not different between WT and *Klrk1*$^{-/-}$ cells in the spleen or LNs of mBMCs (Fig. 2C). To investigate whether WT- or *Klrk1*$^{-/-}$-deficient stroma impacts development of T cells, WT and *Klrk1*$^{-/-}$ mice were lethally irradiated and reconstituted with WT BM. NKG2D deficiency of the nonhematopoietic

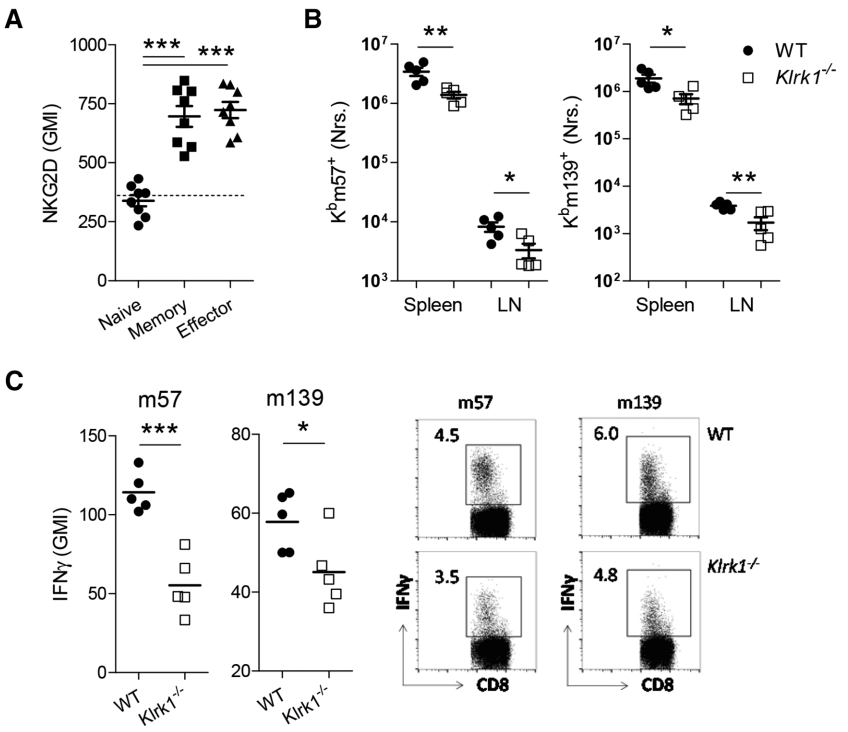

Figure 1. NKG2D-deficient mice have a reduced CD8$^+$ T-cell response to CMV infection. (**A**) WT mice were infected with mCMV. After 7 days, NKG2D expression was determined on Naive (CD62L$^+$CD44$^-$), Memory (K^{b}m57$^+$CD127$^+$), and effector (K^{b}m57$^+$CD127$^-$) CD8$^+$ T cells from spleen by flow cytometry. Dashed line indicates background (NKG2D staining of *Klrk1*$^{-/-}$ cells). (**B**, **C**) WT and *Klrk1*$^{-/-}$ mice were infected with mCMV. After 7 days (**B**) absolute numbers of K^{b}m57$^+$ and K^{b}m139$^+$ cells were determined in spleen and LN. (**C**) IFN-γ production of splenic T cells after restimulation with indicated peptides in vitro. Data shows means ± SEM. Panel **A** shows pooled data of two experiments with four mice per experiment. Data in **B and C** show one of three experiments with five mice per experiment. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ (Student's *t*-test or ANOVA with Bonferroni's post-test). GMI, geometric mean intensity.

environment did not impact thymic subpopulations, nor the amount of CD4$^+$ and CD8$^+$ T cells in the spleen (Fig. 2D). Finally, we investigated the capacity of naïve NKG2D-deficient CD8$^+$ T cells to produce cytokines upon in vitro stimulation. Splenocytes from mBMCs were stimulated with PMA/Ionomycin or agonistic CD3 antibodies. No differences were observed in the number of CD8$^+$ T cells producing IFN-γ, nor in the average level of IFN-γ expression per cell (Fig. 2E). Therefore, we conclude that NKG2D deficiency does not impact development of naïve T cells.

NKG2D deficiency does not affect CD8$^+$ T-cell phenotype or homing

NKG2D deficiency in NK cells affects expression of several inhibitory and activating receptors [15]. We questioned whether altered expression of these receptors on effector cells is responsible for impaired CD8$^+$ responses to mCMV infection. CD45.2$^+$ WT or NKG2D-deficient *Klrk1*$^{\Delta/\Delta}$ OT1 T cells [16] were transferred to WT (CD45.1$^+$) recipients, which were subsequently infected with mCMV expressing the SIINFEKL epitope of ovalbumin (mCMV-SIINFEKL [17]). On day 7 after infection, donor cells were analyzed in spleen. However, no differences were observed between WT and NKG2D-deficient CD8$^+$ T cells for any of the investigated markers (Fig. 3A).

Next, we assessed whether homing of CD8$^+$ T cells is altered in *Klrk1*$^{-/-}$ mice. WT (CD45.1) and *Klrk1*$^{-/-}$ (CD45.2) splenocytes were mixed in a 1:1 ratio, labeled with CFSE, and transferred in WT or *Klrk1*$^{-/-}$ hosts (CD45.2). One day later, donor cell numbers were analyzed. No differences were observed in the ability of cells to enter LNs, spleen, or circulation (Fig. 3B). To assess homing of effector cells, total CD8$^+$ T cells from WT and *Klrk1*$^{-/-}$ mice were isolated 7 days after infection with mCMV, mixed in a 1:1 ratio, CFSE labeled, and injected in coinfected hosts. One day later, CD8$^+$ T cells were analyzed. No differences were observed in the ratio between total or antigen-specific effector WT and *Klrk1*$^{-/-}$ CD8$^+$ T cells in blood, LNs, or spleen (Fig. 3C and D). In summary, NKG2D-deficiency does not affect cell surface marker expression or homing of effector CD8$^+$ T cells.

CD8$^+$ T-cell specific NKG2D deficiency impairs IFN-γ production

NKG2D mediates NK-cell education and its deficiency increases their ability to fight mCMV infection [15]. Lymphocytic choriomeningitis virus (LCMV) is not controlled by NK cells, but by CD8$^+$ T cells [18]. We therefore analyzed effector CD8$^+$ T-cell expansion in WT and *Klrk1*$^{-/-}$ mice 8 days after LCMV infection. We did not observe differences in the percentage of antigen-specific CD8$^+$ T cells in spleen, LNs, and liver and found only a minor reduction in absolute D^{b}Gp33$^+$ cell numbers in spleen (Fig. 4A). This prompted us to investigate viral loads in *Klrk1*$^{-/-}$ mice after mCMV infection. Indeed, we observed a persistent reduction in viral titers both at early and late time points after infection (Fig. 4B) that were lost when NK cells were depleted (Fig. 4C). Thus, differences in NK-cell responses are responsible for changes in antigenic load in *Klrk1*$^{-/-}$ mice after mCMV infection and may impact CD8$^+$ T-cell expansion.

