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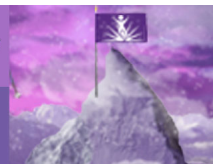
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IL-33 Receptor ST2 Amplifies the Expansion of NK Cells and Enhances Host Defense during Mouse Cytomegalovirus Infection

Tsukasa Nabekura,*,† Jean-Philippe Girard,‡ and Lewis L. Lanier*

NK cells provide important host defense against viruses and can differentiate into self-renewing memory NK cells after infection, alloantigen stimulation, and cytokine stimulation. In this study, we investigated the role of the IL-33 receptor ST2 in the differentiation of NK cells during mouse CMV (MCMV) infection. Although ST2-deficient ($Il1rl1^{-/-}$) Ly49H⁺ NK cells develop normally and differentiate into memory cells after MCMV infection, naive and memory $Il1rl1^{-/-}$ Ly49H⁺ NK cells exhibited profound defects in MCMV-specific expansion, resulting in impaired protection against MCMV challenge. Additionally, IL-33 enhanced m157 Ag-specific proliferation of Ly49H⁺ NK cells in vitro. Thus, an IL-33/ST2 signaling axis in NK cells contributes to host defense against MCMV. *The Journal of Immunology*, 2015, 194: 000–000.

Natural killer cells recognize abnormal or allogeneic cells by using a repertoire of receptors that regulates their activation and effector functions (1). NK cells have adaptive immune features, which include differentiation into self-renewing memory cells after exposure to mouse CMV (MCMV), haptens, cytokines, and alloantigens (2-6). In humans, NKG2C^high NK cells have been identified as memory NK cells based on their specific expansion and persistence for months following human CMV infection (3, 7, 8). Mouse NK cells expressing Ly49H, which recognizes the m157 MCMV glycoprotein on infected cells (9), undergo clonal expansion, contraction, and differentiation into memory cells after MCMV infection (5). The DAP12 and DAP10 adaptor proteins and the costimulatory DNAM-1 receptor are required for optimal expansion of Ly49H⁺ NK cells and their differentiation into memory cells (5, 10). IL-12 is essential for both expansion and memory generation (11); however, the roles of other cytokines for adaptive immune responses mediated by NK cells are largely unknown.

IL-33, a member of the IL-1 cytokine family (12), is expressed constitutively by endothelial and epithelial cells and by fibroblastic reticular cells (FRCs) in secondary lymphoid organs (13). It is released upon tissue damage and necrosis, thus acting as an early inducer of inflammation, and it exacerbates Th2 immune responses in mouse models of arthritis and asthma (14). Binding of IL-33 to its receptor ST2 induces MyD88-dependent activation of NF-κB

and MAPK (14). ST2 is expressed on many immune cells (12, 15, 16), and the IL-33/ST2 signaling pathway augments IL-12–induced IFN-γ production by NK cells (15, 16). ST2 is essential for control of coxsackievirus B5-induced pancreatitis by enhancing IFN-γ production by NK cells, which is associated with viral clearance (17). In the present study, we investigated the role of the IL-33/ST2 signaling axis in the adaptive immune response of NK cells during MCMV infection.

Materials and Methods

Mice and MCMV

C57BL/6 and congenic CD45.1⁺ C57BL/6 mice were purchased from the National Cancer Institute. C57BL/6 ST2-deficient ($Il1rl1^{-/-}$) mice (18), provided by Dr. S. Akira (Osaka University), IL-33–lacZ gene trap reporter ($Il33^{-/-}$) mice (13), and Ly49H-deficient ($Klra8^{-/-}$) mice (19), provided by Dr. S. Vidal (McGill University), and DAP12-deficient ($Tyrobp^{-/-}$) mice (20) were maintained in accordance with the guidelines of the University of California San Francisco Institutional Animal Care and Use Committee. CD45.1⁺ wild-type (WT) and CD45.2⁺ $Il1rl1^{-/-}$ mixed bone marrow (BM) chimeric mice were generated as described (5). Smith strain MCMV was prepared as described (6). Mice were infected by i.p. injection of 1–5 × 10⁵ PFU.

