Cutting Edge: IL-15-Independent NK Cell Response to Mouse Cytomegalovirus Infection

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NK cells respond rapidly during viral infection. The development, function, and survival of NK cells are thought to be dependent on IL-15. In mice lacking IL-15, NK cells are found in severely decreased numbers. Surprisingly, following infection of IL-15- and IL-15Rα-deficient mice with mouse CMV, we measured a robust proliferation of Ly49H-bearing NK cells in lymphoid and nonlymphoid organs capable of cytokine secretion and cytolytic function. Remarkably, even in Rag2<sup>−/−</sup> × Il2rg<sup>−/−</sup> mice, a widely used model of NK cell deficiency, we detected a significant number of NK cells 1 wk after mouse CMV infection. In these mice we measured a >300-fold expansion of NK cells, which was dependent on recognition of the m157 viral glycoprotein ligand and IL-12. Together, these findings demonstrate a previously unrecognized independence of NK cells on IL-15 or other common γ signaling cytokines during their response against viral infection. The Journal of Immunology, 2009, 183: 2911–2914.

**Materials and Methods**

**Mice and infections**

C57BL/6 (B6) and Rag2<sup>−/−</sup> × Il2rg<sup>−/−</sup> B6 mice were purchased from the National Cancer Institute (Frederick, MD) and Taconic, respectively. Il15<sup>−/−</sup>, Il15ra<sup>−/−</sup>, Il15<sup>−/−</sup> × Il15ra<sup>−/−</sup>, and Rag1<sup>−/−</sup> × Il2rb<sup>−/−</sup> B6 mice were bred at the University of California, San Francisco, CA (UCSF). Experiments were done according to the UCSF Institutional Animal Care and Use Committee guidelines. A salivary gland stock of mouse CMV (MCMV; Smith strain) or MCMV-Δm157 was injected i.p. at 5 × 10<sup>6</sup> PFU (21). Neutralizing anti-IL-12 p70 (clone C17.8; 750 μg) was injected i.p. 24 h before infection.

**Flow cytometry and functional assays**

Cells were stained with Abs against NK1.1, CD3, Ly49H, Ly49D, KLRG1, NKp46, NKG2D, CD27, and DX5 (CD49b) (eBioscience or BD Pharmingen). Cells were incubated in tissue culture plates treated with N-[1-(2,3-dioleoyloxy)pro- pyl]-N,N,N-trimethylammonium methylsulfate (Sigma-Aldrich) and coated with anti-NK1.1, anti-Ly49H, or PBS for 5 h at 37°C in the presence of GolgiPlug (BD Pharmingen), followed by staining for lysosome-associated membrane protein (LAMP)-1 and intracellular IFN-γ (BD Pharmingen) (22). NK cells were used as effector cells in a 4-h 51Cr-release assay (23) against Ba/F3 cells and m157-transfected Ba/F3 cells (22).

**Results and Discussion**

**Functional NK cell responses in IL-15Rα- and IL-15 deficient mice**

The spleens of Il15ra<sup>−/−</sup> mice contain <0.1% CD3<sup>−</sup>NK1.1<sup>+</sup> NK cells compared with 2–5% in wild-type (WT) B6 mice (5). The absolute number of NK cells is decreased and the percentage of NK cells bearing the Ly49H receptor is lower in Il15ra<sup>−/−</sup> (~10%) mice compared with WT mice (~50%) (Fig. 1A). During the NK cell response against MCMV in WT mice, the Ly49H<sup>+</sup> NK cells preferentially proliferate during the first several days of infection (21, 24, 25), a response specific for the m157 gene product of MCMV (22, 26). When we infected WT and Il15ra<sup>−/−</sup> mice with MCMV, both mice showed an increase in Ly49H<sup>+</sup> NK cell numbers and comprised >80% of total NK cells at day 7 postinfection (PI) (Fig. 1A). A similar expansion was not observed in the Ly49D<sup>+</sup>Ly49H<sup>−</sup> NK cell subset (Fig. 1A). With precursor numbers of ~2 × 10<sup>4</sup> total Ly49H<sup>+</sup> NK cells in the spleen, the absolute number of

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§ Abbreviations used in this paper: MCMV, mouse cytomegalovirus; γc, IL-2R common γ-chain; LAMP, lysosome-associated membrane protein; PI, postinfection; WT, wild type.

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Ly49H⁺ NK cells in Il15ra⁻/⁻ mice at day 7 PI expanded ~72-fold to become comparable to the numbers found in uninfected WT B6 mice (>10⁶) (Fig. 1B). NK cells from MCMV-infected Il15ra⁻/⁻ mice expressed comparable levels of activating receptors (NK1.1, NKp46, Ly49H, and NKG2D) and activation markers (KLRG1 and CD27) as WT mice (Fig. 1C). When NK cells at day 7 PI were incubated with plate-bound Abs against NK1.1 and Ly49H (or PBS as a control), plots are gated on NK cells (CD3⁻, DX5⁺) expressing LAMP-1 and intracellular IFN-γ. All data presented are representative of at least two independent experiments. αLy49H, Anti-Ly49H; αNK1.1, anti-NK1.1.

