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Cutting Edge: CD8⁺ T Cell Priming in the Absence of NK Cells Leads to Enhanced Memory Responses

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It is uncertain whether NK cells modulate T cell memory differentiation. By using a genetic model that allows the selective depletion of NK cells, we show in this study that NK cells shape CD8⁺ T cell fate by killing recently activated CD8⁺ T cells in an NKG2D- and perforin-dependent manner. In the absence of NK cells, the differentiation of CD8⁺ T cells is strongly biased toward a central memory T cell phenotype. Although, on a per-cell basis, memory CD8⁺ T cells generated in the presence or the absence of NK cells have similar functional features and recall capabilities, NK cell deletion resulted in a significantly higher number of memory Ag-specific CD8⁺ T cells, leading to more effective control of tumors carrying model Ags. The enhanced memory responses induced by the transient deletion of NK cells may provide a rational basis for the design of new vaccination strategies. *The Journal of Immunology*, 2011, 186: 000–000.

In response to infection, Ag-specific CD8⁺ T cells undergo massive expansion followed by a contraction phase in which most of these cells die by apoptosis, whereas the remaining will differentiate into memory cells and survive lifelong (1). It is believed that the contraction phase of T cell responses ensures flexibility to respond against new pathogens and limits immunopathology, which could otherwise occur with the presence of large numbers of activated T cells. Regulation of the clonal expansion, contraction, and stabilization phases of T cell differentiation impact the quantity of memory T cells generated and, therefore, determines the efficacy of secondary immune responses (2).

Recent evidence suggests that in addition to their undisputed role in innate immunity NK cells are important contributors to adaptive immune responses. In line with this idea, we have shown that NK cells are recruited to peripheral reactive lymph nodes and promote CD4⁺ T cell lineage commitment (3).

Furthermore, it has been appreciated that in lymph nodes, NK cells undergo a process of priming, whereby NK cell recognition of IL-15 on dendritic cells (DC) triggers a molecular program, endowing NK cells with lytic capacity (4). However, what the consequences of these effector functions traditionally associated with the innate role of NK cells are in T cell memory differentiation is not known.

NKG2D is expressed on the surface of all NK cells and binds to a family of MHC class I-related molecules (5). Cross-linking of NKG2D by NKG2D ligands (NKG2DL) leads to an activating signal, resulting in cytotoxicity and release of cytokines and chemokines (5). Although NKG2DL can also be expressed on Ag-activated T cells, thus becoming susceptible to autologous NK lysis via NKG2D/NKG2DL interaction and perforin-containing granule exocytosis (6, 7), the in vivo role of NKG2D and perforin on T cell priming is not fully understood.

To test the hypothesis that NK cells regulate CD8⁺ T cell priming, we followed the fate of Ag-primed CD8⁺ T cells in mice in which NK cells were transiently eliminated. We show in this study that early during immune responses, NK cells shape CD8⁺ T cell fate by killing recently activated CD8⁺ T cells in an NKG2D- and perforin-dependent manner. As a result, we found more Ag-specific CD44ʰⁱCCR7⁺CD62L⁺ central memory T cells (T_CM) in NK cell-depleted mice, accounting for a more vigorous recall response when compared with NK cell-sufficient mice.

Materials and Methods

Mice

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Abbreviations used in this article: Ad-OVA, recombinant adenovirus human serotype 5 expressing cDNA for soluble OVA; DC, dendritic cell; DT, diphtheria toxin; NKG2DL, NKG2D ligand; T_CM, central memory T cell; T_EM, effector memory T cell.

Priming of CD8 T cells in vivo

Mice were transferred with OT-I cells (0.1×10^6) i.v. and immunized by s.c. injection of 50 μg OVA protein (Sigma-Aldrich) admixed with 100 ng LPS (Sigma-Aldrich). Recombinant adenovirus human serotype 5 expressing cDNA for soluble OVA (Ad-OVA) has been described elsewhere (11). Endogenous SIINFEKL-specific CD8 T cell priming was induced by an s.c. injection of 10^9 Ad-OVA viral particles.

Flow cytometric analysis

To detect CD8$^+$ T cell expansion, cells were stained with the following Abs: anti-CD3, CD19, anti-CD8α, anti-Vα2 TCR, anti-CD44, anti-CD62L (BD Biosciences); anti-CCR7 (eBioscience); H-2^b/SIINFEKL(OVA)-PE pentamers (ProImmune); and PE-labeled NKG2D tetramers (gift of David Raulet, University of California, San Francisco, San Francisco, CA). IFN-γ production by memory OT-I cells was measured by intracellular staining as described (3).