NK cell enrichment and adoptive transfer

NK cells were enriched by Ab-coated magnetic bead selection or flow cytometry as described (10). Ten million splenocytes or 2 × 10⁵ Ly49H⁺ NK cells from mixed BM chimeric mice were injected i.v. into Ly49H-deficient mice on the day before infection. In some experiments, splenocytes were labeled with 10 μM CellTrace Violet (Invitrogen).

Flow cytometry

Fc receptors were blocked with 2.4G2 mAb before staining with the indicated mAbs or isotype-matched control Abs (BD Biosciences, eBioscience, or BioLegend). Samples were acquired on an LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

In vitro stimulation and proliferation of NK cells

One million splenocytes from mixed BM chimeric mice were incubated in 96-well tissue culture plates coated with anti-NKp46 (29A1.4) as described (5), or with 10 ng/ml mouse IL-12 plus 10 ng/ml mouse IL-18 (R&D Systems), 10 ng/ml PMA plus 1 μg/ml ionomycin (Sigma-Aldrich), or cocultured with 1 × 10⁵ RMA or m157-transfected RMA cells as described (6). One million CellTrace Violet–labeled splenocytes were cocultured with 1 × 10⁵ RMA or m157-transfected RMA cells (fixed in 1% paraformaldehyde) in the absence or presence of 25 ng/ml mouse IL-33 (R&D Systems) and/or 10 ng/ml mouse IL-12 with 50 U/ml human IL-2 (National Cancer Institute) for 4 d at 37°C.

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The abbreviations used in this article: BM, bone marrow; FRC, fibroblastic reticular cell; LEC, lymphatic endothelial cell; MCMV, mouse CMV; p.i., postinfection; WT, wild-type.

Viral load

Ten thousand naive or memory Ly49H$^+$ NK cells were transferred separately into Ly49H-deficient or DAP12-deficient mice and infected with MCMV. Peripheral blood was collected on day 4 postinfection (p.i.), and the right lobe of liver and the spleen were homogenized in DMEM with 10% FCS on day 7 p.i. and DNA was isolated from these specimens. The copy number of MCMV IE1 gene in blood, spleen, and liver was determined as described (10). The copy number of MCMV IE1 gene in the spleen was calculated for the whole organ and the copy number of MCMV IE1 in the liver was adjusted for weight of the tissue.

IL-33 in splenic stromal cells

Splenic stromal cells were prepared as described with minor modifications (21). Spleens were digested with 0.2 U/ml dispase, 0.2 mg/ml collagenase D, and 0.1 mg/ml DNase I (Roche), and stromal cells were enriched by depletion with mAbs against CD4, CD8, CD11b, CD19, and Ter119, and magnetic separation with anti-rat IgG–coated beads (Qiagen). FRCs (CD31$^-$gp38$^+$), lymphatic endothelial cell (LEC)-like cells (CD31$^+$gp38$^+$), double-negative cells (CD31$^-$gp38$^-$), and blood endothelial cells (CD31$^+$gp38$^-$) were gated on 7-aminoactinomycin D$^-$CD45$^-$ cells and purified by flow cytometry. The relative quantity of IL-33 transcripts was determined by quantitative RT-PCR analysis using the following primers: *Actb*, forward, 5′-GGCTGTATTCCCCTCCATCG-3′, reverse, 5′-CCAGTTGGTAACAATGCCATGT-3′. *Il33*, forward, 5′-TCCAACTCCAAGATTTCCCCG-3′, reverse, 5′-CATGCAGTAGACATGGCAGAA-3′.

Statistical analysis

The Student *t* test was used to compare results. The Mann–Whitney *U* test was used to compare MCMV viral titers. Error bars represent SEM.

