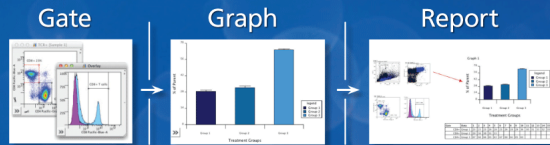


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# NKG2D Induces Mcl-1 Expression and Mediates Survival of CD8 Memory T Cell Precursors via Phosphatidylinositol 3-Kinase

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Memory formation of activated CD8 T cells is the result of a specific combination of signals that promote long-term survival and inhibit differentiation into effector cells. Much is known about initial cues that drive memory formation, but it is poorly understood which signals are essential during the intermediate stages before terminal differentiation. NKG2D is an activating coreceptor on Ag-experienced CD8 T cells that promotes effector cell functions. Its role in memory formation is currently unknown. In this study, we show that NKG2D controls formation of CD8 memory T cells by promoting survival of precursor cells. We demonstrate that NKG2D enhances IL-15–mediated PI3K signaling of activated CD8 T cells, in a specific phase of memory cell commitment, after activation but before terminal differentiation. This signal is essential for the induction of prosurvival protein Mcl-1 and precursor cell survival. In vivo, NKG2D deficiency results in reduced memory cell formation and impaired protection against reinfection. Our findings show a new role for PI3K and the NKG2D/IL-15 axis in an underappreciated stage of effector to memory cell transition that is essential for the generation of antiviral immunity. Moreover, we provide novel insights how these receptors control both effector and memory T cell differentiation. *The Journal of Immunology*, 2013, 191: 000–000.

Upon activation, CD8 T cells form both effector and memory type cells. Lineage choice is influenced by extracellular cues such as cytokines and coreceptors, which promote entry into a differentiation pathway (1). It has been suggested that after memory lineage commitment is initiated, effector to memory transition is a passive process that continues independently of instructions (1). However, it is becoming increasingly clear that this intermediate phase is also subject to regulation. Long after the initial antigenic trigger is cleared from the system, memory precursors continue to differentiate to obtain full memory cell properties (2). Moreover, lack of prosurvival factors in this stage results in a loss of memory cells (3). How effector to memory transition is regulated on a molecular level is poorly understood. Elucidation of factors that play a role in this

process is important, because such factors ultimately control the size and effectiveness of the memory compartment.

NKG2D is a potent activating receptor that is expressed on most cytotoxic cells, including effector and memory  $\alpha\beta$  CD8 T cells (4). NKG2D recognizes MHC class I–like ligands, of which cell surface expression is generally low. In response to stress, such as infection, NKG2D ligands are highly induced (5). Engagement of NKG2D promotes cytotoxic clearance of target cells (6), and this receptor was therefore thought to be exclusively involved in effector functions. During the last few years, evidence accumulated that NKG2D has additional immunomodulatory roles. NKG2D deficiency affects development and responsiveness of NK cells (7). Additionally, NKG2D ligands were found on activated dendritic cells (8), indicating a role for NKG2D in priming of naive T cells. More recently, NKG2D was implied in effector functions of memory CD8 T cells. In the absence of CD4 T cells, the presence of NKG2D ligands during priming rescued recall capabilities of memory CD8 T cells (9).

NKG2D signals in close interaction with the IL-15 receptor. IL-15R stimulation induces NKG2D expression and potentiates cytotoxicity and proliferation of NK and CD8 T cells (10). Moreover, both receptors bind and activate the adaptor molecule Dap10. Transgenic overexpression of a Dap10/ubiquitin fusion protein, which targets Dap10 for proteasomal degradation, greatly impairs both NKG2D and IL-15 signaling in NK cells (11). IL-15 plays a key role in memory cell homeostasis. Mice deficient for IL-15 have a severe reduction in memory cell numbers (12). If and how interactions between NKG2D and IL-15 signaling mediate memory T cell formation are currently unknown.

In this study, we investigated how NKG2D controls memory CD8 T cell formation. We find that NKG2D mediates memory precursor formation in a Dap10-dependent way. NKG2D-deficient mice have curtailed recall responses and reduced viral clearance upon reinfection. NKG2D potentiates IL-15–mediated signaling toward PI3K in a short time window early during memory commitment. This corresponds with increased Mcl-1 protein levels

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Abbreviations used in this article: BM, bone marrow; DKO, double knockout; Eomes, eomesodermin; LCMV, lymphocytic choriomeningitis virus; LM-OVA, OVA-expressing *Listeria monocytogenes*; mBMC, mixed bone marrow chimera; mCMV, murine cytomegalovirus; mTORc, mammalian target of rapamycin complex; PKB, protein kinase B; T<sub>CM</sub>, central memory T; T<sub>EM</sub>, effector memory T; WT, wild-type.

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and enhanced survival of memory precursors. In summary, we present a new role for NKG2D and PI3K in CD8 memory T cell formation and provide more insight in the requirements for effector to memory cell transition.

## Materials and Methods

### Mice

Mice were strictly age- and sex-matched within experiments and handled in accordance with institutional and national guidelines. All animal experiments were performed after approval of our Institute's Animal Ethics Committee. C57BL/6 (B6), OT-1, and B6 Ly5.1 mice were purchased from The Jackson Laboratory. *Hcst*<sup>-/-</sup> mice (13) were obtained from Dr. M Colonna. *Klrkl*<sup>-/-</sup> and *Klrkl*<sup>ΔΔ</sup> mice were generated as described previously (7, 14). In brief, in *Klrkl*<sup>-/-</sup> mice both transcriptional start codons of the *Klrkl* gene have been deleted and exon 3 is replaced by an enhanced GFP cassette. *Klrkl*<sup>ΔΔ</sup> mice were obtained by crossing *Klrkl*<sup>fllox</sup> mice, in which exons 2 and 3 of the *Klrkl* locus are flanked by LoxP sites, with Cre "deleter" mice. Subsequent backcrossing was performed to eliminate the Cre allele and generate homozygous deletion of functional *Klrkl* genes. All lines were kept as breeding colonies in our local animal facility under specific pathogen-free conditions.

### Surface and intracellular staining, Abs, and tetramers

All cell surface Abs were purchased from eBioscience and BD Biosciences, except anti-NKG2D (Novus Biologicals). MHC class I tetramers were generated in-house (Sanquin Research). CFSE, propidium iodide (Molecular Probes), and BrdU (BD Biosciences) labeling and staining were performed according to the manufacturers' protocols. For BrdU labeling, mice were injected i.p. daily with BrdU (0.8 mg). BrdU incorporation in blood CD8 T cells was visualized using the BrdU flow kit (BD Biosciences). Fixation and permeabilization of cells for intracellular stainings was performed using the BD Fix/Perm kit (IFN- $\gamma$ , eBioscience; Mcl-1, Rockland Immunochemicals) or the BrdU flow kit (T-bet, eomesodermin [Eomes], eBioscience). For IFN- $\gamma$  staining, cells were first restimulated 4 h in vitro with 10 ng/ml peptides (Gp33, KAVYNFATC; m57, SCLEFWQRV; m139, TVYGFCLL) or 5  $\mu$ g/ml anti-CD3 (145-2C11) in the presence of brefeldin A (eBioscience). Flow cytometry was measured on a FACScan, FACSCalibur, FACSVerse or FACSARIA (BD Biosciences) and analyzed with FlowJo software (Tree Star).