NK cell depletion and block of NKG2D

NKp46-DTR mice were injected i.p. with 2 μg diphtheria toxin (DT; Calbiochem) and C57BL/6 mice with 200 μg anti-NK1.1 (PK136). To prevent the interaction between NKG2D and NKG2DL, 200 μg anti-NKG2D Ab (12) was given i.v. to mice 2 d before priming.

Purification and adoptive transfer of NK cells

NK cells were negatively sorted from spleen or lymph nodes as indicated by an NK cell isolation kit (Miltenyi Biotec). The purity of the sorted population was typically >90% NKp46$^+$ cells.

In vitro experiments

OT-I CD8$^+$ T cells were cultured for 2 d in vitro in the presence of anti-CD3 plus anti-CD28 beads and labeled with 2 μM CFSE (Molecular Probes-Invitrogen). Polyclonal CD44^{low}CD62L$^+$ naive CD8$^+$ T cells were sorted from naive mice and labeled with 0.5 μM CFSE. Activated OT-I cells and polyclonal CD8$^+$ T cells were cocultured 1:1 (total 1×10^5 cells/well) in the presence or the absence of 3×10^5 NK cells purified from activated lymph nodes.

Recall responses

Eight weeks after vaccination, mice were challenged s.c. with 10 μg OVA protein admixed with 25 μg LPS. A total of 2×10^4 B16-OVA cells were injected s.c., and tumor growth was monitored three times weekly with calipers in two diameters. Mice were killed when the tumors reached 100 mm^2.

Statistical analysis

Data, presented as means ± SD, were analyzed with a paired Student *t* test using the SPSS software (v17) (SPSS). A *p* value <0.05 was considered significant.

Results and Discussion

NK cells modulate CD8$^+$ T cell differentiation

To investigate the role of NK cells in CD8$^+$ T cell priming, we chose the NKp46-DTR mouse model in which NK cells can be transiently eliminated in vivo (13). We observed that the percentage (Fig. 1*A*) and absolute number (Fig. 1*B*) of Ag-primed CD8$^+$ T cells in draining lymph nodes significantly increased in the absence of NK cells ($p < 0.001$). Similar results were obtained in mice in which NK cells were eliminated by administration of anti-NK1.1 Abs (not shown). To extend this observation to a nontransgenic model, endogenous SIINFEKL-specific CD8$^+$ T cells were activated using a replication-defective adenoviral vector encoding the whole OVA protein (11). Similar to our observation with OT-I CD8$^+$ T cells, endogenous anti-OVA CD8$^+$ T cells demonstrated a significant expansion in NK cell depleted mice as compared with controls (Fig. 1*C*). These results show that NK cells modulate CD8$^+$ T cell numbers upon antigenic stimulation. We next examined the phenotype of CD8$^+$ T cells differentiating in NK cell-depleted mice and controls by performing a phenotypic analysis using CD44, CCR7, and CD62L as surface markers. In the presence of NK cells, CD44 was upregulated on Ag-specific CD8$^+$ T cells, and a proportion of these (20–25%) expressed CD62L (Fig. 1*D*) and CCR7 (Supplemental Fig. 1*A*). In sharp contrast, most Ag-specific CD8 T cells expressed CD62L (>70%) and CCR7 (Supplemental Fig. 1*A*) when priming occurred in the absence of NK cells. These data show that NK cell depletion modulates the outcome of T cell differentiation.
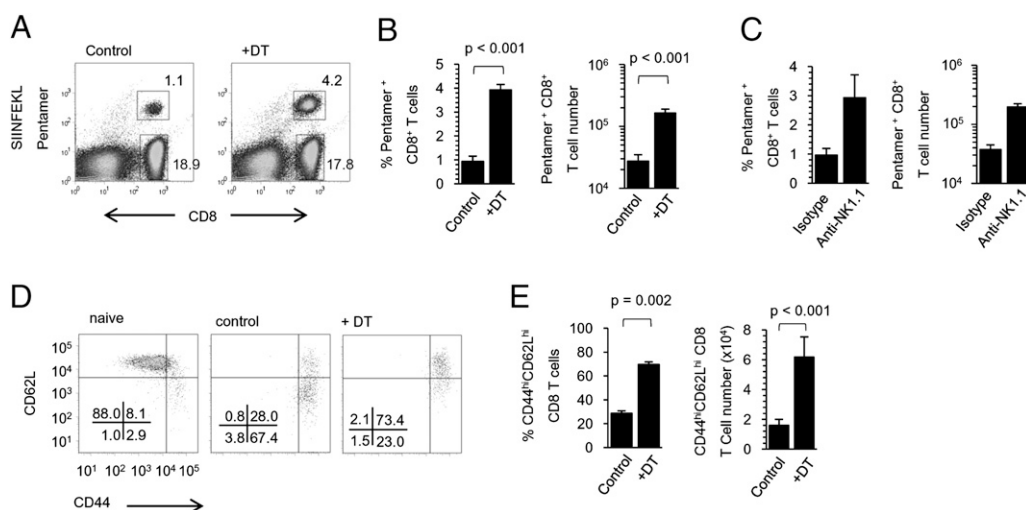