Results

ST2 is dispensable for NK cell development

To determine whether an intrinsic lack of ST2 affects NK cell development and function, we reconstituted lethally irradiated
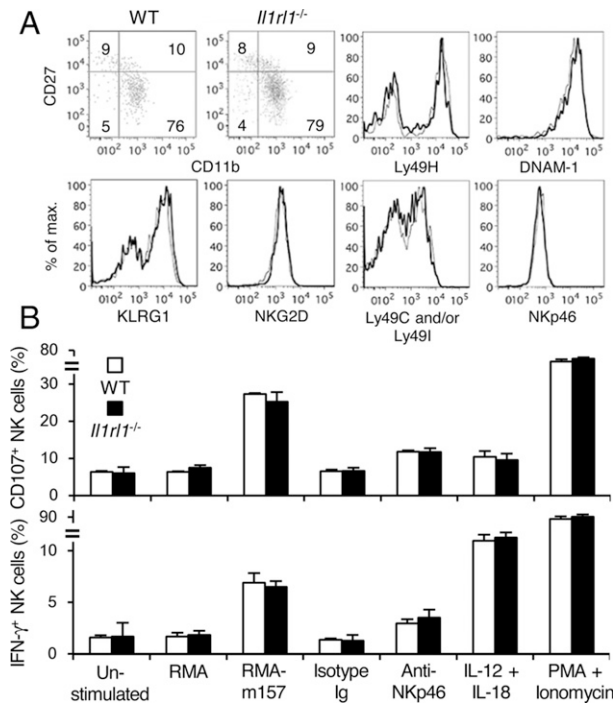

FIGURE 1. Phenotype of *Il1rl1*$^{-/-}$ NK cells. (**A**) Developmental stages as determined by expression of CD11b, CD27, and NK receptors on WT (bold lines) and *Il1rl1*$^{-/-}$ (thin lines) NK cells, gating on TCRβ$^-$NK1.1$^+$ lymphocytes in the blood from mixed BM chimeric mice. Data are representative of three experiments (*n* = 2–3 mice/experiment). (**B**) Degranulation and IFN-γ production of WT (open bars) and *Il1rl1*$^{-/-}$ (filled bars) NK cells after in vitro stimulation. Data are representative of three experiments (*n* = 3–4 in each stimulation).
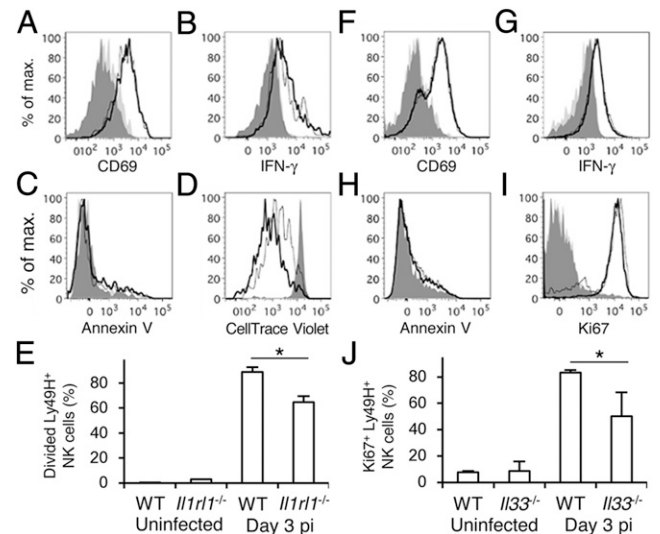
recipient mice with CD45.1$^+$ WT and CD45.2$^+$ *Il1rl1*$^{-/-}$ BM cells and allowed NK cells to reconstitute in the recipient mice for >5 wk. Equivalent frequencies of WT and *Il1rl1*$^{-/-}$ NK cells were detected in the blood, spleen, liver, and BM after reconstitution (data not shown). WT and *Il1rl1*$^{-/-}$ NK cells exhibited similar developmental stages and equivalently expressed KLRG1, NKG2D, Ly49H, Ly49C and/or Ly49I, Ly49G2, DNAM-1, NKp46, NK1.1, IL-2Rα, IL-2Rβ, and IL-7Rα (Fig. 1A and data not shown). Similar frequencies of WT and *Il1rl1*$^{-/-}$ NK cells degranulated and produced IFN-γ when cocultured with m157-transfected RMA cells, stimulated by crosslinking NKp46, cultured with IL-12 plus IL-18, and activated with PMA plus ionomycin (Fig. 1B). Similarly, NK cells in *Il33*$^{-/-}$ mice expressed NK receptors comparably to WT NK cells (data not shown).