### Virus and bacterial infections, NK cell depletions, and in vivo IL-15 stimulation

The bacterial artificial chromosome-derived murine cytomegalovirus (mCMV) strain MW97.01 has previously been shown to be biologically equivalent to the mCMV Smith strain (VR-1399; American Type Culture Collection) and is referred to as wild-type (WT) mCMV. mCMV strains were propagated on mouse embryonic fibroblasts. NK cell depletion was accomplished by i.p. injection of anti-NK1.1 Abs in PBS (clone PK136;

made in-house). Viral titers were determined on mouse embryonic fibroblasts by standard plaque assay. Lymphocytic choriomeningitis virus (LCMV) Armstrong strain (Armstrong E-350; American Type Culture Collection) was propagated on baby mouse kidney cells according to standard protocol. Infection with *Listeria monocytogenes* overexpressing OVA (15) or Gp33 (16), cultured to exponential growth, was performed i.v. at 10<sup>4</sup> CFU per mouse. For in vivo IL-15 stimulation, mice were injected after 24 and 48 h with IL-15/IL-15-R $\alpha$ -Fc (0.2  $\mu$ g/0.93  $\mu$ g; R&D Systems) according to established protocols (17). Proliferation of donor cells was assessed in splenocytes 48 h after the last injection.

### Mixed bone marrow chimeras and adoptive transfers

Bone marrow (BM) recipients were lethally irradiated (9 Gy). Recipient mice received 10<sup>7</sup> donor BM cells i.v. Unless stated otherwise, mixed BM chimeras (mBMCs) received WT and *Klrkl*<sup>-/-</sup> BM mixed in a 1:1 ratio. Experiments were performed at least 8 wk after transfer. OT-1 or *Klrkl*<sup>ΔΔ</sup> OT-1 T cells were transferred i.v. For adoptive transfer of K<sup>b</sup>m57<sup>+</sup> cells, CD8 T cells were purified via magnetic separation (Miltenyi Biotec) and the number of Ag-specific cells was determined by FACS. Total CD8 T cells, containing 5000 K<sup>b</sup>m57<sup>+</sup> cells, were injected i.v. 24 h before infection.

### In vitro stimulations and RT-PCR

CD8 T cells were purified by magnetic cell separation (Miltenyi Biotec). Cells were cultured in RPMI 1640 medium (PAN-Biotech), supplemented with 10% FCS (PAN-Biotech) and 2-ME (Sigma-Aldrich). For in vitro memory differentiation, cells were stimulated for 2 d with 10 pg/ml SIIN-FEKL peptides (GenScript), washed, and cultured for an additional 4 d with 50 ng/ml IL-15 on plates coated with agonistic NKG2D Abs. For phosphorylated stainings, cells were deprived from stimuli for 3 h in medium with 2% FCS, followed by 15 min stimulation with 100 ng/ml IL-15. Cells were fixed with 2% paraformaldehyde, permeabilized in 90% methanol, and stained with fluorescently labeled pStat<sup>Y694</sup> or p-protein kinase B (PKB)<sup>T308</sup> Abs (Cell Signaling Technology). Ly294002 (Invitrogen) was used to inhibit PI3K signaling. For RT-PCR, total RNA was extracted using the TRIzol isolation method (Invitrogen). cDNA was generated using the SuperScript II cDNA synthesis kit (Invitrogen). Transcripts were amplified by PCR.

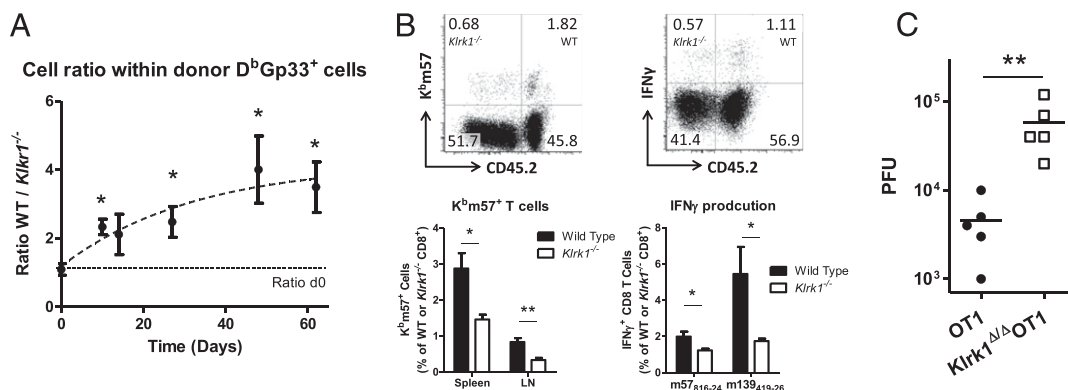
### Statistics

Values shown represent means  $\pm$  SEM, which are depicted using error bars. To analyze statistical significance, Student *t*, Mann-Whitney, and ANOVA tests were used. A *p* value < 0.05 was considered statistically significant.

## Results

### NKG2D promotes memory formation and antiviral protection

To investigate the role of NKG2D in memory CD8 T cell formation, we used mice deficient for this receptor. Unfortunately, studies of immune responses in NKG2D-deficient mice are complicated by



**FIGURE 1.** NKG2D promotes memory formation and antiviral protection. **(A)** mBMCs (WT/*Klrkl*<sup>-/-</sup>, 1:1) were infected with LCMV and changes in the ratio between donor D<sup>b</sup>Gp33<sup>+</sup> WT and *Klrkl*<sup>-/-</sup> CD8 T cells was followed over time (*n* = 6). Dashed line indicates cell ratio within the donor CD8 T cell pool before infection. **(B)** WT and *Klrkl*<sup>-/-</sup> cells of mBMCs (WT/*Klrkl*<sup>-/-</sup>, 1:1) were infected with mCMV. After 50 d, splenocytes were analyzed for donor (*left*) K<sup>b</sup>m57<sup>+</sup>- and (*right*) IFN- $\gamma$ -producing CD8 T cells after in vitro restimulation with m57<sub>816-224</sub> (*n* = 5). Representative plots are gated for donor CD8 T cells. **(C)** Naive recipients received 10<sup>4</sup> OT-1 or *Klrkl*<sup>ΔΔ</sup> OT-1 cells and were infected with 10<sup>4</sup> CFU LM-OVA. On day 42, NK cells were depleted with anti-NK1.1. Twenty-four hours later, mice were infected with mCMV-SIINFEKL. Viral titers were determined after 4 d in spleen (*n* = 5). Data are representative of two (C), three (A), or four (B) experiments. \**p* < 0.05, \*\**p* < 0.005.

altered NK cell development and function in these animals. NK cells limit effector and memory CD8 T cell expansion in a partially NKG2D-dependent way (18, 19). Moreover, NK cells of *Klrk1*<sup>-/-</sup> mice are hyperresponsive, resulting in enhanced viral clearance on day 4 postinfection (7). To avoid an impact of altered viral loads, we decided to study the effect of NKG2D deficiency on CD8 memory formation in mBMCs that contain WT and *Klrk1*<sup>-/-</sup> donor cells in a 1:1 ratio.

mBMCs were infected with LCMV, and the contribution of WT and *Klrk1*<sup>-/-</sup> cells to Ag-specific D<sup>b</sup>Gp33<sup>+</sup> cells was followed over time. Early upon infection, NKG2D-deficient cells contributed equally to the antiviral T cell population. Strikingly, from day 14 onward, the Ag-specific pool was progressively dominated by WT cells as effector cells were lost (Fig. 1A, Supplemental Fig. 1A). Similar observations were made after infection with mCMV (Supplemental Fig. 1B). When antiviral cell numbers were quantified in organs of mice at memory time points, we also observed that *Klrk1*<sup>-/-</sup> cells contributed significantly less to the Ag-specific T cell pool. Similarly, fewer *Klrk1*<sup>-/-</sup> cells produced IFN- $\gamma$  upon in vitro restimulation (Fig. 1B).

