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**Graphical Abstract**

**Highlights**
- CMV-specific memory NK cells arise from KLRG1-negative progenitors
- Host microbiota and T cells regulate the memory NK cell progenitor pool
- Excess IL-15 drives terminal differentiation and depletes memory NK cell progenitors

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**In Brief**
Kamimura and Lanier report that memory NK cells arise from KLRG1-negative progenitors, which are regulated by host microbiota and T cells. Competition between NK cells and T cells for IL-15 prevents depletion of the memory NK cell progenitor pool.
Homeostatic Control of Memory Cell Progenitors in the Natural Killer Cell Lineage

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SUMMARY

Recent studies have demonstrated that natural killer (NK) cells are able to undergo clonal expansion and contraction and to generate self-renewing memory cells after infection with mouse cytomegalovirus (MCMV). It is unclear whether all or only certain subsets preferentially contribute to the generation of memory NK cells. Here, we show that memory NK cells predominantly arise from killer cell lectin-like receptor G1 (KLRG1)-negative NK cell progenitors, whereas KLRG1-positive NK cells have limited capacity for expansion during infection with MCMV. Unexpectedly, the frequency of KLRG1-positive NK cells is significantly affected by the presence of T cells in the host and potentially by the host microbiota. Our findings demonstrate that excessive availability of interleukin (IL)-15 may erode the pool of memory progenitors, resulting in the decreased efficiency of memory generation in the NK cell lineage.

INTRODUCTION

Natural killer (NK) cells are a subset of innate lymphocytes that protect both humans and mice from certain microbial infections and tumors. Until recently, NK cells were considered exclusively as part of the innate immune defenses; however, it becomes increasingly evident that NK cells can exhibit adaptive immune-like features, including the ability to generate long-lived “memory” NK cells in response to various types of antigens (Gillard et al., 2011; O’Leary et al., 2006; Paust et al., 2010; Peng et al., 2013; Sun et al., 2009a). Mouse cytomegalovirus (MCMV) infection is a well-characterized model for studying the mechanisms of host responses against viruses. NK-cell-mediated resistance to MCMV is achieved through Ly49H, an activating NK cell receptor present in MCMV-resistant C57BL/6 (B6) mice but absent in susceptible strains such as BALB/c (Smith et al., 2000). Ly49H recognizes the MCMV-encoded glycoprotein m157 on the surface of infected cells (Arase et al., 2002; Smith et al., 2002) and delivers activating signals through the adaptor proteins DAP10 and DAP12 (Orr et al., 2009). DAP12 is indispensable for stable expression of Ly49H on the cell surface (Arase et al., 2002; Orr et al., 2009).

Ly49H-expressing NK cells (approximately 50% of total NK cells) preferentially expand in response to MCMV infection (Dokun et al., 2001). In the setting of adoptive transfer of NK cells into DAP12- or Ly49H-deficient hosts, Ly49H+ NK cells undergo a robust clonal expansion followed by contraction and surviving NK cells persist for several months (Sun et al., 2009a). These self-renewing mature NK cells undergo secondary expansion in response to rechallenge with MCMV and can protect neonates from MCMV infection about ten times better than naive NK cells (Sun et al., 2009a). Recent studies demonstrated that several factors are critical for the generation of memory NK cells in MCMV infection, including interleukin (IL)-12 (Sun et al., 2012), microRNA-155 (Zawislak et al., 2013), and DNAM-1 (Nabekura et al., 2014). However, whether all Ly49H+ NK cells or only a certain progenitor cell population gives rise to memory NK cells remains to be elucidated.

NK cells share many traits in common with CD8+ T cells (Sun and Lanier, 2011). Naive CD8+ T cells proliferate after antigen-specific activation and develop into short-lived effector and long-lived memory cells. In the CD8+ T cell lineage, KLRG1 has been used as a marker to distinguish short-lived effector (KLRG1+) and long-lived memory (KLRG1−) T cells. Naive CD8+ T cells do not express KLRG1, but it is induced after antigen-specific activation. KLRG1+ T cells expand more robustly and generate more memory T cells than KLRG1− T cells (Sarkar et al., 2008).