ST2 enhances NK cell expansion during MCMV infection

WT and *Il1rl1*$^{-/-}$ Ly49H$^+$ NK cells were isolated from BM chimeric mice, adoptively transferred into Ly49H-deficient recipient mice, and infected with MCMV. During the early phase of infection, donor WT and *Il1rl1*$^{-/-}$ Ly49H$^+$ NK cells were activated equivalently, as evidenced by upregulation of CD69 on day 1.5 p.i. and KLRG1 on day 7 p.i., and they produced similar amounts of IFN-γ on day 1.5 p.i. (Fig. 2A, 2B and data not shown). A similar frequency of *Il1rl1*$^{-/-}$ Ly49H$^+$ NK cells stained for annexin V in uninfected and infected recipient mice, comparably to WT Ly49H$^+$ NK cells (Fig. 2C). However, *Il1rl1*$^{-/-}$ Ly49H$^+$ NK cells proliferated less than did WT Ly49H$^+$ NK cells on day 3 p.i.

FIGURE 2. ST2 enhances Ly49H$^+$ NK cell proliferation during MCMV infection. Ly49H$^+$ NK cells (**A** and **B**) or CellTrace Violet–labeled splenocytes (**C–E**) from WT and *Il1rl1*$^{-/-}$ mixed BM chimeric mice were transferred into Ly49H-deficient mice and infected with 1 × 10^5 PFU MCMV. (A) CD69 and (B) IFN-γ on day 1.5 p.i., and (C) annexin V and (D) CellTrace Violet on day 3 p.i. of WT (bold lines) and *Il1rl1*$^{-/-}$ (thin lines) Ly49H$^+$ NK cells in the spleen. Dark gray–shaded and light gray–shaded histograms represent naive WT and *Il1rl1*$^{-/-}$ Ly49H$^+$ NK cells, respectively. Data are representative of two experiments (*n* = 3 mice/experiment). (E) Percentages of divided Ly49H$^+$ NK cells were quantified. Data are pooled from two experiments (*n* = 6 mice). (**F–J**) WT and *Il33*$^{-/-}$ mice were infected with 5 × 10^5 PFU MCMV. (F) CD69 and (G) IFN-γ on day 1.5 p.i. and (H) annexin V and (I) Ki67 on day 3 p.i. of WT (bold lines) and *Il33*$^{-/-}$ (thin lines) Ly49H$^+$ NK cells in the spleen. Dark gray–shaded and light gray–shaded histograms represent naive WT and *Il33*$^{-/-}$ Ly49H$^+$ NK cells, respectively. Data are representative of two experiments (*n* = 2–4 mice/experiment). (J) Percentages of Ki67$^+$ Ly49H$^+$ NK cells were quantified. Data are pooled from two experiments (*n* = 6 mice). *p < 0.05.

(Fig. 2D, 2E). Consistent with the phenotype of $Il1rl1^{-/-}$ NK cells, Ly49H$^+$ NK cells in $Il33^{-/-}$ mice activated, produced IFN-γ, and stained for annexin V early after infection comparably to WT mice (Fig. 2F–H). However, a smaller percentage of Ki67$^+$Ly49H$^+$ NK cells was observed in $Il33^{-/-}$ mice compared with WT mice (Fig. 2I, 2J). These findings demonstrate that ST2 and IL-33 are dispensable for activation, IFN-γ production, and survival of NK cells, but they augment expansion of Ly49H$^+$ NK cells during MCMV infection.