To see whether *Klrk1*<sup>-/-</sup> memory CD8 T cells also provided reduced antiviral protection, NKG2D-deficient *Klrk1* <sup>$\Delta\Delta$</sup>  animals (14) were crossed on OT-1 TCR transgenic mice (20). WT mice were transferred with OT-1 or *Klrk1* <sup>$\Delta\Delta$</sup>  OT-1 cells and infected with OVA-expressing *L. monocytogenes* (LM-OVA). Fifty days after primary infection, mice were infected with mCMV-SIINFEKL and viral titers were quantified after 4 d. Mice transferred with *Klrk1* <sup>$\Delta\Delta$</sup>  OT-1 cells showed a >10-fold increase in viral titers compared with mice that received OT-1 cells (Fig. 1C).

Thus, NKG2D is important for the formation of CD8 T cell memory and NKG2D deficiency results in impaired antiviral immunity.

#### NKG2D impairs central memory precursor cell formation

Next, we investigated whether NKG2D-deficient memory cells are only reduced in numbers, or also functionally impaired on a per cell basis. When equal numbers of OT-1 or *Klrk1* <sup>$\Delta\Delta$</sup>  OT-1 memory cells were transferred to naive recipients, no differences were observed in their capacity to clear virus (Fig. 2A). Also, homeostatic proliferation of NKG2D-deficient memory cells was not affected (Supplemental Fig. 1C). Subsequently, we investigated recall capabilities of *Klrk1*<sup>-/-</sup> memory CD8 T cells. mBMCs were primed with LCMV. After 65 d, mice were reinfected with LM-Gp33, which generates a recall response, or with LM-OVA, which does not. After 7 d, the ratio between D<sup>b</sup>Gp33<sup>+</sup> donor cells was determined. Significantly more WT cells were present in the D<sup>b</sup>Gp33<sup>+</sup> effector CD8 T cell population after infection with LM-Gp33. However, equal ratios between D<sup>b</sup>Gp33<sup>+</sup> WT and *Klrk1*<sup>-/-</sup> cells were observed after LM-Gp33 and LM-OVA infection (Fig. 2B). This indicates that secondary expansion of memory cells is not affected by NKG2D deficiency. To test this in a second model, WT and *Klrk1*<sup>-/-</sup> mice were infected with mCMV. Fifty-four days later, CD8 T cells were purified and equal numbers of K<sup>b</sup>m57 binding cells were injected in naive recipients. Subsequently, mice were infected with mCMV. Eight days after infection, *Klrk1*<sup>-/-</sup> cells had expanded equally as WT cells (Supplemental Fig. 1D). These findings indicate that NKG2D deficiency affects memory T cell formation rather than memory T cell function.

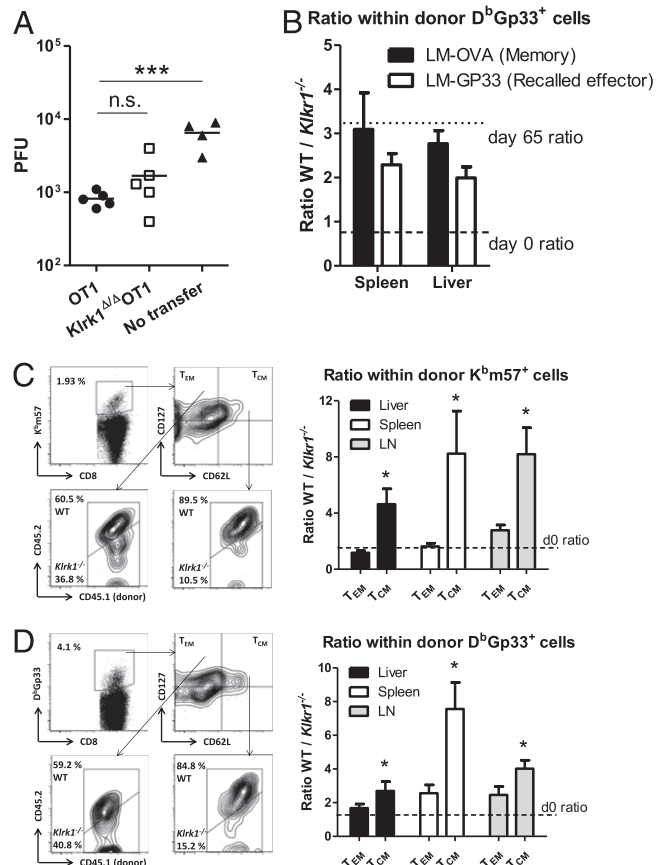
Our findings prompted us to investigate the effect of NKG2D deficiency on CD8 memory T cell precursor subpopulations during priming. Eight days after mCMV infection of mBMCs, *Klrk1*<sup>-/-</sup> cells contributed equally to both the effector and effector memory T (T<sub>EM</sub>) cell pools (Fig. 2C and data not shown). In contrast, central memory T (T<sub>CM</sub>) precursor cells, which are enriched for

cells that provide long-lasting immunity (1), contained up to 10-fold more WT than *Klrk1*<sup>-/-</sup> cells (Fig. 2C). Similar observations were made after infection with LCMV (Fig. 2D). To corroborate our observations in a third model, OT-1 (CD45.1/2) and *Klrk1* <sup>$\Delta\Delta$</sup>  OT-1 (CD45.2) cells were mixed in a 1:1 ratio and injected in WT (CD45.1) recipients. Again, NKG2D-deficient cells contributed significantly less to the donor T<sub>CM</sub> precursor cell pool after mCMV-SIINFEKL infection (Supplemental Fig. 1E).

Thus, NKG2D deficiency does not affect memory cell function but impairs T<sub>CM</sub> precursor cell formation.