FIGURE 1. NK cell depletion impacts CD8$^+$ T cell priming. *A,* Transgenic OT-I CD8$^+$ cells were adoptively transferred into NKp46-DTR mice and primed with whole OVA+LPS. NK cell depletion was achieved by injecting DT. Shown is the frequency of Ag-specific CD8$^+$ T cells in the draining lymph nodes of control and NK cell-depleted mice 6 d after priming. *B,* Frequency and absolute cell number ± SD of SIINFEKL-specific CD8$^+$ T cells of three independent experiments as in *A. C,* C57BL/6 mice were treated with anti-NK1.1 or isotype as control and primed with Ad-OVA particles. Frequency and absolute cell number ± SD of endogenous SIINFEKL-specific CD8$^+$ T cells of two independent experiments. *D,* Expression of CD44 and CD62L in endogenous naive CD8$^+$ T cells (*left panel*) and on Ag-specific (pentamer$^+$) CD8$^+$ T cells in draining lymph nodes of control (*middle panel*) or DT-treated (*right panel*) mice 6 d after priming. *E,* Mean frequency and absolute cell number ± SD of CD44^{hi}CD62L^{hi} SIINFEKL-specific CD8$^+$ T cells in control mice and NK cell-depleted mice of three independent experiments.

CD8 T cell differentiation depends on NKG2D and perforin expressed on NK cells

Following interaction with mature DC, NK cells acquire the ability to kill MHC-mismatched B cell targets (14) and melanoma cells (15) in lymph nodes. Therefore, we next asked whether NK cell effector functions could modulate CD8+ T cell differentiation and addressed the contribution of NKG2D and perforin in these events. Fig. 2A shows that, similar to what was observed in NK cell-depleted mice, blocking the ligation of NKG2DL by NKG2D results in increased expansion of Ag-specific CD8+ T cells. In agreement with previous reports, NKG2DL was absent on naive T cells but was transiently induced on T cells 24–48 h after activation (6) (Supplemental Fig. 1B), before they acquired phenotypic features of T_{CM} or effector memory (T_{EM}) CD8+ T cells. As NKG2D may be expressed on cells other than NK cells, we next tested that the CD8 T cell phenotype observed directly correlated with NKG2D expressed on NK cells. For this, we induced the activation of transgenic CD8+ T cells in $NKG2D^{-/-}$ mice that were adoptively transferred with NK cells expressing or lacking NKG2D. Fig. 2B shows that CD8+ T cells demonstrated a significant expansion ($p < 0.001$) in environments in which NKG2D is lacking. Remarkably, the number of CD8+ T cells was reduced when $NKG2D^{-/-}$ mice were adoptively transferred with $NKG2D^+$ but not $NKG2D^-$ NK cells. To assess the role of perforin, CD8+ T cells were activated in NK cell-depleted mice that were replenished with perforin+ or perforin− NK cells. Fig. 2C shows that more CD8+ T cells were induced in NK cell-depleted mice that were transferred with perforin+ but not perforin− NK cells. Next, in vitro experiments were set to confirm that activated CD8+ T cells were indeed killed by NK cells. Resting, polyclonal CD8+ T cells were cocultured at a 1:1 ratio with previously activated transgenic CD8+ T cells, in the presence or the absence of NK cells. Fig. 2D shows that addition of NK cells to these cocultures reduced the transgenic/polyclonal T cell ratio due to a diminished number of activated CD8+ T cells. In contrast, NK cells lacking NKG2D or perforin (Fig. 2E) did not alter this ratio. Altogether, these experiments show that CD8 T cell expansion and the phenotype of differentiating CD8+ T cells (Supplemental Fig. 2) is modulated by NKG2D and perforin expressed by NK cells. Importantly, the similar number of DC observed in the lymph nodes of control and NK cell-depleted mice (not shown) and the lack of expression of NKG2DL on DC (not shown) suggest that NK cells shape CD8+ T cell differentiation in vivo by directly killing activated CD8 T cells and not DC.