Il15⁻/⁻ mice are also deficient in NK cells (3). On day 7 PI, we observed robust expansion of Ly49H⁺ NK cells in the spleens of MCMV-infected Il15⁻/⁻ mice (Fig. 2A). Expression of KLRG1, a NK cell activation marker (27), was comparable in WT and Il15⁻/⁻ mice (supplemental Fig. 1). With <10⁵ total Ly49H⁺ NK cells in the spleen before infection, the absolute number of Ly49H⁺ NK cells in Il15⁻/⁻ mice at day 7 PI was >10⁶, representing 50-fold increase in absolute numbers (Fig. 2A). We tested the ability of Ly49H⁺ NK cells from Il15⁻/⁻ mice to kill m157-bearing target cells. Ly49H⁺ NK cells isolated at day 7 PI from MCMV-infected Il15⁻/⁻ mice were able to efficiently lyse m157-bearing target cells (Fig. 2B).

To test whether a specific viral ligand (and not inflammation alone) is required to drive NK cell proliferation, we infected Il15⁻/⁻ mice with MCMV or a mutant strain lacking m157 (MCMV-Δm157). Unlike MCMV-infected Il15⁻/⁻ mice, which contained a large percentage and absolute number of Ly49H⁺ NK cells at day 7 PI (45.3-fold expansion), infection of Il15⁻/⁻ mice with MCMV-Δm157 did not generate many NK cells (1.7-fold expansion) compared with uninfected controls (Figs. 2, C and D). The diminished proliferation of NK cells during infection with MCMV-Δm157 is not due to defective replication, as this mutant virus is equally or more virulent than WT MCMV (28). Adoptive transfer of WT NK cells into Il15⁻/⁻ recipient mice results in the rapid loss of the transferred NK cells (1–10); however, during infection with MCMV we measured large numbers of transferred NK cells (CD45.1⁺) at day 7 PI in spleen and liver of the Il15⁻/⁻ recipients (supplemental Fig. 2, A and B). At later time points after MCMV infection (days 15 and 30 PI) transferred NK cells were difficult to recover (data not shown), suggesting that following the resolution of infection, NK cells again require IL-15 for survival. Expansion and survival of adoptively transferred WT NK cells were not observed in Il15⁻/⁻ mice infected with MCMV-Δm157 (supplemental Fig. 2, A and B). Altogether, these experiments demonstrate that both viral infection and m157 are required for robust NK cell proliferation in the setting of IL-15 deficiency.

NK cell responses in Rag2⁻/⁻ × Il2rg⁻/⁻ mice

The Rag2⁻/⁻ × Il2rg⁻/⁻ mouse is currently the best model of NK cell deficiency. Without the common γ-chain (γC), NK cells cannot receive signals from any cytokine of the γC family, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. In naive Rag2⁻/⁻ × Il2rg⁻/⁻ mice, NK cells were barely detectable (0.05% in spleen and 0.2% in liver) (Fig. 3A and supplemental Fig. 3). When we infected WT and Rag2⁻/⁻ × Il2rg⁻/⁻ mice with MCMV and measured NK cell responses at day 7 PI, Rag2⁻/⁻ × Il2rg⁻/⁻ mice showed an increase in total NK cell numbers in spleen (comprising 1.6% of splenocytes) and liver (comprising 1% of hepatic lymphocytes) (Fig. 3A and supplemental Fig. 3). In Rag2⁻/⁻ × Il2rg⁻/⁻ mice, as with the other models of IL-15 deficiency, only the NK cells expressing Ly49H (and not Ly49D⁺ Ly49H⁻ NK cells) expanded vigorously, up-regulating KLRG1 (Fig. 3A). With <1000 total Ly49H⁺ NK cells in the spleens of uninfected Rag2⁻/⁻ × Il2rg⁻/⁻ mice, the absolute number of Ly49H⁺ NK cells at day 7 PI became >10⁵ (320-fold expansion).