To negate the effect of differential NK-cell functionality, mBMCs were generated and infected with mCMV. Eight days after infection, no differences were observed between WT and *Klrk1*$^{-/-}$

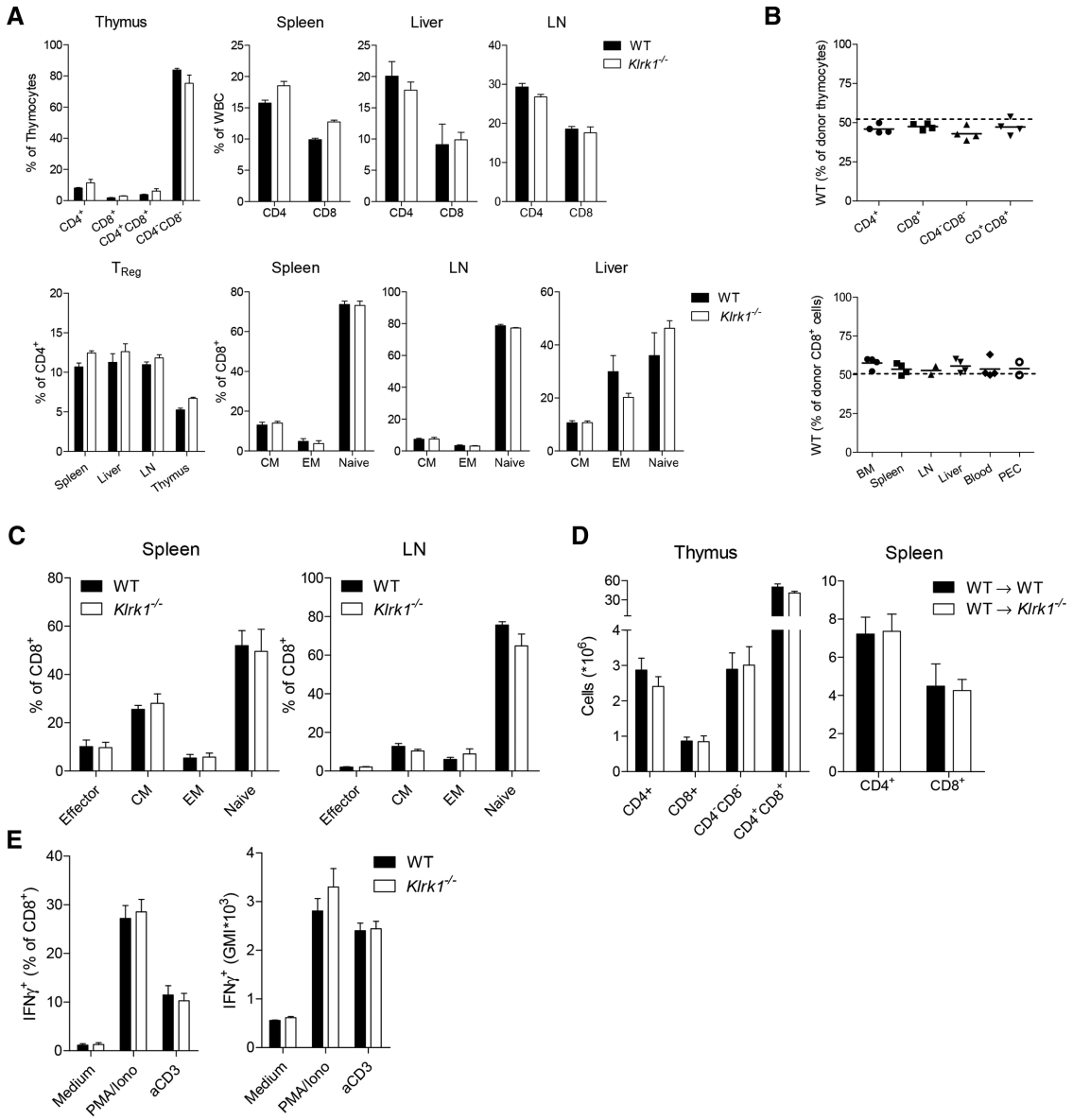

Figure 2. NKG2D does not affect CD8$^+$ T-cell development (**A**) T cells of indicated organs were quantified by flow cytometry in 8-week-old WT and *Klrk1*$^{-/-}$ mice. (**B**, **C**) mBMCs were generated using WT (CD45.1) and *Klrk1*$^{-/-}$ (CD45.1/2) BM mixed in a 1:1 ratio and injected in CD45.2 recipients. Eight weeks after reconstitution (**B**) the fraction of WT donor cells was determined in (top) thymocytes and (bottom) the CD8$^+$-cell populations of the indicated organs. (**C**) The composition of CD8$^+$ donor subpopulations was determined in spleen and LN. (**D**) WT or *Klrk1*$^{-/-}$ (CD45.2) recipients were lethally irradiated and reconstituted with WT (CD45.1) BM. After 8 weeks, donor cell numbers were quantified in (left) thymus and (right) spleen. (**E**) Splenocytes from mBMCs (WT: *Klrk1*$^{-/-}$, 1:1) were stimulated in vitro with PMA/Ionomycin or αCD3 antibodies and IFN-γ production of CD8$^+$ T cells was assessed. Data show means ± SEM. Panels show a representative of two (**B–D**) or three (**A**, **E**) experiments using three to five mice per experiment. GMI, geometric mean intensity; WBC, white blood cells; effector, (CD127$^-$); CM, central memory (CD127$^+$CD62L$^+$CD44^{Bright}); EM, effector memory (CD127$^+$CD62L$^-$CD44$^+$); naïve, (CD127$^+$CD62L$^+$CD44$^{Dim/-}$).

CD8$^+$ T-cell expansion (Fig. 4D). NKG2D has been shown to promote cell division in T cells after stimulation in vitro [19]. To test this in vivo, mBMCs were infected with mCMV and injected every second day with BrdU. On day 8 after infection, BrdU incorporation was analyzed in effector CD8$^+$ T cells. We observed strong labeling of BrdU, indicative of rapid proliferation in all effector cells. However, no differences in BrdU incorporation were observed between WT and *Klrk1*$^{-/-}$ donor cells (Fig. 4E). Thus, NKG2D deficiency does not impair effector CD8$^+$ T-cell proliferation in response to mCMV infection.

Next, we assessed the functionality of *Klrk1*$^{-/-}$ CD8$^+$ T cells in mBMCs 8 days after mCMV infection. In vitro restimulation with CD3ε antibodies or viral peptides showed no differences in granzyme B expression (Fig. 4F). However, NKG2D-deficient cells produced significantly less IFN-γ in response to restimulation (Fig. 4G). To ensure a sufficient amount of WT help for