ST2 enhances expansion of naive and memory Ly49H$^+$ NK cells and control of MCMV

WT and $Il1rl1^{-/-}$ Ly49H$^+$ NK cells from BM chimeric mice were adoptively transferred into Ly49H-deficient recipient mice and infected with MCMV. $Il1rl1^{-/-}$ Ly49H$^+$ NK cells proliferated less than did WT Ly49H$^+$ NK cells in the spleen and liver at the peak of the NK cell response on day 7 p.i. (Fig. 3A, 3B). Consistent with the phenotype of $Il1rl1^{-/-}$ Ly49H$^+$ NK cells, Ly49H$^+$ NK cells in $Il33^{-/-}$ mice had a defect in proliferation after the infection compared with WT mice (Fig. 3C). WT and $Il1rl1^{-/-}$ Ly49H$^+$ NK cells adoptively transferred into Ly49H-deficient recipient mice generated a long-lived subset in the blood and spleen 1 mo p.i. with MCMV (Fig. 3D, 3E), although $Il1rl1^{-/-}$ Ly49H$^+$ NK cells showed pronounced impairment in expansion in the blood on day 7 p.i. (Fig. 3D). These $Il1rl1^{-/-}$ Ly49H$^+$ NK cells displayed a memory-associated phenotype characterized as CD11b$^+$CD27$^-$KLRG1^{high}DNAM-1^{low}, as described previously (5, 6, 10) (Fig. 3F). Memory $Il1rl1^{-/-}$ Ly49H$^+$ NK cells that were isolated
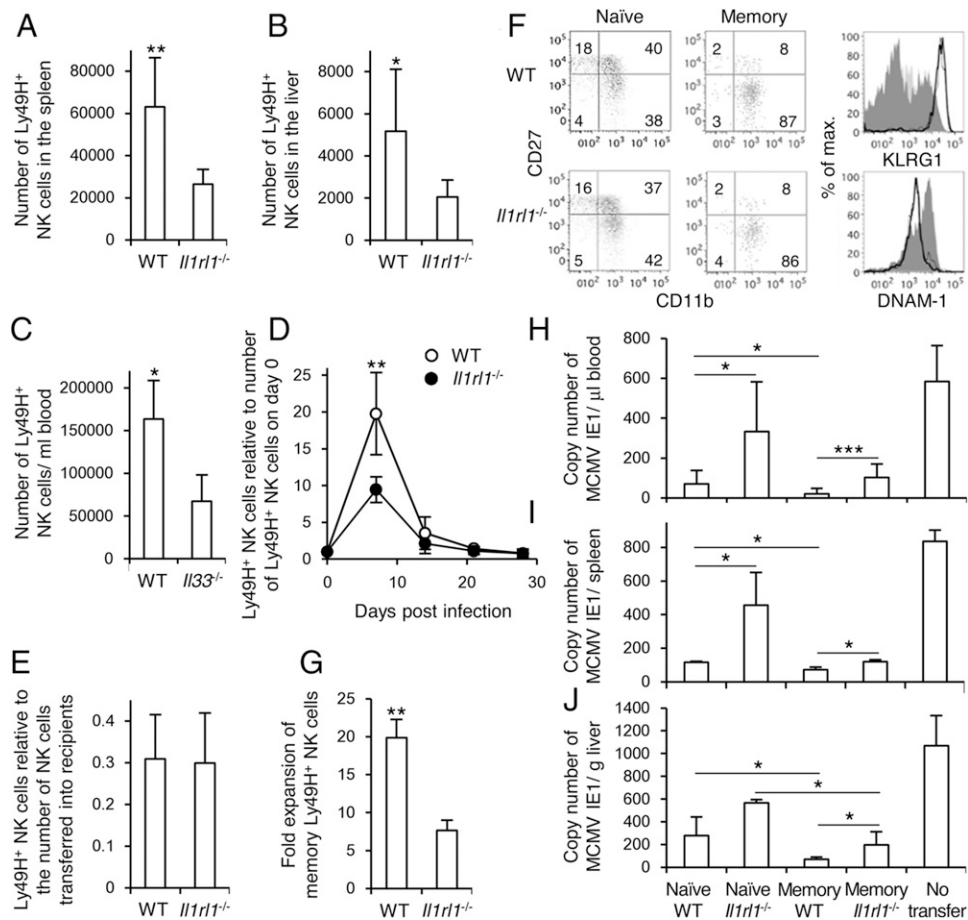