Increased memory CD8+ T cell responses in NK cell-depleted mice

To assess the fate of CD8+ T cells generated in the presence or the absence of NK cells, we measured the number of pentamer+ CD8+ T cells at different time points after priming. As
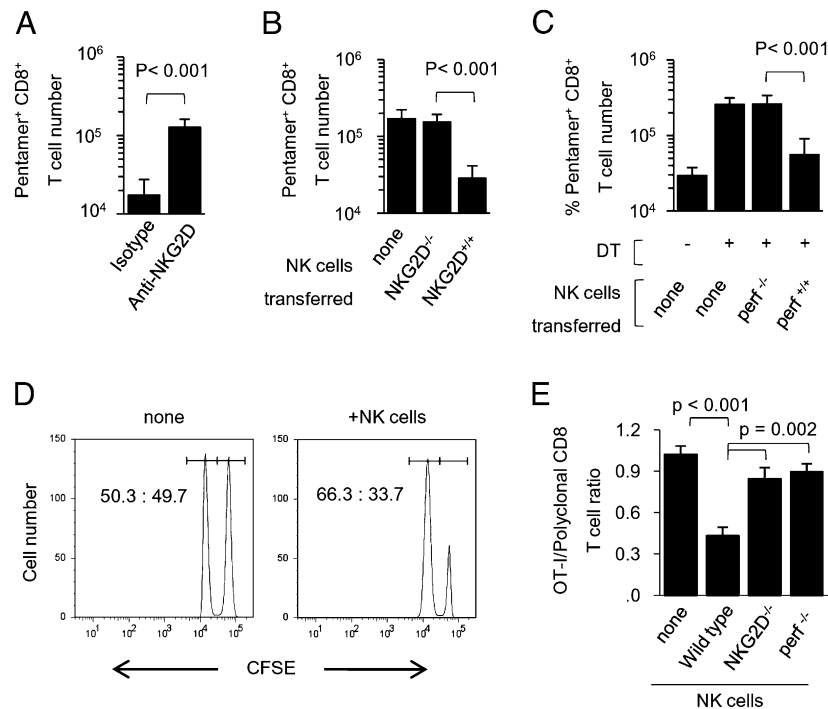

FIGURE 2. NK cell-mediated modulation of CD8+ T cell priming depends on NKG2D–NKG2DL interactions and on perforin expression by NK cells. *A*, Transgenic OT-I CD8+ cells were transferred into wild-type C57BL/6 mice and primed with whole OVA+LPS. Groups of animals were injected i.v. with 200 μg anti-NKG2D Abs or isotype as controls. Shown is the absolute SIINFEKL-specific CD8+ T cell number ± SD of three independent experiments. *B*, $NKG2D^{-/-}$ mice were adoptively transferred with OT-I CD8+ T cells and primed as in *A*. Groups of mice received i.v. highly purified spleen $NKG2D^+$ or $NKG2D^-$ NK cells. Shown is the absolute SIINFEKL-specific CD8+ T cell number ± SD of three independent experiments. *C*, NKp46-DTR mice were adoptively transferred with OT-I CD8+ T cells, primed as before, and depleted of NK cells with DT. Groups of mice were transferred with NK cells enriched from the spleen of perforin+/+ and perforin−/− mice. Six days after priming, draining lymph nodes were analyzed. Shown is the absolute SIINFEKL-specific CD8+ T cell number ± SD of two independent experiments. *D*, Naive polyclonal CD8+ T cells (CFSE^low) and activated SIINFEKL-specific CD8+ T cells (CFSE^hi) were cocultured in the presence or the absence of wild-type NK cells that were enriched from draining lymph nodes. The numbers inside the squares represent the proportion of CFSE^hi and CFSE^low CD8+ T cells 6 h after addition of wild-type NK cells. *E*, OT-I/polyclonal CD8+ T cell ratio ± SD, from two experiments, after coculture with the indicated NK cells.