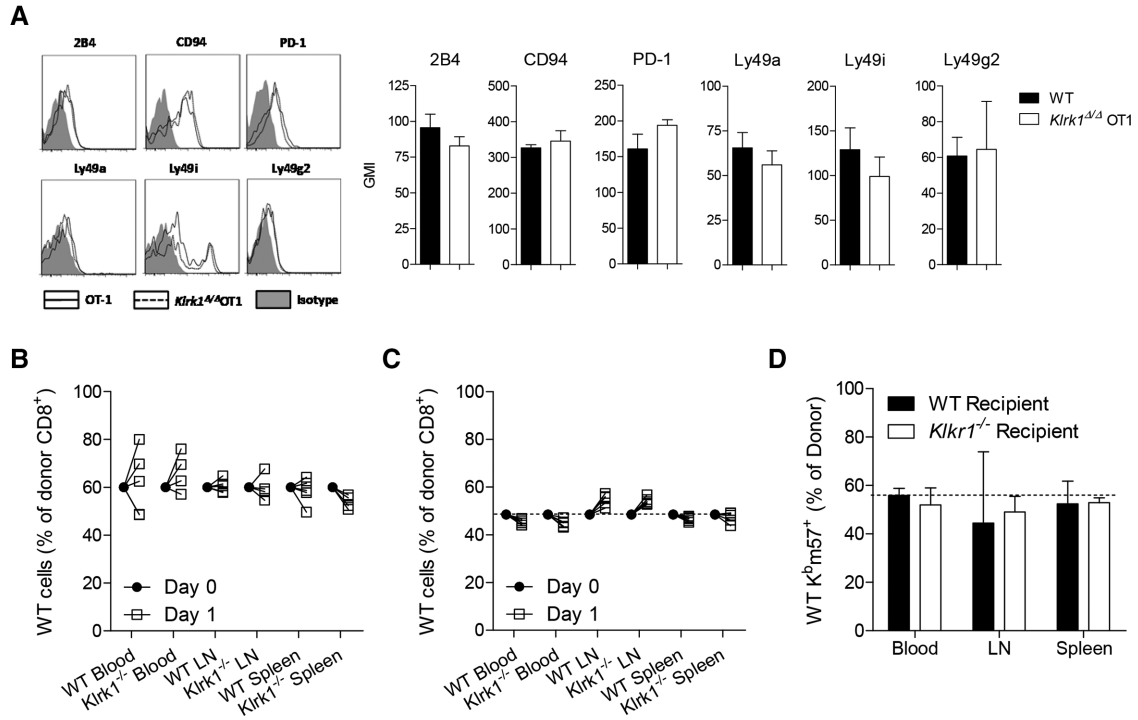

Figure 3. NKG2D does not affect surface receptor expression or homing. (**A**) Recipient mice (CD45.1) received OT-1 or Klrk1$^{\Delta/\Delta}$OT1 cells (both CD45.2) and were infected with mCMV-SIINFEKL 1 day later. After 7 days, the phenotype of donor cells in spleen was determined by FACS. Shown is geometric mean intensity (GMI) of which background signal is subtracted. (**B**) WT (CD45.1) and *Klrk1*$^{-/-}$ (CD45.2) splenocytes were mixed in a 1:1 ratio, labeled with CFSE and transferred in naive WT or *Klrk1*$^{-/-}$ hosts (CD45.2). Twenty four hours after transfer (day 1), donor cell numbers were analyzed. Shown is the percentage of WT cells within the donor (CFSE$^+$) CD8$^+$ T-cell pool in blood, LN, and spleen before (day 0) and after injection. (**C**, **D**) WT (CD45.1) and *Klrk1*$^{-/-}$ (CD45.2) mice were infected with mCMV. Seven days later, splenocytes were isolated, mixed in a 1:1 ratio, labeled with CFSE and transferred in coinfected (CD45.2) WT or *Klrk1*$^{-/-}$ hosts. Twenty four hours after transfer, the percentage of donor WT cells within (**C**) total CD8$^+$ T cells and (**D**) CFSE$^+$K^{b}m57$^+$ effector cells was determined in the indicated organs and compared with the percentage before injection (dashed line). Data shows means ± SEM and are representative of two experiments with three to five mice per experiment.

expanding CD8$^+$ T cells, mBMCs were generated using WT and *Klrk1*$^{-/-}$ BM mixed in a 3:1 ratio. Also under these conditions, total CD8$^+$ T-cell expansion was unaffected by deficiency for NKG2D, whereas IFN-γ production was reduced upon mCMV infection (data not shown). Thus, IFN-γ production, but not CD8$^+$ T-cell expansion is reduced in absence of NKG2D.

To investigate the impact of NKG2D-deficiency on CD8$^+$ T cells in a second viral model, effector cell responses in mBMCs were analyzed 8 days after LCMV infection. Again, we did not observe changes in the ratio between WT and *Klrk1*$^{-/-}$ donor cells of virus-specific CD8$^+$ T cells compared to the ratio before infection (Fig. 4H). In contrast, we observed a large reduction in the ability of *Klrk1*$^{-/-}$ CD8$^+$ T cells to produce IFN-γ, both after stimulation with CD3ε antibodies and viral peptides (Fig. 4I). Thus, NKG2D promotes IFN-γ production by antiviral CD8$^+$ effector T cells also after infection with LCMV.

In summary, NKG2D deficiency does not affect antiviral CD8$^+$ T-cell expansion, but reduces IFN-γ expression in these cells.

T-cell specific NKG2D deficiency impairs cytokine production, but not proliferation of CD8$^+$ T cells

Next, we investigated whether the impaired ability of NKG2D-deficient CD8$^+$ T cells to produce cytokines was also observed in T-cell specific NKG2D-deficient mice. Therefore, animals carrying an allele of NKG2D flanked by LoxP sites [20] were crossed with mice expressing cre recombinase behind the CD4$^+$ promotor. NKG2D$^{Fl/Fl}$CD4^{Cre} (NKG2D^{CKO}) mice and NKG2D$^{Fl/Fl}$ littermates were infected with mCMV and the CD8$^+$ T-cell response was analyzed 7 days later. Again, we did not observe any differences in the relative and absolute numbers of virus-specific CD8$^+$ T cells, both for the K^{b}m57 and K^{b}m139 epitopes (Fig. 5A). In contrast, in vitro restimulation with peptides or with CD3ε antibodies showed that mice deficient for NKG2D produced significantly less IFN-γ and TNF (Fig. 5B and C). Interestingly, after stimulation with CD3 antibodies, but not with peptides, NKG2D mice also showed a reduced capacity to produce granzyme B (Fig. 5D). This suggests that NKG2D deficiency may lead to reduced cytotoxic potential of effector CD8$^+$ T cells only after a strong antigenic stimulus.