KLRG1 has also been used as a marker for mature NK cells (Hayakawa and Smyth, 2006; Huntington et al., 2007; Robbins et al., 2004). Approximately 30%–50% of NK cells in resting, uninfected mice express KLRG1 (Huntington et al., 2007) at intermediate levels (KLRG1int+), and KLRG1− NK cells give rise to KLRG1+ NK cells after adoptive transfer into Rag2−/−Il2rg−/− mice (Huntington et al., 2007). After infection with MCMV, 90%–100% of NK cells express KLRG1 in very high amounts (KLRG1high) (Fogel et al., 2013; Robbins et al., 2002, 2004). Fogel et al. (2013) have noted that Ly49H+ NK cell that have specifically responded to MCMV express high levels of KLRG1, with down-regulation of SCA-1 and CD27. Furthermore, we have observed that this high level of KLRG1 is stably maintained on MCMV-specific memory NK cells for months after infection (Sun et al., 2009a; Nabekura et al., 2014). KLRG1 is not required for NK cell maturation or effector functions because Klrgr1−/− C57BL/6 mice demonstrate normal NK cell development, cytolytic activity, and production of cytokines and mount a normal protective response to MCMV infection (Gründemann et al.,
Both the Ly49H+ KLRG1+ memory NK cells in wild-type (WT) and Klrg1−/− mice after infection with MCMV (Y.K., unpublished data). Nonetheless, KLRG1 provides an informative marker to distinguish subsets of NK cells based on maturational status. Here, we demonstrate that memory NK cells are predominantly derived from the KLRG1-negative progenitors, whereas the KLRG1int+ NK cells in healthy, uninfected mice have limited capacity for expansion or memory formation in response to MCMV infection. Further, our studies revealed that the presence of T cells and possibly commensal bacteria in the host might influence the generation of memory NK cells by influencing the abundance of these KLRG1− memory NK cell progenitors.

RESULTS

KLRG1− NK Cells Preferentially Generate Memory NK Cells

KLRG1− NK cells are capable of activation and degranulation when cocultured in vitro with target cells expressing m157, whereas KLRG1int− NK cells are potent memory progenitors, whereas the KLRG1int+ NK cells that develop during steady state in naive mice are effector cells with limited potential for expansion, resembling KLRG1− CD8+ T cells. To compare the capacity of
Percentages of transferred Ly49H+ NK cells isolated from WT and Ly49H+ NK cells. This result suggests that the marked proliferation of Ly49H+ NK cells into Ly49H-deficient hosts and infected with KLRG1 on day 3 (Figure 1C), consistent with their equivalent ability to degranulate when stimulated with m157-bearing target cells (Figure S1B). However, Ly49H-deficient mice receiving KLRG1− Ly49H+ NK cells showed decreased viral loads in oral lavage on day 14 compared to those receiving KLRG1int+ Ly49H+ NK cells. This result suggests that the marked proliferation by KLRG1− memory NK cell progenitors may be important for control of persistent, but not acute, virus infection.

**T Cells Preserve the Progenitors for Memory NK Cells**

We observed that the percentage of KLRG1int+ NK cells in spleen and peripheral blood isolated from T-cell-deficient mice, e.g., Rag1−/− mice and Tcra−/− mice, but not B-cell-deficient mice (μMT), is higher than from syngeneic WT mice (Figure 2A). To directly compare their proliferation and memory generation, we cotransferred equal numbers of CD45-congenic WT and Rag1−/− Ly49H+ NK cells into Ly49H-deficient mice. After MCMV infection, Rag1−/− Ly49H+ NK cells expanded less and generated fewer memory NK cells than WT NK cells (Figure 2B). Similarly, Ly49H+ NK cells isolated from Tcra−/− mice were isolated from these chimeric mice and cotransferred with an equal number of WT (CD45.1+) Ly49H+ NK cells. Graph shows percentages of Ly49H+ NK cells within the total NK cell population at the indicated time points after MCMV infection. (E) Percentages of KLRG1int+ cells within the total NK cell population in WT and Rag1−/− mice and in mixed bone marrow chimeric mice (1:1 mixture of WT [CD45.1+] and Rag1−/− [CD45.2+]) cells. BM, bone marrow. (F) WT and Rag1−/− NK cells were isolated from the mixed BMC mice (E), adoptively transferred into Ly49H-deficient hosts, and then infected with MCMV. Percentages of WT and Rag1−/− NK Ly49H+ NK cells after MCMV infection are shown. (G) Percentages of KLRG1int+ cells within the total NK cell population in WT and Rag1−/− mice and in Rag1−/− mice given WT spleen cells (5 x 10^7 cells).