#### NKG2D enhances IL-15 responsiveness of memory precursors via *Dap10*

Next, we investigated how NKG2D controls memory cell formation. In effector CD8 T cells, NKG2D is thought to signal ex-



**FIGURE 2.** NKG2D impairs central memory precursor cell formation. (A) Naive recipients (CD45.1) received  $5 \times 10^3$  OT-1 or *Klrk1* <sup>$\Delta\Delta$</sup>  OT-1 cells (CD45.2) and were infected with mCMV-SIINFEKL. Fifty-four days later CD45.2<sup>+</sup> cells were isolated and naive recipients received 5000 CD45.2<sup>+</sup> cells or PBS (WT). After 24 h, NK cells were depleted and mice were infected with mCMV-SIINFEKL. Viral titers were determined after 4 d in spleen ( $n = 5$ ). (B) mBMCs (WT/*Klrk1*<sup>-/-</sup>, 1:1) were infected with LCMV. Sixty-five days later, mice were infected with  $10^4$  CFU LM-OVA or LM-Gp33. The ratio between WT and *Klrk1*<sup>-/-</sup> cells was determined within the splenic D<sup>b</sup>Gp33<sup>+</sup> CD8 T cell population ( $n = 6$ ). (C) mBMCs (WT/*Klrk1*<sup>-/-</sup>, 1:1) were infected with mCMV. After 8 d, ratios between WT and *Klrk1*<sup>-/-</sup> cells were determined within K<sup>b</sup>m57<sup>+</sup> CD8 T<sub>EM</sub> (CD62L<sup>-</sup>CD127<sup>+</sup>) and T<sub>CM</sub> (CD62L<sup>+</sup>CD127<sup>+</sup>) cells. Dashed line indicates ratio within the total donor CD8 T cell pool before infection. Representative plots are gated for donor CD8<sup>+</sup> cells ( $n = 5$ ). (D) mBMCs (WT/*Klrk1*<sup>-/-</sup>, 1:1) were infected with LCMV. After 8 d, ratios between WT and *Klrk1*<sup>-/-</sup> cells were determined within D<sup>b</sup>Gp33<sup>+</sup> CD8 T<sub>EM</sub> and T<sub>CM</sub> cells. Dashed line indicates ratio within the total donor CD8 T cell pool before infection. Representative plots are gated for CD8<sup>+</sup> cells. Data are representative of two (A, B), three (D), or five (C) experiments. \* $p < 0.05$ , \*\*\* $p < 0.0005$ .

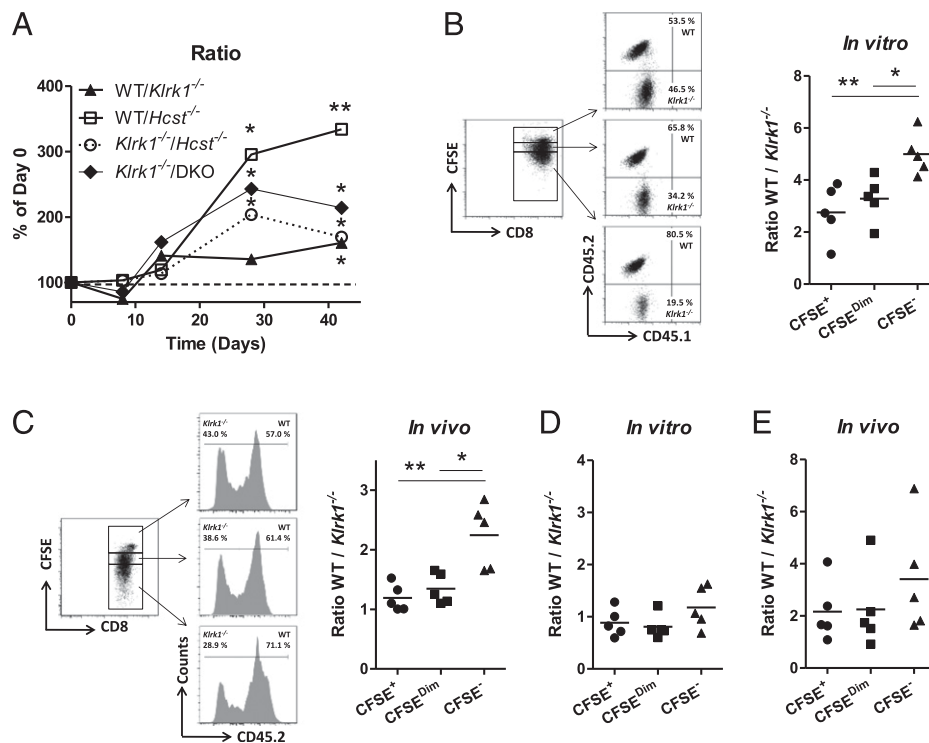


clusively via the adaptor molecule Dap10 (13). We questioned whether Dap10 is also essential for NKG2D-mediated memory formation. mBMCs were generated using WT, *Klrk1*<sup>-/-</sup>, *Hcst*<sup>-/-</sup> (Dap10-deficient), or double knockout (DKO) mice. After reconstitution, mice were infected with LCMV, and donor cell ratios within the T<sub>CM</sub> cell compartment were followed. To directly compare groups, relative changes in cell ratio compared with the initial values was followed over time. These showed that WT and *Klrk1*<sup>-/-</sup> cells both have a competitive advantage over *Hcst*<sup>-/-</sup> cells (Fig. 3A). This is expected, because Dap10 is responsible for signal transduction of many receptors (11, 21) and its deletion is therefore likely to have a broader impact than the absence of NKG2D. Second, WT cells have a larger advantage over *Hcst*<sup>-/-</sup> cells than do *Klrk1*<sup>-/-</sup> cells over DKO cells. Because this difference can only be explained by the presence of Dap10-dependent NKG2D signaling in WT cells (Supplemental Fig. 1F), this indicates that Dap10 is required for at least part of the effects of NKG2D on memory formation. If NKG2D is also capable of signaling independently of Dap10 in memory precursors, *Hcst*<sup>-/-</sup> cells would perform better than double-deficient cells. However, *Klrk1*<sup>-/-</sup> cells had no greater advantage over DKO than over *Hcst*<sup>-/-</sup> cells. Thus, NKG2D signals exclusively through Dap10 when mediating its effects on T<sub>CM</sub> cell formation.

Dap10 physically interacts with both NKG2D and the IL-15R (11). Because IL-15 is important for memory CD8 T cell formation (12), we investigated whether NKG2D mediates IL-15 re-

sponsiveness in memory precursors. mBMCs were infected with mCMV and after 8 d splenocytes were labeled with CFSE, stimulated in vitro with IL-15, and proliferation was assessed. Strikingly, WT cells were specifically enriched in the highly proliferated CFSE<sup>-</sup> cell population (Fig. 3B). No difference in staining of the IL-15R subunits CD122 and CD132 was observed, excluding altered receptor expression as a cause of reduced IL-15 responsiveness (Supplemental Fig. 1G). Additionally, IL-15 enhanced peptide responsiveness of antiviral WT but not *Klrk1*<sup>-/-</sup> cells (Supplemental Fig. 1H). Similar observations were made upon in vitro restimulation of splenocytes from mBMCs 8 d after LCMV infection (data not shown). Interestingly, in vitro restimulation of splenocytes with IL-15 did not lead to induction of NKG2D ligands (Supplemental Fig. 1I). Accordingly, changes in ratios within the CFSE<sup>-</sup> population were still observed, even when NKG2D signaling was blocked with Abs (Supplemental Fig. 1J). These findings suggest that NKG2D can enhance IL-15 responsiveness of memory CD8 precursor cells even in the absence of active NKG2D signaling.