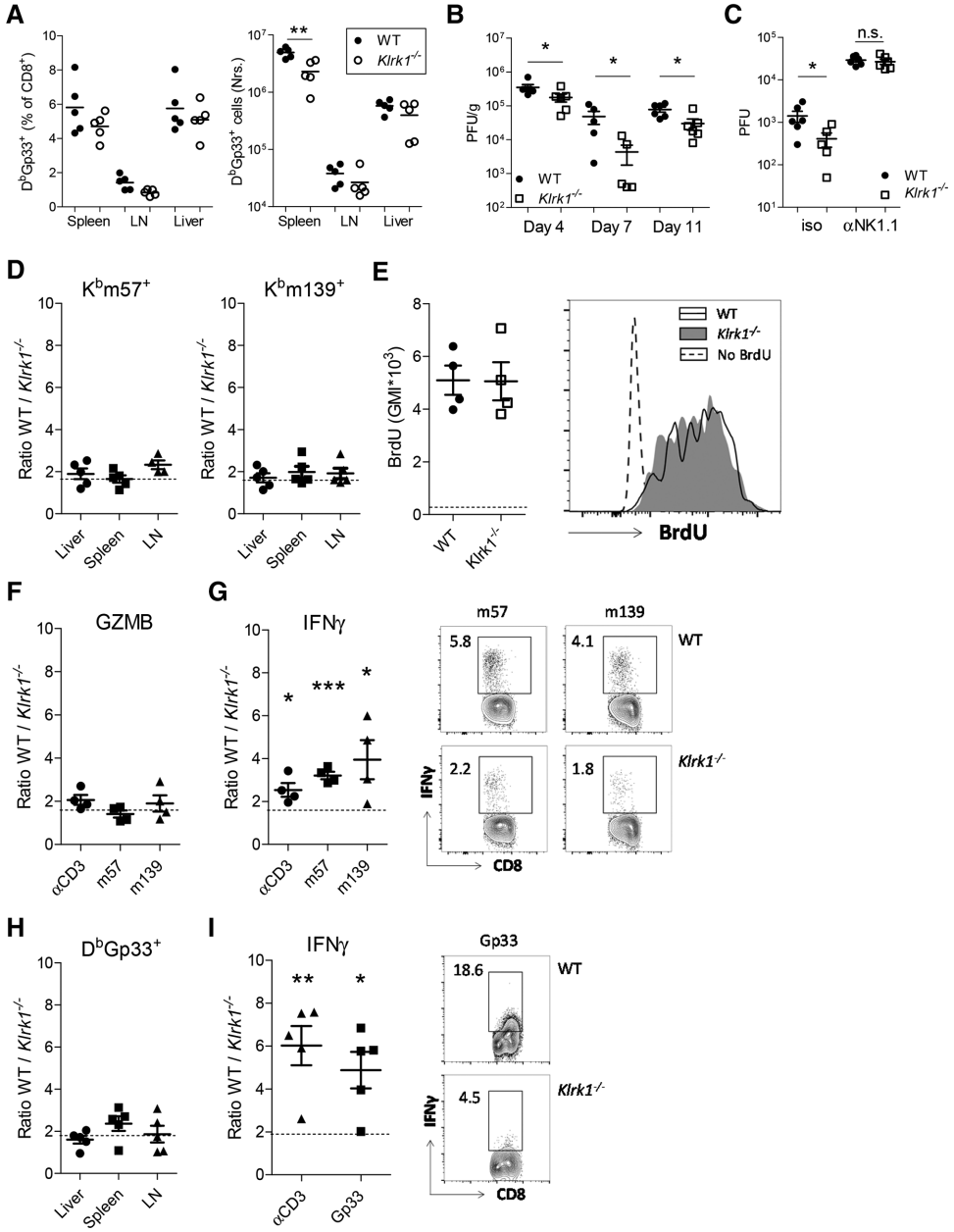

Figure 4. NKG2D deficiency impairs IFN-γ production but not proliferation of mCMV-specific CD8$^+$ T cells. (**A**) WT and *Klrk1*$^{-/-}$ mice were infected with LCMV. After 8 days, the percentage and absolute number of antigen-specific CD8$^+$ T cells was determined in spleen, inguinal LNs, and liver. (**B**) WT and *Klrk1*$^{-/-}$ mice were infected with mCMV and kinetics of viral load was followed over time in the liver. (**C**) Mice were treated with isotype or NK-cell depleting antibodies. After 1 day, mice were infected with mCMV. Four days after infection, virus titers were determined in spleen. (**D**) mBMCs (WT:*Klrk1*$^{-/-}$, 1:1) were infected with mCMV and after 8 days, ratios between WT and *Klrk1*$^{-/-}$ cells were determined within K^{b}m57$^+$ and K^{b}m139$^+$ effector cells. Dashed lines indicate CD8$^+$ T-cell ratio in blood before infection. (**E**) mBMCs were infected with mCMV. On days 1, 3, 5, and 7 after infection, mice were injected with BrdU. On day 8 after infection, BrdU labeling of effector CD8$^+$ T cells was determined in blood. Dashed line indicates BrdU staining of CD8$^+$ T cells from animals that were not injected with BrdU. (**F and G**) mBMCs were infected with mCMV. On day 8 after infection, the ratio within (**F**) granzyme B (GZMB) and (**G**) IFN-γ-producing cells after in vitro restimulation of splenic T cells with anti-CD3ε antibodies or with m57 or m139 peptides. FACS plots are gated for CD8$^+$ cells. (**H–I**) mBMCs (WT:*Klrk1*$^{-/-}$, 1:1) were infected with LCMV and after 8 days, ratios between WT and *Klrk1*$^{-/-}$ cells were determined in spleen. Dashed lines indicate CD8$^+$ T-cell ratio in blood before infection. (**H**) Ratio within K^{b}Gp33$^+$ effector cells. (**I**) Ratio within IFN-γ-producing cells after in vitro restimulation with anti-CD3ε antibodies or Gp33 peptides. FACS plots are gated for CD8$^+$ cells. Data shows mean ± SEM and are representative of two (**A–C, E, H–I**), or four (**D, F**) experiments with four to five mice per experiment. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ (Student's *t*-test or ANOVA with Bonferroni's post-test).

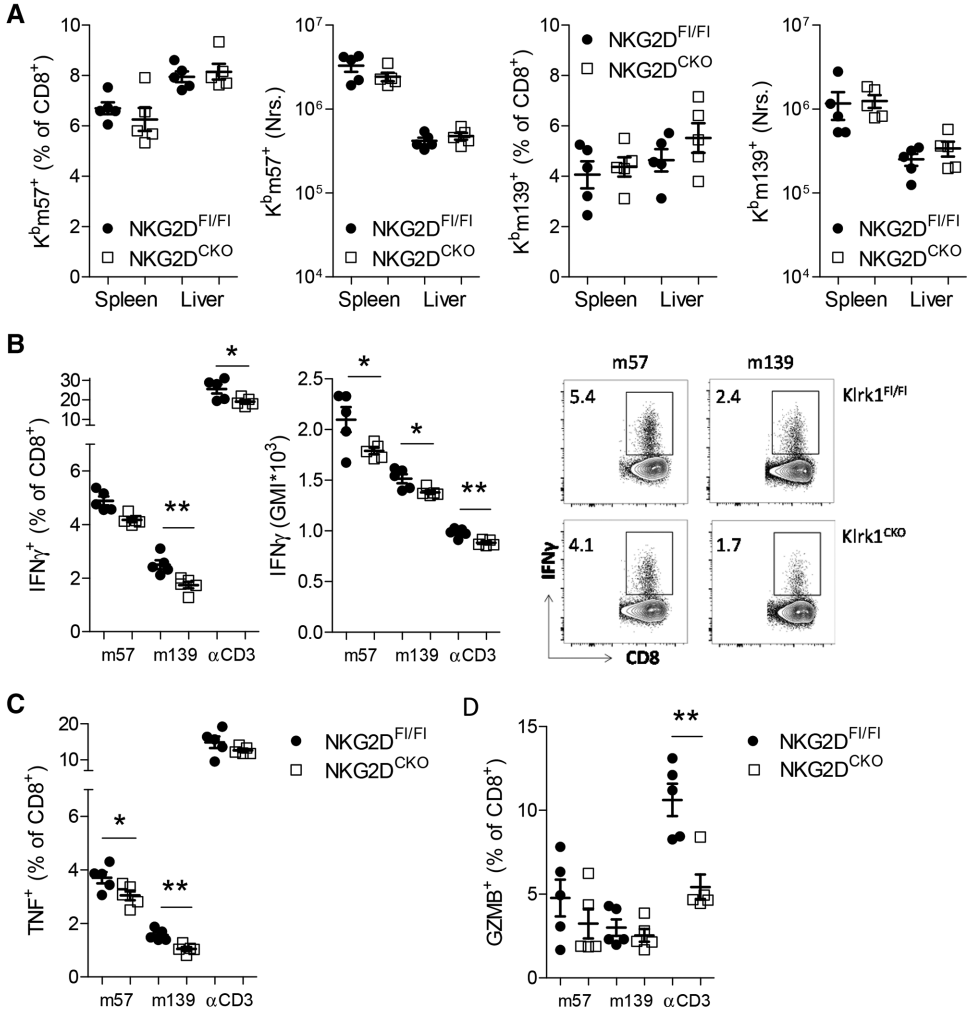

Figure 5. T-cell specific NKG2D deficiency impairs cytokine production, but not proliferation of CD8$^+$ T cells. NKG2D$^{Fl/Fl}$CD4^{Cre} (NKG2D^{CKO}) mice and NKG2D$^{Fl/Fl}$ littermates were infected with mCMV and antiviral responses were analyzed after 7 days. (**A**) Relative and absolute K^{b}m57- and K^{b}m139-specific CD8$^+$ T-cell numbers. (**B**) IFN-γ, (**C**) TNF, and (**D**) granzyme B production was analyzed after in vitro restimulation of splenic T cells, of NKG2DFl/Fl (closed spheres) or NKG2Dcko (open squares) mice with viral peptides or CD3ε antibodies. Data show means ± SEM and are representative of two experiments with five mice per experiment. $^*p < 0.05$, $^{**}p < 0.005$ (Student's *t*-test).