Data are representative of two (C, I, and J), three (E and F), and four (A and B) independent experiments. Error bars indicate SEM (n = 3–5). See also Figure S2.
KLRG1<sup>int+</sup> NK cells in that commensal bacteria might be involved in the induction of C57BL/6 mice obtained from different vendors or animal body on the day of WT splenocytes transfer in order to deplete all mature endogenous NK cells. This allowed newly emerging endogenous <i>Rag1</i><sup>−/−</sup> NK cells to be exposed to mature WT T and B cells. At the same time, control WT and <i>Rag1</i><sup>−/−</sup> mice (not receiving adoptively transferred splenocytes) were also treated with anti-NK1.1 antibody. After 8 weeks, the percentage of KLRG1<sup>int+</sup> NK cells derived from these endogenous <i>Rag1</i><sup>−/−</sup> NK cell progenitors was comparable to NK cells in WT mice (Figure 2G) and the response to MCMV was rescued (Figure 2H). These findings indicate that T cells suppress the induction of KLRG1<sup>int+</sup> NK cells during steady state in WT mice and maintain memory NK cell progenitors.

To further determine whether KLRG1<sup>−</sup> NK cells from <i>Rag1</i><sup>−/−</sup> mice possess the same capacity for expansion and memory generation as KLRG1<sup>−</sup> NK cells from WT mice, we sorted KLRG1<sup>−</sup> NK cells from WT (CD45.1<sup>+</sup>) and <i>Rag1</i><sup>−/−</sup> mice and cotransferred them (mixed 1:1) into Ly49H-deficient hosts followed by MCMV infection. Both NK cells showed equivalent expansion and memory generation (Figure 2I). We observed equivalent results using a mixture of NK cells from WT (CD45.1<sup>+</sup>) and <i>Tcra</i><sup>−/−</sup> mice (Figure 2J). These results indicate that it is the frequency of KLRG1<sup>int+</sup> NK cells in these strains that is the important factor for differential NK cell expansion and memory generation and not an intrinsic property of the NK cells in these strains.

### Nucleotide-Binding Oligomerization Domain Sensors in the Host Affect the Progenitors for Memory NK Cells

Because the frequency of KLRG1<sup>int+</sup> NK cells in naive, uninfected mice can be influenced by extrinsic influences, we investigated whether commensal bacteria affect the generation of KLRG1<sup>int+</sup> NK cells at steady state. The initial clue that commensal bacteria might be involved was our observation that C57BL/6 mice obtained from different vendors or animal facilities displayed distinct percentages of KLRG1<sup>int+</sup> NK cells in naive, uninfected mice (Figure S2). Hence, we hypothesized that commensal bacteria might be involved in the induction of KLRG1<sup>int+</sup> NK cells in <i>Rag1</i><sup>−/−</sup> mice. To test this, we cohoused WT mice with <i>Rag1</i><sup>−/−</sup> mice, where the transfer of microbiota can be expected. After cohousing for 3 weeks, splenic NK cells from WT mice cohoused with <i>Rag1</i><sup>−/−</sup> mice showed an increased frequency of KLRG1<sup>int+</sup> NK cells compared to the frequency of KLRG1<sup>int+</sup> NK cells in WT mice (Figure 3A). To further address the involvement of commensal bacteria in the generation of KLRG1<sup>int+</sup> NK cells at steady state, we depleted commensal bacteria by treatment with a cocktail of broad-spectrum antibiotics (designated as Abx) in the drinking water. Treatment with Abx water significantly reduced the frequency of KLRG1<sup>int+</sup> NK cells compared to treatment with control water (Figure 3B). Of note, any single Abx treatment was not effective (data not shown). To compare their responses to MCMV, we cotransferred an equal number of Ly49H<sup>+</sup> NK cells from control water-treated mice (CD45.2<sup>+</sup>) and Abx-treated mice (CD45.1<sup>+</sup>) into Ly49H-deficient recipients (receiving control water). After MCMV infection, NK cells from Abx-treated mice proliferated more robustly and generated memory NK cells more efficiently than NK cells from control water-treated mice (Figure 3C).