FIGURE 3. ST2 and IL-33 augment expansion of naive and memory Ly49H$^+$ NK cells and control of MCMV. (**A** and **B**) WT and $Il1rl1^{-/-}$ Ly49H$^+$ NK cells from mixed BM chimeric mice were transferred into Ly49H-deficient mice and infected with 1×10^5 PFU MCMV. The number of Ly49H$^+$ NK cells in the spleen (A) and liver (B) on day 7 p.i. is shown. Data are pooled from two experiments (n = 6 mice). (**C**) WT and $Il33^{-/-}$ mice were infected with 5×10^5 PFU MCMV. The number of Ly49H$^+$ NK cells in the blood on day 7 p.i. is shown. Data are pooled from two experiments (n = 4 mice). (**D** and **E**) WT and $Il1rl1^{-/-}$ Ly49H$^+$ NK cells were transferred into Ly49H-deficient mice and infected with MCMV. (D) The number of Ly49H$^+$ NK cells in the blood is represented as the ratio relative to the number of Ly49H$^+$ NK cells in the blood on day 0 (before infection). (E) The y-axis represents the number of memory Ly49H$^+$ NK cells detected in the spleen on day 29 p.i. compared with the number of naive Ly49H$^+$ NK cells adoptively transferred into recipient mice on day 0. Data are pooled from three experiments (n = 8 mice). (**F**) Phenotype of WT and $Il1rl1^{-/-}$ memory Ly49H$^+$ NK cells on day 29 p.i. in the spleen. Bold and thin lines represent memory WT and $Il1rl1^{-/-}$ Ly49H$^+$ NK cells, respectively. Dark gray–shaded and light gray–shaded histograms represent naive WT and $Il1rl1^{-/-}$ Ly49H$^+$ NK cells, respectively. Data are representative of two experiments (n = 2–3 mice/experiment). (**G**) Memory Ly49H$^+$ NK cells were isolated 29 d p.i., transferred into naive Ly49H-deficient mice, and infected with 1×10^5 PFU MCMV. Expansion of memory Ly49H$^+$ NK cells in the spleen on day 7 after secondary infection is represented as the fold expansion relative to the number of memory Ly49H$^+$ NK cells detected in the spleen of mice adoptively transferred with memory Ly49H$^+$ NK cells but not infected. Data are pooled from three experiments (n = 9 mice). *$p < 0.05$, **$p < 0.01$ versus $Il1rl1^{-/-}$ or $Il33^{-/-}$. (**H–J**) WT and $Il1rl1^{-/-}$ memory NK cells were purified 25–32 d p.i., and WT and $Il1rl1^{-/-}$ naive NK cells were purified from BM chimeric mice. WT and $Il1rl1^{-/-}$ naive and memory Ly49H$^+$ NK cells were transferred separately into Ly49H-deficient or DAP12-deficient mice and infected with 1×10^5 PFU MCMV. The copy number of MCMV IE1 gene in the blood (H) on day 4 p.i. and in the spleen (I) and liver (J) on day 7 p.i. was analyzed by quantitative PCR. (H) Data were pooled from four experiments (n = 7–11 mice/group) and (I and J) data were pooled from two experiments (n = 4 mice/group). *$p < 0.05$, ***$p < 0.005$.

from infected mice and adoptively transferred into naive Ly49H-deficient recipients also expanded poorly when rechallenged with MCMV (Fig. 3G). Moreover, both naive and memory $Il1rl1^{-/-}$ Ly49H$^+$ NK cells adoptively transferred into Ly49H-deficient mice or DAP12-deficient mice, which lack functionally competent Ly49H$^+$ NK cells and are unable to control early replication of MCMV (5), showed a poor protective effect against MCMV challenge compared with WT Ly49H$^+$ NK cells in the blood, spleen, and liver, consistent with the impaired proliferation of $Il1rl1^{-/-}$ Ly49H$^+$ NK cells (Fig. 3H–J). Thus, ST2 is necessary not only for the optimal expansion of naive and memory Ly49H$^+$ NK cells during MCMV infection, but also for efficient control of MCMV.