To investigate the effect of NKG2D ablation on IL-15 responsiveness of memory precursors in vivo, purified CD8 cells from mBMCs, infected 8 d previously with mCMV, were CFSE labeled and transferred to naive recipients. Next, mice were injected twice with a mixture of IL-15 and IL-15Rα-Fc, which works as an IL-15 superagonist for memory precursors (17). Two days after the last injection, proliferation of donor cells was investigated. Again,



**FIGURE 3.** NKG2D enhances IL-15 responsiveness of memory precursors via Dap10. **(A)** mBMCs, containing bone marrow of WT and *Klrk1*<sup>-/-</sup>, WT and *Hcst*<sup>-/-</sup>, *Klrk1*<sup>-/-</sup> and *Hcst*<sup>-/-</sup>, or *Klrk1*<sup>-/-</sup> and DKO cells mixed in a 1:1 ratio were infected with LCMV. The ratio between donor populations within the D<sup>b</sup>Gp33<sup>+</sup> central memory cell pool, normalized to day 0 values, was followed over time ( $n = 5$ ). Statistical differences are in comparison with the starting ratio. **(B)** mBMCs (WT/*Klrk1*<sup>-/-</sup>, 1:1) were infected with mCMV. Eight days later, splenocytes were CFSE labeled and stimulated in vitro with 50 ng/ml IL-15. After 4 d, the cell ratio within cells that had proliferated 0 (CFSE<sup>+</sup>), 1–2 (CFSE<sup>dim</sup>), or  $\geq 3$  (CFSE<sup>-</sup>) times was determined. CD8 T cells are gated ( $n = 5$ ). **(C)** mBMCs (WT/*Klrk1*<sup>-/-</sup>, 1:1) were infected with mCMV. After 8 d, total CD8 T cells were purified, CFSE-labeled, and transferred to naive recipients. Twenty-four and 48 h later, mice were injected with IL-15/IL-15Rα-Fc ( $n = 5$ ). Two days after the last injection, the cell ratio between donor CFSE<sup>+</sup>, CFSE<sup>dim</sup>, and CFSE<sup>-</sup> cells was determined. CD8<sup>+</sup> donor cells are gated ( $n = 5$ ). **(D)** and **(E)** mBMCs (WT/*Klrk1*<sup>-/-</sup>, 1:1) were infected with mCMV and after 50 d, splenocytes were isolated. **(D)** Total splenocytes were stimulated in vitro with IL-15. After 4 d, the cell ratio between donor cells within CFSE<sup>+</sup>, CFSE<sup>dim</sup>, and CFSE<sup>-</sup> cells was determined ( $n = 5$ ). **(E)** CD8 T cells were purified and transferred to WT naive recipients. Twenty-four and 48 h later, mice were injected with IL-15/IL-15Rα-Fc ( $n = 5$ ). Two days after the last injection, the cell ratio between donor CFSE<sup>+</sup>, CFSE<sup>dim</sup>, and CFSE<sup>-</sup> cells was determined ( $n = 5$ ). Data are representative of one (A), two (C, E), or five (B, D) experiments. \* $p < 0.05$ , \*\* $p < 0.005$ .

WT cells were significantly enriched in the dividing cell fraction (Fig. 3C). Also, no differences in CD122 expression were observed (data not shown). Next, IL-15 responsiveness of *Klrk1*<sup>-/-</sup> memory cells was investigated. mBMCs were infected with mCMV and, after 56 d, IL-15 responsiveness was analyzed in vitro and in vivo. Surprisingly, proliferation differences were almost completely lost in both models (Fig. 3D, 3E).

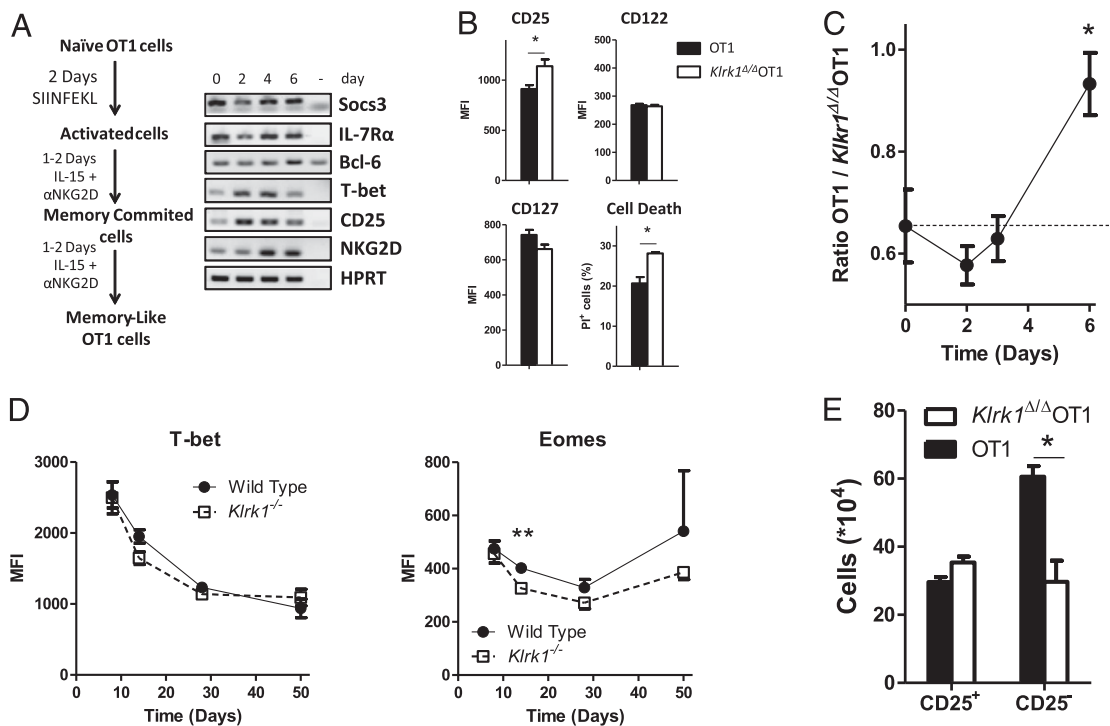
In summary, NKG2D increases IL-15 responsiveness of memory precursor CD8 T cells during priming.

#### *NKG2D* promotes survival of CD8 memory precursors

The reduced number of *Klrk1*<sup>-/-</sup> cells within the CFSE<sup>-</sup> pool after IL-15 stimulation may be the result of reduced proliferation or of impaired survival of activated cells. To investigate the effect of NKG2D deficiency on CD8 T cell proliferation, mBMCs were infected with LCMV, pulsed daily with BrdU from day 4 to 12 after infection, and chased for 50 d. No differences in BrdU labeling could be discerned at any time within virus-specific cells (Supplemental Fig. 2A), suggesting that NKG2D deficiency impairs survival of proliferating cells. Because apoptosis is intrinsically difficult to study in vivo, we set up an in vitro memory differentiation model. Purified, CFSE-labeled OT-1 T cells were stimulated for 2 d with OVA-peptides. Subsequently, cells were washed, transferred to plates coated with agonistic NKG2D-Abs and cultured in medium with 5, 50, or 500 ng/ml IL-15 for an additional 4 d. IL-15 at 5 ng/ml was unable to sustain viability (Supplemental Fig. 2B). IL-15 at 500 ng/ml resulted in effector-like CD25<sup>bright</sup> CD127<sup>dim</sup> cells, whereas 50 ng/ml IL-15 induced a CD25<sup>dim</sup> CD127<sup>bright</sup> memory-like phenotype (Supplemental Fig. 2C).