NKG2D deficiency impairs control of viral replication by CD8$^+$ T cells

To allow direct analysis of the antiviral capacity of NKG2D-deficient CD8$^+$ T cells, OT1 T cells were mixed with *Klrk1*$^{\Delta/\Delta}$OT1 cells in a 1:1 ratio and transferred to WT recipients. Next, recipients were infected with mCMV-SIINFEKL and donor cell expansion was analyzed after 8 days. Donor cells had strongly expanded and formed a significant fraction of the total CD8$^+$ T-cell pool (Fig. 6A). However, similar to our observations using mBMCs, NKG2D deficiency did not impact the ability of CD8$^+$ T cells to expand in response to viral infection (Fig. 6B). IFN-γ production, in contrast, was significantly impaired in *Klrk1*$^{\Delta/\Delta}$OT1 cells (Fig. 6C). Thus, also in this transfer model, cytokine production but not proliferation is impaired in CD8$^+$ cells lacking NKG2D.

To investigate the cytotoxic capacity of NKG2D-deficient CD8$^+$ T cells, OT1 or *Klrk1*$^{\Delta/\Delta}$OT1 cells were transferred to WT (CD45.1) recipients and animals were infected with mCMV-SIINFEKL. After 8 days, mice were injected with CD45.1$^+$ splenocytes pulsed with SIINFEKL, m57, or influenza peptides. After 16 h, killing of CD45.1$^+$ cells was analyzed. The endogenous response resulted in a reduction of m57-pulsed cells, though it was much less potent than the SIINFEKL-directed response that is for more than 90% dependent on donor cells (Fig. 6C). However, OT1 or *Klrk1*$^{\Delta/\Delta}$OT1 cells displayed no difference in their capacity to kill SIINFEKL-pulsed cells (Fig. 6D). Thus, NKG2D deficiency does not reduce cytotoxicity of CD8$^+$ T cells.

To investigate the ability of NKG2D-deficient CD8$^+$ T cells to control viral replication, mice were transferred with OT1 or *Klrk1*$^{\Delta/\Delta}$OT1 cells. Next, animals were infected with mCMV-SIINFEKL or mCMV and virus titers were followed over time

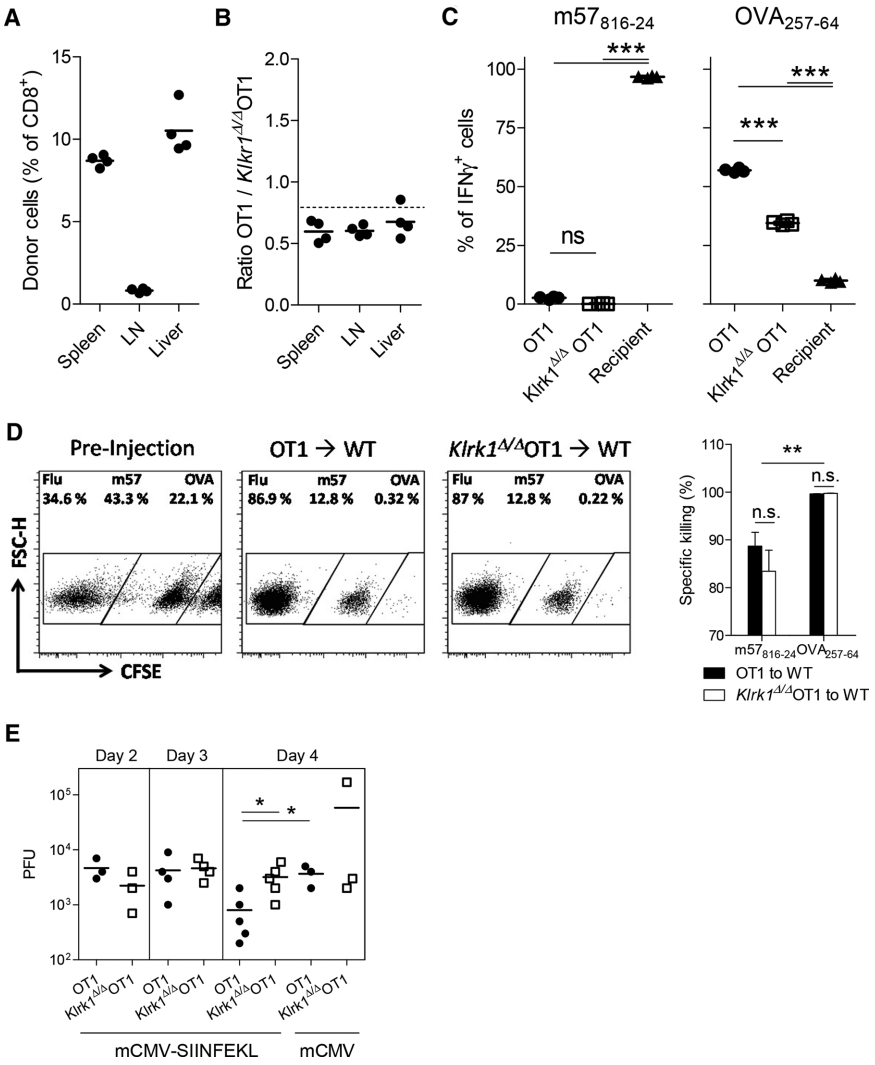

Figure 6. NKG2D deficiency impairs control of viral replication by CD8$^+$ T cells. (A–C) OT1 (CD45.1/2) and *Klrk1*$^{\Delta/\Delta}$OT1 (CD45.2) cells were mixed in a 1:1 ratio and cells were transferred to WT recipients (CD45.1). After 24 h, mice were infected withPFU mCMV-SIINFEKL and 8 days later CD8$^+$ effector T-cell responses were analyzed. (**A**) Fraction of donor cells within the total CD8$^+$ T-cell pool (**B**) Ratio between OT1 and *Klrk1*$^{\Delta/\Delta}$OT1 effector cells. (**C**) IFN-γ production of CD8$^+$ T cells from spleen restimulated in vitro with m57 or Ova peptides. (**D**) OT1 or Klrk1$^{\Delta/\Delta}$OT1 cells (both CD45.2) were transferred in naïve WT (CD45.2) recipients. Twenty four hours after transfer, mice were infected with mCMV-SIINFEKL. Eight days after infection, splenocytes of naïve WT mice (CD45.1) were labeled with high, intermediate, or no levels of CFSE and pulsed with SIINFEKL, m57, or Flu peptides, respectively, and mixed in a 1:1:1 ratio. Cells were injected in infected animals and after 4 h, the ratio within donor cells was determined in the spleens of infected mice. Gates were set on CD45.1$^+$CD45.2$^-$ cells. (**E**) OT1 or *Klrk1*$^{\Delta/\Delta}$OT1 cells were transferred in naïve WT recipients. Next, NK cells were depleted with αNK1.1 antibodies. Twenty four hours later, mice were infected with mCMV or mCMV-SIINFEKL. Viral titers were determined in spleens. Data show means ± SEM and are representative of two (**D**, **E**) or three (**A–C**) experiments with three to five mice per experiment. $^{**}p < 0.005$, $^{***}p < 0.001$ (Student's *t*-test, ANOVA with Bonferroni's post-test or Mann–Whitney test).

in the spleen. On day 4 after infection, mice infected with mCMV-SIINFEKL showed significantly lower virus titers in the spleen compared to animals receiving mCMV (Fig. 6E). Strikingly, *Klrk1*$^{\Delta/\Delta}$OT1 cells failed to provide protection against mCMV-SIINFEKL infection. Thus, NKG2D deficiency impairs the capacity of CD8$^+$ T cells to control viral replication when strong antigens such as SIINFEKL are expressed by a pathogen. In summary, NKG2D deficiency does not affect the direct killing capacity of CD8$^+$ T cells, but reduces its ability to control viral replication, most likely as a result of their impaired ability to produce cytokines.