*The online version of this article contains supplemental material.
NK cell response in II15<sup>−/−</sup> × II15ra<sup>−/−</sup> mice dependent on IL-12

IL-12 is produced by dendritic cells and granulocytes in response to viral and bacterial infection and is required for the generation of Th1 cells, as well as for inducing proliferation and IFN-γ in activated CD8<sup>+</sup> T cells and NK cells (reviewed in Ref. 29). Additionally, IL-12 plays an important role in NK cell production of IFN-γ and NK cell blastogenesis during MCMV infection (30, 31), and NK cell proliferation in response to MCMV infection is somewhat impaired in II12<sup>−/−</sup> mice (32, 33). To address whether IL-12 contributes to NK cell expansion in the setting of IL-15 deficiency, we injected II15<sup>−/−</sup> mice with a neutralizing anti-IL-12 Ab before infection. Uninfected II15<sup>−/−</sup> mice have very few peripheral Ly49H<sup>+</sup> NK cells, but 7 days following infection, significant numbers and percentages of Ly49H<sup>+</sup> NK cells were detected in the spleen (78%) and liver (91%) (Fig. 4A). However, absolute numbers of Ly49H<sup>+</sup> NK cells at day 7 PI were ~30-fold less in anti-IL-12 treated mice compared with control mice (Fig. 4B). The overall expansion of Ly49H<sup>+</sup> NK cells in II15<sup>−/−</sup> × II15ra<sup>−/−</sup> mice was ~70-fold vs a 2-fold increase in anti-IL-12-treated II15<sup>−/−</sup> × II15ra<sup>−/−</sup> mice (Fig. 4B). Thus, IL-12 contributes greatly to the overall NK cell response following MCMV infection in mice lacking the ability to produce or respond to IL-15.

Future studies are required to determine whether the small number of NK cells that do proliferate during MCMV infection represent a unique IL-15-independent subset or new bone marrow emigrants that are rescued from death by IL-12 and...
inflammatory cytokine signaling. Moreover, although we have shown that IL-12 is involved in NK cell expansion in the absence of IL-15, other factors might also contribute to their proliferation and survival. In conclusion, our surprising findings contribute added insight into the cytokines (or lack thereof) that NK cells require during an immune response against viral infection.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

References

SUPPLEMENTAL FIGURES

Supplemental Figure 1. NK cell expansion in wild-type and \( II15^{-/-} \) mice. Wild-type and \( II15^{-/-} \) mice were infected with MCMV and NK cells (CD3\(^{-}\), NK1.1\(^{+}\)) from spleen and liver were analyzed at 7 days PI for expression of Ly49H and KLRG1.

Supplemental Figure 2. Expansion and survival of adoptively transferred NK cells in \( II15^{-/-} \) mice. (A) Schematic of experiment: NK cells from wild-type CD45.1\(^{+}\) mice were purified and transferred into \( II15^{-/-} \) mice. Following infection with MCMV or MCMV-\(\Delta m157\), the NK cells (CD3\(^{-}\), NK1.1\(^{+}\)) were analyzed at day 7 PI. (B) Flow cytometric plots show the percentages of host (CD45.1\(^{-}\)) and donor (CD45.1\(^{+}\)) Ly49H\(^{+}\) NK cells in the spleens and livers of \( II15^{-/-} \) mice infected with MCMV or MCMV-\(\Delta m157\) (compared with uninfected mice).

Supplemental Figure 3. Liver NK cell expansion in wild-type and \( Rag2^{-/-} \times II2rg^{-/-} \) mice. Wild-type and \( Rag2^{-/-} \times II2rg^{-/-} \) mice were infected with MCMV and percentages of NK cells (CD3\(^{-}\), NK1.1\(^{+}\)) in the liver were determined at 7 days PI (compared to uninfected mice). Liver NK cells were analyzed for expression of Ly49H, Ly49D, and KLRG1.

Supplemental Figure 4. Expansion of Ly49H\(^{+}\) NK cells in \( Rag1^{-/-} \times II2rb^{-/-} \) mice. (A) \( Rag1^{-/-} \times II2rb^{-/-} \) mice were infected with MCMV and NK cells (CD3\(^{-}\), NK1.1\(^{+}\)) from spleen and liver were analyzed at 7 days PI (compared to uninfected mice) for expression
of Ly49H and Ly49D. (B) The absolute numbers of Ly49H⁺ NK cells (left graph) and Ly49H⁻ NK cells (right graph) in the spleens and livers of uninfected and day 7 PI Rag1⁻/⁻ × Il2rb⁻/⁻ mice. Error bars on graph display s.e.m. (n = 3-4). Fold expansion of Ly49H⁺ or Ly49H⁻ NK cells over the first 7 days of infection are shown. (C) NK cells from the spleen of Rag1⁻/⁻ × Il2rb⁻/⁻ mice at day 7 PI were analyzed for expression of NKG2D, KLRG1, and DX5. All data presented are representative of at least 2 independent experiments.
Supplemental Figure 1
Supplemental Figure 2

A

Wildtype NK cells (CD45.1) → MCMV or MCMV-lam157

↓

IL-12 (CD45.2)

→ Measure Ly49H

NK cell response

B

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Supplemental Figure 3

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Supplemental Figure 4

A

B

C