expected, the absolute number of Ag-primed CD8$^+$ T cells increased during the first week after priming, to progressively decline until day 40 (Fig. 3A). Between day 40 and 60 after stimulation, the number of CD8$^+$ T cells remained constant. Although the number of CD8$^+$ T cells that expanded during the first week in the absence of NK cells largely exceeded the number of CD8$^+$ T cells primed in control mice (Fig. 1), the contraction and stabilization phases of CD8$^+$ T cells in both experimental conditions were parallel (Fig. 3A). These results were similar whether monitored in draining lymph nodes, spleen, or nondraining lymph nodes (Fig. 3B, 3C). Next, we evaluated the ability of memory CD8$^+$ T cells to elicit recall responses. Following in vivo challenge, memory CD8$^+$ T cells activated in NK-sufficient mice expanded >10-fold with respect to unchallenged controls (Fig. 3D). This expansion was similar to that observed in CD8$^+$ T cells monitored in NK cell–depleted mice. Moreover, the percentage of Ag-specific CD8$^+$ T cells that produced IFN-γ 4 d poststimulation was similar whether priming occurred in the presence or the absence of NK cells (Fig. 3E). Because T$_{CM}$ CD8$^+$ T cells confer superior antitumor immunity when compared with T$_{EM}$ CD8$^+$ T cells (16), we examined immunized mice for their ability to restrain the s.c. growth of B16 tumor cells expressing

OVA in this system. Fig. 3F shows that B16-OVA tumors became detectable 10–12 d after inoculation in naive mice and grew rapidly and progressively thereafter, with all mice euthanized by day 21 when the tumor reached 100 mm^2. Mice vaccinated in the presence of NK cells exhibited a markedly slower rate of tumor growth and prolonged survival that was further improved in mice that were immunized in the absence of NK cells. Together, these data show that the transient deletion of NK cells at the time of T cell priming results in more memory cells that can exert more efficient recall responses with respect to NK-sufficient controls.