Because 50 ng/ml IL-15 induced a phenotype that most closely resembled that of memory cells, we used this culture condition for further experiments. Next, expression of key proteins in CD8 T cell differentiation was investigated. Two days of peptide stimulation induced a clear “activated” T cell phenotype, characterized by reduced transcription of CD127, *Socs3*, and *Bcl-6* and an increase of CD25 and T-bet (Fig. 4A). When cells were switched to IL-15/anti-NKG2D, they acquired a “memory-committed” phenotype and induced expression of CD127, *Socs3*, and NKG2D. Reciprocally, transcript levels of T-bet and its direct target CD25 were reduced. Further culture under these conditions generated cells that highly resembled memory cells, as shown by induction of *Bcl-6*. Therefore we characterized this state as “memory-like.”

OT-1 and *Klrk1*<sup>ΔΔ</sup> OT-1 cells were directly compared in this culture model. No differences were observed in activated T cells. During memory commitment, we observed a transient increase of CD122 expression in *Klrk1*<sup>ΔΔ</sup> OT-1 cells (Supplemental Fig. 2D). Markedly, when cells had differentiated into memory-like cells, we observed significantly impaired survival of NKG2D-deficient cells. Additionally, these cells displayed a reduced ability to down-regulate CD25 (Fig. 4B). Proliferation, as measured by CFSE dilution, was not different between OT-1 and *Klrk1*<sup>ΔΔ</sup> OT-1 cells (Supplemental Fig. 2E). To investigate whether NKG2D deficiency affects competitive fitness in our culture system, OT-1 cells were mixed with *Klrk1*<sup>ΔΔ</sup> OT-1 cells and changes in ratios were followed over time. Indeed, WT cells obtained a numerical advantage upon memory differentiation (Fig. 4C). Thus, NKG2D deficiency reduces competitive fitness of activated cells upon IL-15 stimulation due to impaired survival.



**FIGURE 4.** NKG2D enhances survival of memory precursor cells. (A–C) Experimental model for in vitro memory T cell differentiation. Naive OT-1 cells were cultured for 2 d with 10 pg/ml SIINFEKL peptide. Next, cells were washed and cultured on plates coated with agonistic NKG2D Abs for an additional 4 d in the presence of 50 ng/ml IL-15. (A) Gene transcription analyzed by RT-PCR. (B) Cell surface marker expression and viability of OT-1 and *Klrk1*<sup>ΔΔ</sup> OT-1 cells after 6 d of culture. Cell death was assessed by propidium iodide (PI) exclusion ( $n = 4$ ). (C) OT-1 (CD45.1/2) and *Klrk1*<sup>ΔΔ</sup> OT-1 (CD45.2) cells were mixed, and changes in the cell ratio within the PI<sup>-</sup> population were followed over time ( $n = 4$ ). (D) mBMCs (WT/*Klrk1*<sup>-/-</sup>, 1:1) were infected with mCMV and expression of T-bet and Eomes was followed over time within CD8<sup>+</sup>K<sup>b</sup>m57<sup>+</sup> cells ( $n = 5$ ). (E) Naive recipients (CD45.1) were transferred with 10<sup>4</sup> OT-1 or *Klrk1*<sup>ΔΔ</sup> OT-1 cells (CD45.2) and infected with mCMV-SIINFEKL after 24 h. Five days later, donor cells in spleen were analyzed for CD25 expression ( $n = 4$ ). Data are representative of two (D, E), four (C), or eight (A, B) experiments. \* $p < 0.05$ , \*\* $p < 0.005$ .

Recently, it was shown that in the absence of CD4 T cell help, NKG2D hyperstimulation can affect expression of T-bet in CD8 T cells (9). Therefore, we investigated whether NKG2D only promotes survival or also gene transcription in our culture system. No differences were observed in the expression of genes that affect memory differentiation, such as *Socs3*, *Id2*, *Id3*, *Bcl-6*, *Eomes*, and *CD127*. We did find a small increase in expression of T-bet and CD25 (Supplemental Fig. 2F). To investigate whether NKG2D deficiency affects T-bet expression *in vivo*, mBMCs were infected with mCMV and T-bet expression was followed over time. Both at effector and memory time points, no differences in T-bet expression could be discerned within Ag-specific T cells (Fig. 4D). Notably, from day 14 after infection,  $K^b$ m57<sup>+</sup> WT cells expressed higher levels of *Eomes*, which corresponds with a higher fraction of memory cell precursors.

Enhanced T-bet expression promotes effector cell differentiation and impairs memory formation (22). If NKG2D functions primarily to repress T-bet in memory precursors, its ablation will increase effector cell numbers while simultaneously reducing memory precursor formation. If it primarily promotes T<sub>CM</sub> cell survival, NKG2D deficiency will only negatively impact memory cell numbers. OT-1 or *Klrk1*<sup>Δ/Δ</sup> OT-1 cells were transferred into naive recipients, which were subsequently infected with mCMV-SIINFEKL and analyzed after 5 d. At this time point, low CD25 expression is associated with memory differentiation, whereas high CD25 expression defines cells with effector potential (23). Strikingly, NKG2D-deficient cells generated significantly fewer

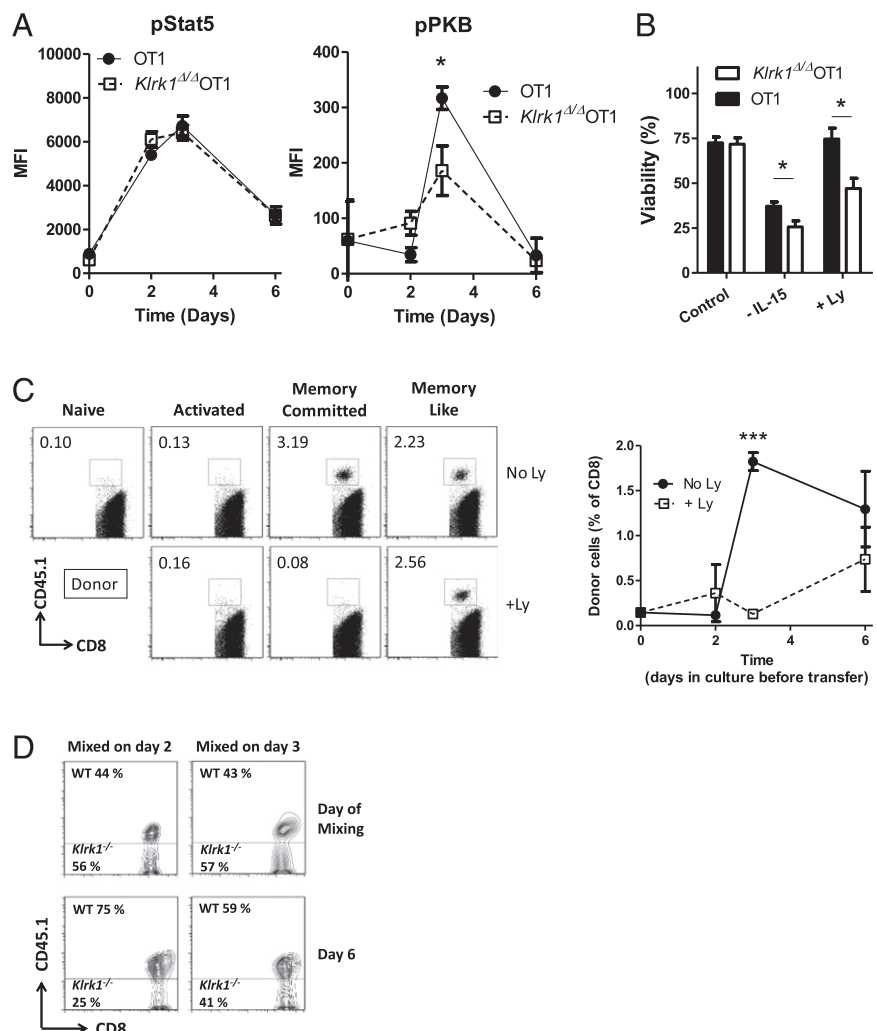
CD25<sup>low</sup> memory precursors, whereas the number of CD25<sup>high</sup> effectors was not affected (Fig. 4E).