NKG2D stimulation during priming promotes cytokine production

To investigate directly whether NKG2D stimulation during priming regulates the ability of CD8$^+$ T cells to produce cytokines we used an in vitro model for effector cell differentiation [21]. Previously, we generated 3T3 cells overexpressing Rae1δ, Rae1ε, Mult1, and H60 (Fig. 7A and [22, 23]), the NKG2D ligands present in C57BL/6 mice. Purified OT-1 cells were stimulated with different concentrations of SIINFEKL peptide, after which they were placed on a monolayer of our 3T3 cell lines in the presence of IL-2. After 2 days, cytokine production was assessed upon peptide restimulation. Rae1δ, Rae1ε, and H60, but not Mult-1 promoted IFN-γ and TNF production in OT-1 T cells after suboptimal TCR stimulation (Fig. 7B–D). This effect was mediated through transcriptional regulation, since IFN-γ mRNA levels closely correlated with protein expression (Fig. 7C and E). Finally, we questioned whether CD8$^+$ T cells are primed through Dap10 or Dap12. T cells of WT, Dap10$^{-/-}$, or Dap12$^{-/-}$ mice were stimulated in vitro with anti-CD3ε antibodies, after which they were cultured for 2 days on 3T3 or 3T3-Rae1δ cells in the presence of IL-2. Peptide restimulation showed that Rae1δ promoted IFN-γ production in WT and Dap12$^{-/-}$ cells, but failed to achieve this effect in Dap10$^{-/-}$ cells (Fig. 7F).

Thus, Dap10-dependent NKG2D stimulation during priming regulates the ability of effector CD8$^+$ T cells to produce cytokines through transcriptional control.

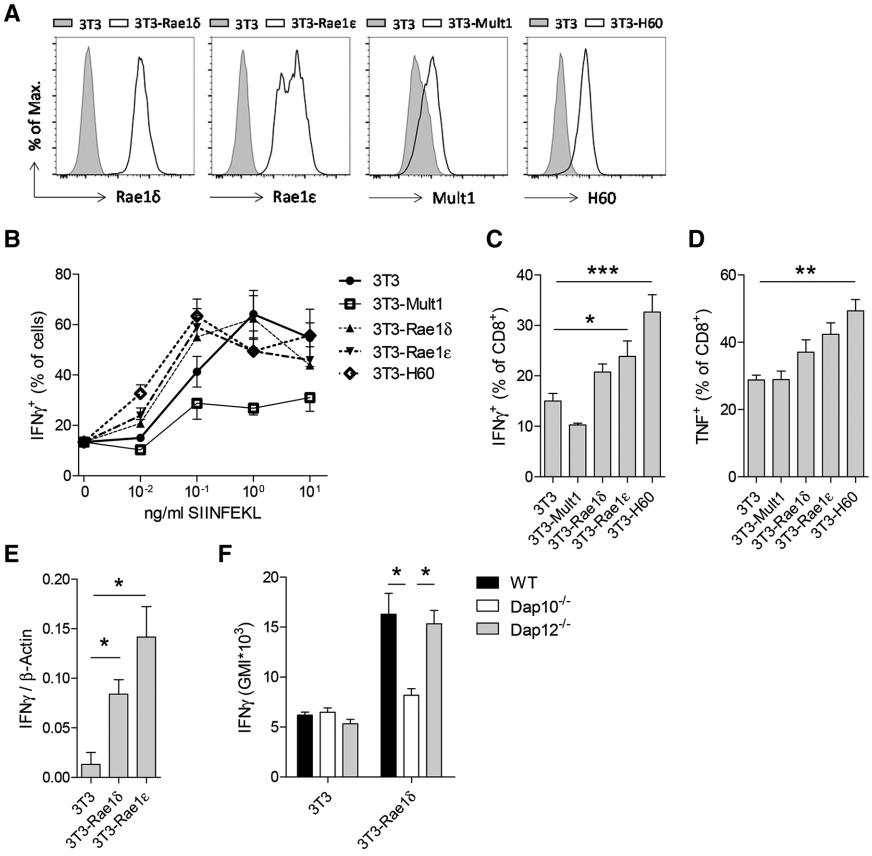

Figure 7. NKG2D stimulation during priming promotes cytokine production. (**A**) 3T3 cells or 3T3 cells overexpressing Rae1δ, Rae1ε, Mult-1, and H60 were stained for surface expression of Rae1δ, Rae1ε, Mult-1, and H60, respectively. Shown are representative FACS plots. (**B–E**) OT-1 T cells were purified and primed with different concentrations of SIINFEKL. Next, cells were transferred to wells with a monolayer of the indicated 3T3 cells and cultured with IL-2. After 2 days, cytokine production was analyzed by intracellular flow cytometry upon SIINFEKL restimulation. (**B**) IFN-γ production at indicated concentrations of peptide during priming. (**C**) IFN-γ and (**D**) TNF production of cells stimulated with 0.01 ng/mL SIINFEKL during priming. (**E**) IFN-γ mRNA levels of cells primed with SIINFEKL and costimulated with indicated 3T3 cell lines. Values were normalized to β-actin. (**F**) WT, Dap10$^{-/-}$, and Dap12$^{-/-}$ splenocytes were primed with anti-CD3ε antibodies. Next, cells were transferred to wells with a monolayer of 3T3 or 3T3-Rae1δ cells and cultured 2 days with IL-2. IFN-γ production was analyzed by intracellular flow cytometry 4 h after PI restimulation. Data show means ± SEM and are representative of one (**E**), or two (**A–D**, **F**) experiments with three mice per experiment. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ (Student's t-test or ANOVA with Bonferroni's post-test).

IL-15 superagonist rescues the reduced ability of *Klrk1*$^{-/-}$ CD8^{+} T cells to produce IFN-γ

We next investigated whether CD8^{+} T cells stimulate NKG2D in cis, or whether other immune cell subsets are involved. In vitro generated effector CD8^{+} T cells did not express NKG2D ligands (Fig. 8A). Therefore, we measured NKG2D ligands on classical dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), macrophages and B cells, which represent the main APC subsets, in mice infected 40 h prior with mCMV or in uninfected controls. Noninfected mice did not express NKG2D ligands on any of the investigated populations (Fig. 8B and C and data not shown). However, infection resulted in upregulation of H60 on a subset of cDCs. H60-positive cDCs expressed lower levels of CD11c (Fig. 8D), which is associated with an activated phenotype [24]. Thus, in vivo, NKG2D on CD8^{+} T cells is likely triggered in trans by H60 on cDCs during priming.

NKG2D potentiates IL-15 signaling toward PI3 kinase to promote memory CD8^{+} T-cell formation [16]. We questioned whether enhanced IL-15 signaling during priming in vivo could overcome the reduced capacity of NKG2D-deficient effector CD8^{+} T cells to produce IFN-γ. WT and *Klrk1*$^{-/-}$ mice were infected with mCMV. After 2 days, mice were injected with PBS or with a mixture of IL-15 and IL-15Rα-F_c fusion protein, which forms an IL-15 superagonist (IL-15SA) [25]. Eight days after infection, the ability of CD8^{+} T cells to produce IFN-γ was analyzed. IL-15SA did not increase the capacity of WT CD8^{+} T cells to produce IFN-γ in response to peptide restimulation, indicating already optimal IL-15 access. However, production of IFN-γ by *Klrk1*$^{-/-}$ cells was restored to WT levels after administration of IL-15SA (Fig. 8E).

Thus, IL-15 stimulation is able to overcome the reduced ability of *Klrk1*$^{-/-}$ effector CD8^{+} T cells to produce IFN-γ.