Splenic stromal cells upregulate IL-33 during MCMV infection

FRCs, a stromal cell subset in secondary lymphoid organs, constitutively express IL-33, and IL-33 is upregulated in inflamed tissues (13). We investigated whether stromal cell subsets modulate expression of IL-33 during MCMV infection. Frequencies of splenic stromal cell subsets, as determined by staining CD45$^-$ cells with CD31 and gp38, did not change dramatically during MCMV infection (data not shown). However, FRCs, LEC-like cells, and double-negative cells, but not blood endothelial cells, highly upregulated IL-33 transcripts in the early course of infection (Fig. 4).

IL-33 enhances m157-dependent proliferation of Ly49H$^+$ NK cells

IL-33 synergizes with IL-12 for IFN-γ production by NK cells (15, 16). To address whether IL-33 has a direct effect on proliferation, WT and $Il1rl1^{-/-}$ Ly49H$^+$ NK cells were cocultured with RMA or m157-transfected RMA cells in the absence or presence of IL-12 and IL-33 with a low dose IL-2. IL-33 had little impact on Ag nonspecific cell divisions of Ly49H$^+$ NK cells induced by IL-2, or the m157-driven proliferation of Ly49H$^+$ NK cells in the absence of IL-12 (Fig. 5A, 5B). Alternatively, in the presence of IL-12, IL-33 enhanced proliferation of WT Ly49H$^+$ NK cells, but not $Il1rl1^{-/-}$ Ly49H$^+$ NK cells (Fig. 5C, 5D).

Discussion

The quantity of bioactive IL-33 is transcriptionally and post-translationally regulated (12, 14). IL-33 transcription is induced by poly(I:C), an agonist of TLR3, which is essential in the innate
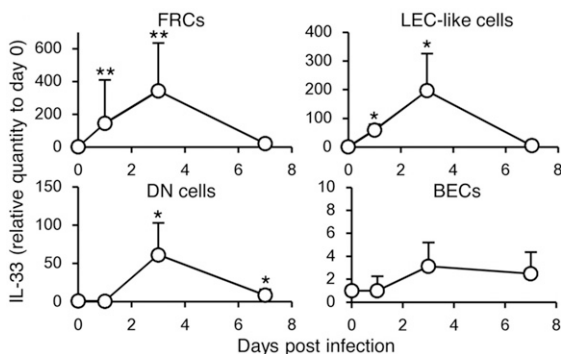

FIGURE 4. FRCs and LEC-like cells upregulate IL-33 during MCMV infection. Splenic stromal cell subsets were purified and IL-33 transcripts were quantified by quantitative RT-PCR. Relative quantity of IL-33 transcripts in each stromal subset in infected mice with 5×10^5 PFU MCMV was represented as the ratio relative to the quantity in uninfected mice (day 0). Data were pooled from three experiments (n = 6 mice/day). *$p < 0.05$, **$p < 0.01$ versus day 0. BECs, blood endothelial cells; DN, double-negative.
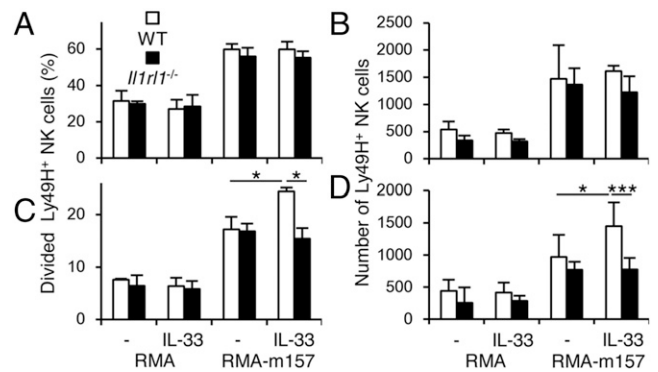