Taken together, these data indicate that under normal conditions NKG2D promotes survival of memory precursors rather than affecting proliferation or memory differentiation.

#### *NKG2D promotes survival by enhancing IL-15–mediated PI3K signaling and induction of Mcl-1*

NKG2D signals to PI3K via its adaptor molecule Dap10 (24). Additionally, Dap10 associates with CD132 on the IL-15R and its absence affects Stat5 phosphorylation (11). We questioned which signaling pathway mediates prosurvival effects in our *in vitro* memory differentiation model. Therefore, phosphorylation of Stat5 and PKB, a downstream target of PI3K, was measured in response to acute IL-15 stimulation. Activation of T cells enhanced Stat5 signaling, which was further increased upon memory commitment. However, no differences were observed between OT-1 and *Klrk1*<sup>Δ/Δ</sup> OT-1 cells. In contrast, *Klrk1*<sup>Δ/Δ</sup> OT-1 cells showed significantly reduced PKB phosphorylation upon IL-15 stimulation, specifically during memory commitment (Fig. 5A, Supplemental Fig. 3A). Similar observations were made in *Hest*<sup>-/-</sup> cells (Supplemental Fig. 3B). Thus, NKG2D enhances PI3K signaling during memory commitment. Interestingly, increased PI3K signaling impairs CD122 expression (25), which corresponds with our observation that *Klrk1*<sup>-/-</sup> cells have higher CD122 expression during memory commitment (Supplemental Fig. 2D).

**FIGURE 5.** NKG2D-mediated PI3K signaling promotes memory precursor survival. (A–D) Cells were cultured under memory differentiating conditions. (A) On the indicated days, cells were starved from stimulation for 3 h. Next, cells were stimulated for 15 min with 100 ng/ml IL-15 and analyzed for pStat5 and pPKB. (B) On day 2 of culture, PI3K signaling was blocked with 2 μM LY294002 (+Ly), or cells were cultured in absence of IL-15 (–IL-15). Viability was assessed on day 3 (*n* = 3). (C) OT-1 cells (CD45.1/2<sup>+</sup>) were transferred to naive (CD45.2) recipients on day 0 (naive), 2 (activated), 3 (memory-committed), or 6 (memory-like) of culture. To investigate the effect of PI3K inhibition on memory differentiation, cells were cultured with (+Ly) or without (–Ly) 10 μM Ly on days 0–2 (for activated cells), days 2–3 (for memory-committed cells), or days 4–6 (for memory-like cells). Transferred cells were normalized based on cells cultured without Ly. After 30 d, recipients were infected with mCMV-SIINFEKL. Recall capacity was assessed 8 d later. CD8<sup>+</sup> T cells from lymph node are gated (*n* = 3–4). (D) OT-1 and *Klrk1*<sup>Δ/Δ</sup> OT-1 cells were cultured separately under memory-differentiating conditions and mixed on day 2 or 3 of culture. The change in ratios was assessed on day 6 of culture. Data are representative of two (C) or three (A, B, D) experiments. \**p* < 0.05, \*\*\**p* < 0.0005.



To investigate whether memory-committed *Klrk1*<sup>-/-</sup> cells have altered sensitivity to PI3K signaling, its activity was blocked with the specific inhibitor Ly294002 (26). Notably, memory-committed *Klrk1*<sup>-/-</sup> cells were much more sensitive to PI3K inhibition or IL-15 depletion than were WT cells and they showed increased cell death (Fig. 5B, Supplemental Fig. 3C). Accordingly, in a mixed culture of OT-1 and *Klrk1*<sup>ΔΔ</sup> OT-1 cells, NKG2D-sufficient cells displayed a significant survival advantage when PI3K signaling was inhibited during memory commitment (Supplemental Fig. 3D).

Our findings are remarkable, because PI3K is thought to enhance effector and impair memory cell differentiation (25). In our experiments, IL-15-induced PI3K signaling plays a vital role early during memory commitment. To investigate whether PI3K is essential for memory formation, its activity was blocked during T cell activation (days 0–2), memory commitment (days 2–4), or terminal memory differentiation (days 4–6) in our in vitro system. After each stage, cells were transferred in naive hosts and 30 d later, recall capacity was investigated. Both naive and activated T cells failed to generate a measurable recall response, in contrast to memory-committed and memory-like cells. Importantly, only when PI3K signaling was inhibited during memory commitment, recall capacity was severely impaired (Fig. 5C). Thus, PI3K is essential for IL-15-dependent memory cell formation early during memory commitment.

Next, we analyzed whether NKG2D primarily mediates its effects during memory commitment. OT-1 and *Klrk1*<sup>ΔΔ</sup> OT-1 cells were activated separately and mixed in a 1:1 ratio before (day 2) or after (day 3) memory commitment. Ratios were analyzed on day 6. When cells were mixed on day 2, NKG2D-deficient cells contributed much less to the final cell population, compared with mixtures equilibrated at a later stage (Fig. 5D). When cells were mixed after memory differentiation, no changes in recalled cell numbers were observed 30 d after transfer in naive hosts (Supplemental Fig. 3E).

Finally, we investigated which prosurvival protein is induced by NKG2D during memory commitment. Previously, it was shown that, in response to IL-3, PI3K signaling enhances levels of the antiapoptotic protein Mcl-1 (27). Additionally, IL-15 mediates survival of NK cells by sustaining Mcl-1 levels, which partially depends on PI3K signaling (28). Therefore, we investigated whether NKG2D affects Mcl-1 during memory commitment in our culture system. As previously reported (29, 30), T cell activation leads to a rapid increase of Mcl-1 protein levels (Fig. 6A, 6B). Culture in the presence of IL-15 further enhances Mcl-1 protein, which is impaired by PI3K inhibition (Supplemental Fig. 3F). Strikingly, NKG2D-deficient cells failed to increase Mcl-1 protein levels in response to IL-15 (Fig. 6A, 6B). This could partially, but not completely, be overcome by increasing IL-15 concentrations (Fig. 6C). In memory-like cells, differences in Mcl-1 were almost completely lost.