Discussion

NKG2D was shown to be able to promote CD8^{+} T-cell proliferation, cytotoxicity, and cytokine proliferation in various experimental settings [19, 26]. However, due to the broad impact of NKG2D deficiency on key antiviral immune cell subsets [15], until now it was unclear whether NKG2D is a costimulatory molecule for CD8^{+} T cells in vivo, or only targets them to cells expressing NKG2D ligands. We show that during viral infection, NKG2D is not required for CD8^{+} T-cell expansion. Also, NKG2D deficiency had a minor impact on cytotoxic potential, which only became apparent following strong stimuli. However, we demonstrate that NKG2D has a nonredundant costimulatory role on effector CD8^{+} T cells that potentiates their ability to produce cytokines.

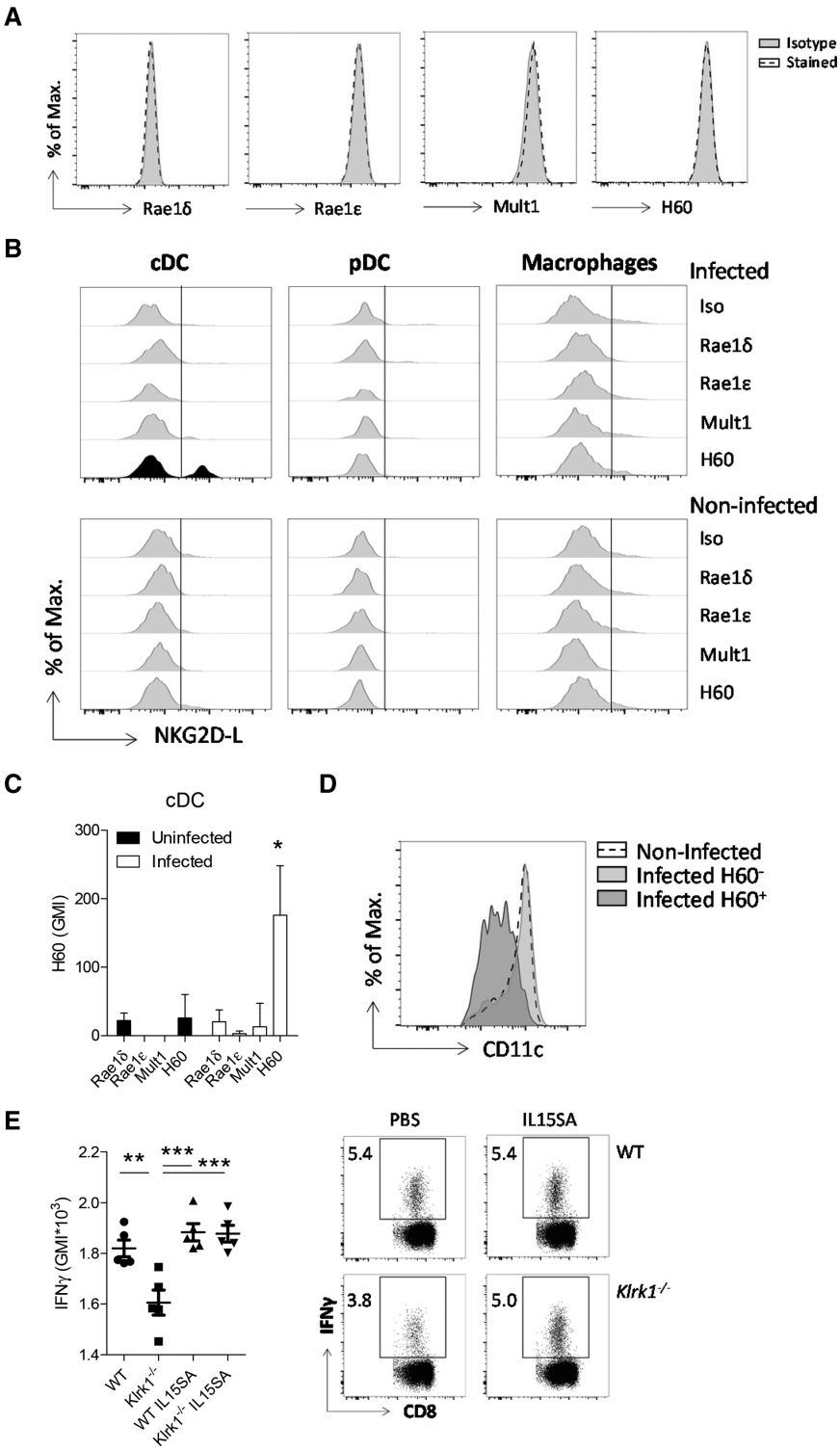

Figure 8. IL-15 superagonist rescues deficient IFN-γ production of *Klrk1*$^{-/-}$ cells. (**A**) OT-1 T cells were stimulated as described for Fig. 7B. NKG2D ligand expression was assessed by flow cytometry. Shown are representative histograms. (**B–D**) Mice were injected i.v. with PBS or mCMV. After 40 h NKG2D ligand expression was assessed in splenic cDC (MHC-II$^+$CD11c$^+$CD11b$^+$F4/80$^-$PDCA-1$^-$), pDC (MHC-II$^{+/dim}$CD11c$^{+/dim}$CD11b$^-$F4/80$^-$PDCA-1$^+$), and macrophages (CD11b$^+$F4/80$^+$MHC-II$^+$CD19$^-$GR1$^-$) by flow cytometry. (**B**) Shown are representative FACS plots (**C**) quantification of H60 expression on cDC's. (**D**) CD11c expression on total cDC's of noninfected mice and H60$^+$ and H60$^-$ Cdc's of infected mice. (**E**) WT and *Klrk1*$^{-/-}$ mice were infected with mCMV. After 48 h, mice were injected once with PBS or IL-15 superagonist. Seven days after infection IFN-γ production was analyzed after in vitro restimulation of splenocytes with m57 peptides. Data show means ± SEM and are representative of two experiments with five mice per experiment. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ (Student's *t*-test or ANOVA with Bonferroni's post-test).

The impact of a particular costimulatory signal depends both on the regulation of ligands on APCs and on receptor expression on T cells. For example, CD27 and CD28 are both constitutively expressed on CD8$^+$ T cells [27]. However, whereas CD28 ligands are broadly expressed, the CD27 ligand CD70 is only transiently induced on a restricted number of cells [27, 28]. Other costimulatory receptors, such as OX40 are only induced several days after infection [29]. CD28 is therefore important for the activation and expansion of naïve CD8$^+$ T cells, whereas OX40 promotes survival of activated effector CD8$^+$ T cells several days after initial antigen encounter [29]. Differential regulation of costimulatory ligands and their receptors appears to be a way

in which the efficiency and magnitude of the effector response is fine-tuned to the requirements of a specific infection [30, 31]. Our findings indicate that, in the case of viral infection, the costimulatory role of NKG2D is mostly restricted to regulation of cytokine production. Interestingly, infected mBMCs did not show a difference in granzyme B production between WT- and NKG2D-deficient cells after infection, whereas NKG2D^{CKO} mice did. This may reflect an unappreciated role for NKG2D in CD4$^+$ helper T-cell function.

The discrepancy of our data with in vitro studies that show a role for NKG2D in CD8$^+$ T-cell expansion [11, 19, 26] is possibly the result of differences in intensity of NKG2D stimulation. In CD8$^+$ T cells, NKG2D is linked to DAP10 and signals to PI3 kinase (PI3K) [8]. PI3K is a potent signaling nexus that is able to promote proliferation, cytotoxicity, and cytokine production via various downstream signaling cascades [32]. The signal intensity upstream of PI3K controls which downstream pathways are activated. Low-intensity PI3K activation drives phosphorylation of Akt at Thr308, which is sufficient to induce cytokine production [33]. High-intensity PI3K stimulation results in activation of the mTOR signaling cascade, phosphorylating a second site on Akt (Ser473) that enhances proliferation and induces expression of cytotoxic molecules [2, 34]. Our findings suggest that CD8$^+$ T cells encounter sufficient NKG2D ligands during priming to promote cytokine production, yet not enough to also drive proliferation in vivo. Since IL-15 is a potent inducer of PI3K-mediated effector cell functions [2], this might explain why IL-15 superagonists were able to overcome the effects of NKG2D deficiency. In addition, cytokine production depends more on PI3K signaling than cytotoxicity. In NK cells, loss of the PI3K subunit p110δ prevented cytokine production, whereas cytotoxicity was preserved [35].