In summary, to our knowledge, we show for the first time that NK cells regulate the amplitude and outcome of CD8$^+$ T cell differentiation in vivo. A detailed analysis of Ag-specific CD8$^+$ T cells primed in the presence or the absence of NK cells demonstrates that innate cells limit clonal T cell expansion and switch CD8$^+$ T cell differentiation toward a T$_{EM}$ phenotype. Mechanistically, we showed that recently activated, Ag-primed CD8 T cells express NKG2DL and are killed by NK cells in an NKG2D- and perforin-dependent manner. An otherwise unpredicted and striking consequence of these findings is that the number of memory T$_{CM}$ CD8$^+$ T cells is higher when naive CD8$^+$ T cells were primed in the
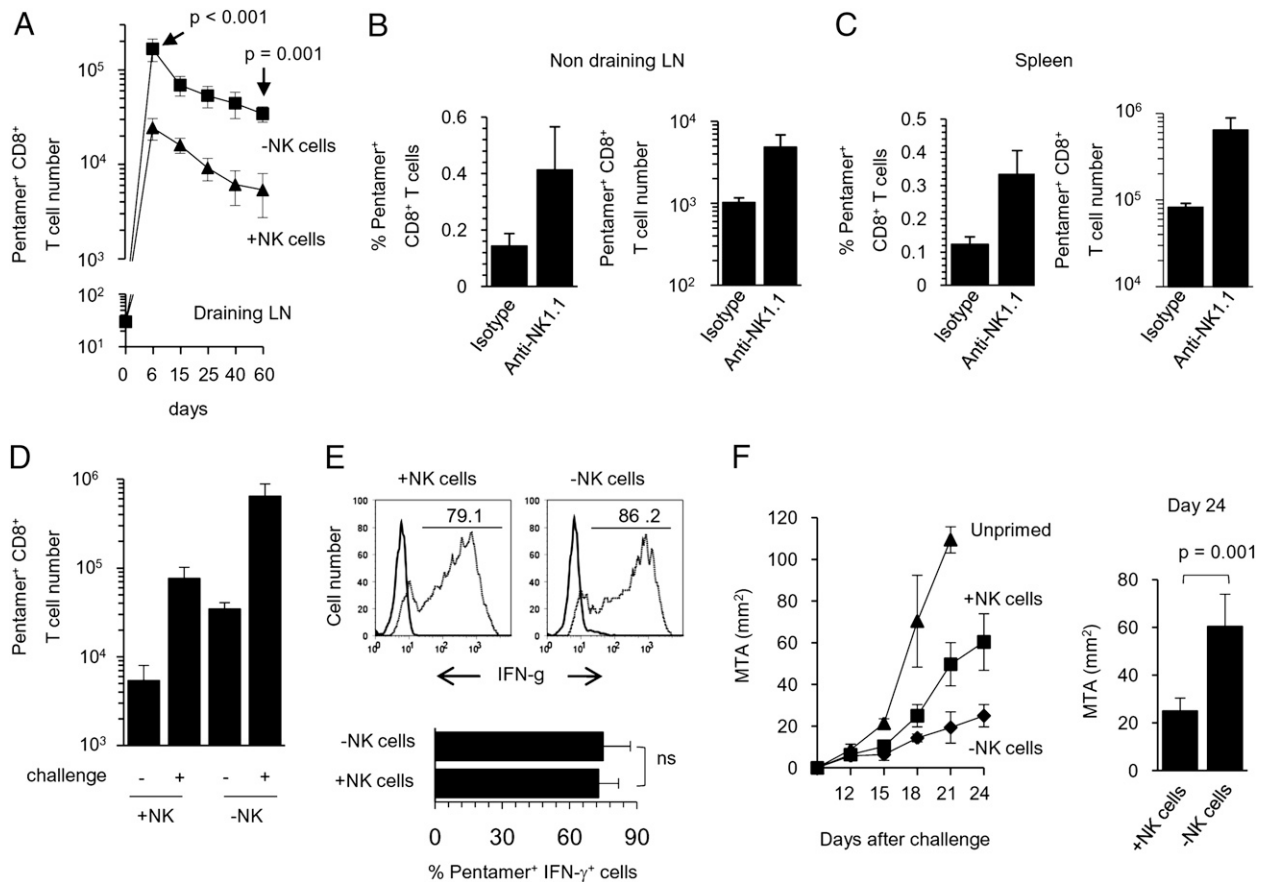
FIGURE 3. Depletion of NK cells before priming impacts CD8$^+$ T cell memory responses. *A*, On day 0, groups of control or anti-NK1.1–treated C57BL/6 mice were adoptively transferred with OT-I CD8$^+$ T cells and primed as before. Shown is the absolute number of Ag-specific CD8 T cells in draining lymph nodes at the indicated time points after priming. Frequency and absolute OT-I CD8$^+$ T cell number in nondraining lymph nodes (*B*) and spleen (*C*) of mice primed 8 wk before in the presence or the absence of NK cells. *D*, Eight weeks after priming, mice were challenged with OVA protein in LPS. Shown is the absolute OT-I CD8$^+$ T cell number 4 d after challenge. *E*, Intracellular IFN-γ production by pentamer$^+$ CD8$^+$ T cells 4 d after challenge. *F*, Groups of mice that were vaccinated in the presence (+NK cells) or the absence (−NK cells) of NK cells were challenged with 2 × 10^4 B16-F10-OVA tumor cells and monitored for tumor development thereafter. Naive, unprimed mice were used as controls. Data shown represent mean tumor area (MTA; in square millimeters) ± SEM. Results are representative of two independent experiments.

absence of NK cells, leading to enhanced recall responses and more effective control of tumor cells carrying model Ags. Although the precise mechanism that resulted in T_{CM} versus T_{EM} cell generation has yet to be defined, different DC/CD8$^+$ T cell ratios would limit Ag availability on DCs, thereby supporting the concept that T cell precursor frequency and competition for resources are important physiological regulators of memory CD8$^+$ T cell lineage commitment (17). Of note, NKG2DL was detected on Ag-primed T cells 24–48 h after activation, but not later. This suggests that NK cell-mediated control of T cell differentiation in lymph nodes occurred before any signs of polarization to the T_{CM} or T_{EM} subsets can be phenotypically defined. Therefore, by increasing the number of CD8 T cells, the absence of NK cells would indirectly promote T_{CM} CD8$^+$ T cell differentiation rather than directly targeting NKG2DL expression on T_{CM} or T_{EM} CD8$^+$ T cells. The preferential generation of T_{CM} CD8$^+$ T cells in NK cell-depleted mice may pave the way for improving new vaccine strategies against tumors and infectious disease targets.

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Disclosures

The authors have no financial conflicts of interest.

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