FIGURE 5. IL-33 enhances m157-dependent proliferation of Ly49H$^+$ NK cells. CellTrace Violet–labeled splenocytes from WT and $Il1rl1^{-/-}$ mixed BM chimeric mice were cocultured with RMA or m157-transfected RMA cells in the absence or presence of IL-33 and/or IL-12. Percentages of divided Ly49H$^+$ NK cells and the number of Ly49H$^+$ NK cells in the cultures with or without IL-33 in the absence (**A** and **B**) or presence of IL-12 (**C** and **D**) are shown. Data are representative of two experiments (n = 3–4 in each stimulation). *$p < 0.05$, ***$p < 0.005$.

immune defense against MCMV (22, 23). IL-33 is released by tissue damage or infection and subsequently cleaved into the biological active form by proteases released during inflammation (12). A previous study using an MCMV strain encoding GFP has demonstrated that MCMV infects FRCs and spreads in the spleen within 48 h p.i. (24). Interestingly, NK cells migrate to the white pulp of the spleen and are in contact with FRCs in the T cell area by 24 h after MCMV infection, prior to the expansion of Ly49H$^+$ NK cells (25). Taken together, a high concentration of bioactive IL-33 at the local interface of NK cells with stromal cells would ensure efficient ST2 signaling in NK cells, which may contribute to the preferential expansion of Ly49H$^+$ NK cells.

An IL-12/STAT4 signaling pathway is critical for both expansion and memory generation of NK cells in response to MCMV infection and alloantigen stimulation (6, 11). Unlike IL-12, the IL-33/ST2 signaling pathway augments the expansion of Ly49H$^+$ NK cells, but it is not required for differentiation into memory cells. Although IL-33 synergizes with IL-12 for IFN-γ production by NK cells in vitro (15, 16), production of IFN-γ by NK cells in vivo early after MCMV infection does not require IL-33 or ST2, indicating that other cytokines produced during infection might compensate. Moreover, previously we demonstrated that Ly49H$^+$ NK cells do not require IFN-γ to undergo expansion during MCMV infection (11), suggesting that the robust proliferation of NK cells requires IL-12–dependent signals and is enhanced by IL-33–dependent signals, but not IFN-γ–mediated signaling. Both IL-12–deficient and STAT4-deficient Ly49H$^+$ NK cells have a severe defect in expansion during MCMV infection (11), whereas an IL-33/ST2 signaling deficiency has a lesser impact. IL-18 and IL-1β, which are other members of the IL-1 cytokine family, are known to synergize with IL-12 for IFN-γ production by NK cells in vitro and in vivo (15, 16, 26). A recent study has demonstrated that an IL-18/IL-18R signaling axis is required for the optimal IFN-γ production, expansion, and memory differentiation of Ly49H$^+$ NK cells during MCMV infection (27). The authors show that MyD88-deficient Ly49H$^+$ NK cells exhibit the same defects as IL-18R–deficient Ly49H$^+$ NK cells (27). In contrast, IL-1R–deficient Ly49H$^+$ NK cells normally expand and differentiate into memory NK cells after the infection (27). In the present study, ST2-deficient Ly49H$^+$ NK cells exhibit impairment in MCMV-specific expansion of naive and memory Ly49H$^+$ NK cells, but neither in IFN-γ production nor in differentiation into memory

NK cells. Interestingly, IL-18R signaling is dispensable for the secondary expansion of memory Ly49H^{+} NK cells when rechallenged with MCMV. These results suggest that IL-33 is released by damaged cells in the early phase of MCMV infection and that ST2 signaling transiently enhances MyD88 signaling to augment the proliferation of naive and memory Ly49H^{+} NK cells, whereas IL-18 more broadly impacts NK cell responses in the course of MCMV infection.

Our findings indicate that multiple cytokines and their downstream signaling pathways differentially modulate the adaptive immune features of NK cells. Further studies of spatiotemporal regulation of cytokine production, as well as the adaptor molecules through which cytokine receptors signal, will be required to understand fully the molecular mechanisms underlying the differentiation of memory NK cells.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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