Our findings do not downplay the important role of NKG2D in control of viral infection. CMV expresses several immunoevasins, such as m152, which actively prevent surface expression of NKG2D ligands [36]. Infection with m152-deficient viruses therefore results in reduced viral titers, partially because of an enhanced T-cell response [37]. Indeed, NKG2D-deficient CD8$^+$ T cells did have a reduced capacity to produce cytokines and had impaired capacity to control viral replication, both after mCMV and LCMV infection. Importantly, NKG2D-deficient effector CD8$^+$ T cells were hyporesponsive to TCR engagement compared to WT cells, even in the absence of overt NKG2D ligation. Thus, NKG2D mediates both priming and direct target engagement of CD8$^+$ T cells.

Our findings should be viewed within the limitations of our study, such as the restricted number of antigen-specific responses investigated. As shown in Fig. 7B, the effect of NKG2D-priming is optimal within a window of T-cell stimulation strength. Figure 5D shows that NKG2D may have a similar impact on regulation of cytotoxicity. We believe that our experiments show the role of NKG2D on CD8$^+$ T-cell priming in the most physiological models to date. However, we should not disregard the discrepancy between the costimulatory potential of NKG2D shown by others [9, 10] and the actual role that we observe in vivo, which may depend on the antigens studied. It will be interesting to see whether NKG2D has a more potent role in CD8$^+$ T-cell priming against other epitopes, most notably those of a tumor origin.

In summary, we show that during viral infection, the role of NKG2D extends beyond the direct killing of ligand-expressing target cells and actively contributes to priming of effector CD8$^+$ T cells. Our findings have important implications for vaccine strategies against viruses and tumors that aim to exploit NKG2D signaling in CD8$^+$ T cells to enhance their effectiveness.

Materials and methods

Mice

Mice were age- and sex-matched within experiments and handled according institutional and national guidelines. All animal experiments were performed after approval of our Institute's Animal Ethics Committee. C57BL/6 (B6), OT-1, B6 CD45.1, and CD4^{Cre} mice were purchased from The Jackson Laboratory. Dap10$^{-/-}$ and Dap12$^{-/-}$ mice were provided by M. Colonna. *Klrk1*$^{-/-}$, *Klrk1*$^{Fl/Fl}$, and *Klrk1*$^{\Delta/\Delta}$ mice were generated as described previously [15, 20]. All lines were kept as breeding colonies in our local animal facility under specific pathogen-free conditions.

Surface and intracellular staining, Abs, and tetramers

Cell surface mAbs were purchased from eBioscience and BD Biosciences, except for Rae1ε (R&D systems). Antibodies against Rae1δ, Mult1, and H60 were made in-house [22, 23]. MHC class I tetramers were a kind gift of Dr. Anja ten Brinke (Sanquin Research). CFSE (Molecular Probes) and BrdU (BD Biosciences) labeling and staining were performed according to manufacturers' protocols. For BrdU labeling, mice were injected i.p. every second day with BrdU (0.8 mg). Intracellular staining was performed using the BD Fix/Perm kit (cytokines) or the BrdU flow kit (BrdU). Flow cytometry was measured on a FACScan, FACSCalibur, FACSVerse, or FACSAria (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Viral infections and in vivo experiments

The BAC-derived murine CMV strain MW97.01 is biologically equivalent to the mCMV Smith strain (VR-1399; ATCC) and is referred to as mCMV [38]. mCMV strains were propagated on MEF. NK-cell depletion was accomplished by i.p. injection of anti-NK1.1 Abs (clone PK136; made in-house). Viral titers were determined on MEF by standard plaque assay. LCMV (Armstrong) was propagated on BMK cells according to standard protocol.

For in vivo IL-15 stimulation, mice were injected 48 h after infection with IL-15/IL-15-Ra-Fc (0.2 mg/0.93 mg; R&D Systems) as described [25]. BM recipients were lethally irradiated (9 Gy). Recipient mice received 10^7 donor BM cells i.v. mBMCs received WT and *Klrk1*$^{-/-}$ BM mixed in a 1:1 ratio. Experiments were performed at least 8 weeks after transfer. OT1 or *Klrk1*$^{\Delta/\Delta}$OT1 T cells were transferred i.v. using 10 000 cells per recipient. For homing experiments, splenocytes were isolated from naïve mice or mice infected 7 days prior with mCMV. Donor cells were mixed in a 1:1 ratio and labeled with CFSE. Cells were injected i.v. in naïve or coinfected mice. After 24 h, cell ratios were determined in recipients. For in vivo killing experiments, naïve CD45.1 splenocytes were labeled with high (10 μg/mL), intermediate (1 μg/mL), or no CFSE and pulsed with peptides (1 μg/mL). Populations were mixed in 1:1:1 ratios and injected in CD45.2 recipients. Ratio between donor cells was determined after 16 h.

In vitro stimulations

For in vitro generation of effector cells, CD8$^+$ T cells were purified from spleens by positive selection using the MACS cell separation system (Myltenyi), CFSE labeled, and cultured for 27 h with SIINFEKL peptide (Genscript) or agonistic anti-CD3ε (145-2C11) and anti-CD28 (37.51, eBioscience) antibodies in RPMI-1640 medium (PAN-Biotech), supplemented with 10% FCS (PAN-Biotech) and 2-ME (Sigma-Aldrich). Next, cells were washed and cultured for 2 days with 100 ng/mL IL-2. 3T3 and NKG2D-L 3T3 cells were generated previously [22, 23]. In costimulation assays, cells were cultured for 2 h in presence of Mytomycin C (10 μg/mL) to block proliferation. Next, cells were washed and plated out as a monolayer 24 h before transfer of activated CD8$^+$ T cells. For cytokine staining, cells were first restimulated 4 h in vitro with 10 ng/mL peptides (Gp33-KAVYNFATC; m57-SCLEFWQRV; m139-TVYGFCLL; Ova-SIINFEKL) or 5 mg/mL anti-CD3ε (145-2C11) in the presence of Brefeldin A (eBioscience).

Acknowledgments: We thank S. Slavić-Stupac and M. Samsa for technical assistance. This work was supported by the Netherlands Organization for Scientific Research (91614029 to F.M.W.), the European Commission (FP7-PEOPLE-2010-IEF-274995 and PCIG14-GA-2013-630827 to F.M.W.), a University of Rijeka Support grant to F.M.W. (865.10.2101) and the European Social Fund for Croatia (HR.3.2.01-0263 to B.P.).

Conflict of interest: The authors declare no commercial or financial conflict of interest.

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Abbreviations: **cDCs**: classical DCs · **Klrk1**: killer cell lectin like receptor K1 · **LCMV**: lymphocytic choriomeningitis virus · **mBMC**: mixed bone marrow chimera · **mCMV**: mouse CMV · **NKG2D**: NK group 2 member D

Full correspondence: Dr. Felix Wensveen, Department of Histology and Embryology, Brace Branchetta 20, 51000, Rijeka, Croatia
e-mail: Felix.Wensveen@gmail.com

Received: 11/11/2016
Revised: 28/2/2017
Accepted: 30/3/2017
Accepted article online: 4/4/2017