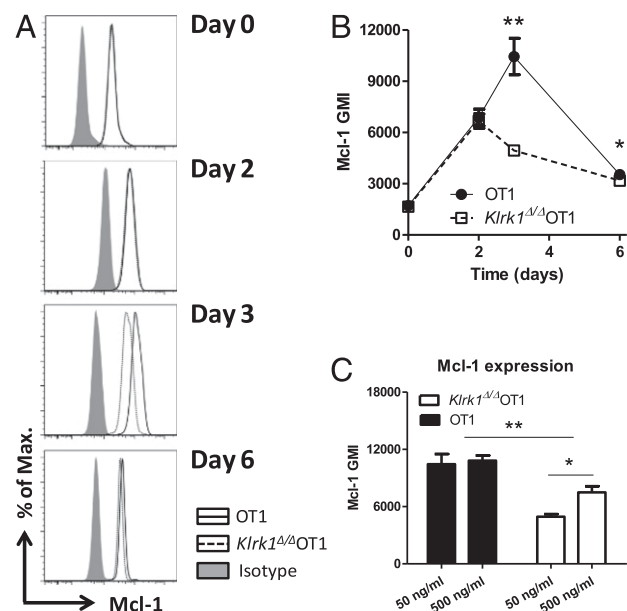
Thus, NKG2D promotes survival of memory precursors in a specific transition phase from activated to memory-committed cells, which correlates with enhanced IL-15-mediated induction of Mcl-1 protein levels via the PI3K signaling pathway.

## Discussion

NKG2D is well known to enhance CD8 effector T cell functions. Recently, NKG2D-ligand overexpression was shown to improve effector functions of memory CD8 T cells in immune-deficient mice (9). In this study, we demonstrate that NKG2D also mediates memory cell formation. NKG2D potentiates IL-15 signaling toward PI3K, which increases Mcl-1 protein levels and enhances memory precursor cell survival. NKG2D deficiency therefore results in impaired memory CD8 T cell formation and reduced

protection against reinfection. Thus, NKG2D provides a vital signal for early memory precursors that allows transition from effector to memory phenotype.

When CD8 cells differentiate into effector or memory cells, key proteins actively inhibit differentiation into the other lineage (1). PI3K signaling in particular is associated with effector cell differentiation. By way of PKB, PI3K induces mammalian target of rapamycin complex (mTORc) signaling, which results in enhanced Foxo1 phosphorylation. This allows T-bet expression and impairs Eomes-mediated memory differentiation (31). Indeed, overexpression of PI3K impairs CD8 memory formation and enhances effector responses (25). It is therefore surprising that NKG2D, which signals through PI3K in T cells (32), promotes both effector function and memory formation via this protein. Key to this dual capability appears to be the cumulative intensity of stimuli converging in an activated T cell. In our in vitro model, only intermediate concentrations of IL-15 in combination with NKG2D stimulation resulted in memory cell differentiation, whereas high or low levels induced effector differentiation or cell death, respectively. This supports a previously proposed model that lineage decisions depend on the (right) amount of proinflammatory signals (1). In the presence of many stimuli, multiple signaling pathways converge on PI3K, which activates mTORc and induces effector differentiation. Under these conditions, for example in the presence of transgenic NKG2D ligand expression (9), NKG2D functions as an instructive, proinflammatory receptor that enhances effector functions (Supplemental Fig. 4A). When few inflammatory signals reach an activated cell, mTORc is not activated, which allows memory differentiation (31). In this situation, NKG2D functions as a permissive factor that promotes survival of precursors by enhancing PI3K-mediated PKB phosphorylation and Mcl-1 stabilization (27). A similar dual effect was shown for the IL-2 receptor, which promotes effector or memory differentiation, dependent on the levels of IL-2 (23). The signal



**FIGURE 6.** NKG2D enhances Mcl-1 protein levels during memory commitment. (A–C) Cells were cultured under memory-differentiating conditions ( $n = 3–4$ ). (A) Representative histograms of Mcl-1 staining. (B) Mean Mcl-1 expression of OT-1 and *Klrk1*<sup>ΔΔ</sup> OT-1 cells under memory-differentiating conditions. (C) Mcl-1 expression in OT-1 and *Klrk1*<sup>ΔΔ</sup> OT-1 cells on day 3 of culture after 24 h of stimulation with 50 or 500 ng/ml IL-15. Data are representative of two (C) or three (A, B) experiments. \* $p < 0.05$ , \*\* $p < 0.005$ .



intensity model also explains why *Klrkl*<sup>-/-</sup> cells form T<sub>EM</sub> cells equally well as do WT cells. Similar to effector cells, effector memory formation is induced by proinflammatory signals (33).

An important question is why the NKG2D/IL-15R axis mediates its effect on memory cell formation in such a narrow time window, in a phase we called “memory commitment.” Naturally, our categorization of differentiation stages draws artificial lines through a continuous process in which effector function is gradually lost and long-term memory potential gained. However, our categories correspond remarkably well with expression of the IL-2, IL-7, and IL-15 receptors. Naive and memory cells are characterized by high surface levels of CD127 (IL-7R $\alpha$ ), and their deficiency of IL-7 signaling results in a dramatic reduction of cell numbers (3). CD25, the IL-2R $\alpha$ -chain, is highly induced upon T cell activation, and the effects that IL-2 has on activated CD8 T cells are many (34). However, CD25 is expressed for only a few days after T cell activation. In fact, the earliest memory cell precursors are characterized by specific loss of CD25 expression several days before CD127 is re-expressed, but when CD122 and NKG2D levels are high (Ref. 23) and this study). Thus, memory differentiation involves a transition phase from IL-2 to IL-7 dependency, during which activated cells highly rely on IL-15/NKG2D signaling for their survival (Supplemental Fig. 4B). Indeed, NKG2D deficiency leads to a specific reduction of early, CD25<sup>dim</sup> memory precursors. Surprisingly, NKG2D was able to enhance IL-15 responsiveness of memory precursors even in the absence of active NKG2D signaling. This suggests that, in addition to its receptor function, NKG2D also operates as a mediator of signal transduction. Possibly it executes this role by bringing Dap10 into the IL-15 signaling complex, as was previously suggested (11). Interestingly, IL-15 was recently shown to mediate transition from glycolytic to oxidative metabolism during memory differentiation by inducing mitochondrial biogenesis (35). Thus, a model appears in which the NKG2D/IL-15R axis facilitates the transition from effector to memory stage.

In summary, we demonstrate a new function of PI3K and NKG2D in memory CD8 T cell formation. NKG2D promotes Mcl-1 induction and enhances survival early during memory cell commitment. In accordance with our observations, mice deficient for Noxa, the proapoptotic antagonist of Mcl-1, generate more memory cells upon influenza infection (36), stressing the importance of these proteins in memory cell formation. Our findings indicate that T cell vaccines will benefit greatly from persistent low-level NKG2D signaling to maximize the number of memory precursors that is allowed to form long-term memory.

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## Disclosures

The authors have no financial conflicts of interest.

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