To investigate how commensal bacteria might induce KLRG1<sup>int+</sup> NK cells, we screened informative mutant mice lacking innate pattern-recognition receptors and their signaling molecules, which included mice lacking MyD88 and Ticam1 (also known as Trif), Nod1, Nod2, or Nod1 and Nod2. Only Nod-deficient mice showed a decreased percentage of KLRG1<sup>int+</sup> NK cells at steady state (data not shown; Figure 3D). NK cells isolated from Nod1<sup>−/−</sup> (Figure 3E) and Nod2<sup>−/−</sup> B6 mice (Figure 3F) proliferated more vigorously and generated more memory NK cells than NK cells isolated from WT B6 mice. To determine whether intrinsic or extrinsic factors in Nod-deficient mice regulate KLRG1 expression, we generated mixed BMC mice with bone marrow cells from WT (CD45.1<sup>+</sup>) and Nod1<sup>−/−</sup> or Nod2<sup>−/−</sup> mice (CD45.2<sup>+</sup>) introduced into lethally irradiated WT recipients. The difference in KLRG1 expression between WT and Nod-deficient NK cells was abolished in mixed BMC mice (data not shown). This indicates that Nod sensing is not NK cell intrinsic but is due to Nod-dependent sensors on nonhematopoietic cells in the host. To compare the expansion and memory generation, we isolated WT and Nod-deficient NK cells from these chimeric mice and adoptively transferred the NK cells into Ly49H-deficient hosts. After MCMV infection, although Nod1<sup>−/−</sup> NK cells showed slightly decreased expansion, both Nod1<sup>−/−</sup> and Nod2<sup>−/−</sup> NK cells demonstrated equivalent generation of memory cells compared to WT NK cells (Figures 3G and 3H). To determine whether KLRG1<sup>−</sup> NK cells from WT and Nod2<sup>−/−</sup> mice possess the same capacity for expansion and memory generation, we sorted KLRG1<sup>−</sup> NK cells from WT (CD45.1<sup>+</sup>) and <i>Tcra</i><sup>−/−</sup> mice and cotransferred an equal number of Ly49H<sup>−</sup> NK cells into Ly49H-deficient hosts. Both WT and Nod2<sup>−/−</sup> KLRG1<sup>−</sup> Ly49H<sup>−</sup> NK cells expanded and generated memory in the same manner (Figure 3I). These results suggest that commensal bacteria might promote the induction of KLRG1<sup>int+</sup> NK cells and erode the reservoir for memory NK cell progenitors.

### Antibiotic Treatment Suppresses Homeostatic Proliferation of NK Cells

Our results indicate that T cells suppress the induction of KLRG1<sup>int+</sup> NK cells, whereas in WT mice, commensal bacteria might promote the generation of KLRG1<sup>int+</sup> NK cells. To test whether a T-cell-deficient environment is sufficient to explain the increased frequency of KLRG1<sup>int+</sup> NK cells, we transferred WT CD45.1-congenic NK cells into WT, <i>Rag1</i><sup>−/−</sup>, or <i>Tcra</i><sup>−/−</sup> mice. Donor NK cells transferred into <i>Rag1</i><sup>−/−</sup> or <i>Tcra</i><sup>−/−</sup> mice, but not WT mice, demonstrated a dramatic increase in the percentage of KLRG1<sup>int+</sup> NK cells (Figure 4A, upper panels). Unexpectedly, donor WT NK cells underwent homeostatic proliferation in <i>Rag1</i><sup>−/−</sup> and <i>Tcra</i><sup>−/−</sup> mice, which contain normal numbers of endogenous NK cells (Figure 4A, upper panels). This increased baseline proliferation in <i>Rag1</i><sup>−/−</sup> and <i>Tcra</i><sup>−/−</sup> mice
was further confirmed by the increased expression of Ki67 in NK cells isolated from these mice compared to those from WT mice (Figure S3). Furthermore, donor WT NK cells transferred into Abx water-treated Rag1<sup>−/−</sup> or Tcrα<sup>−/−</sup> mice showed a decreased frequency of KLRG1<sup>int+</sup> NK cells and less homeostatic proliferation compared to NK cells from control Rag1<sup>−/−</sup> or Tcrα<sup>−/−</sup> mice (Figure 4A, lower panels). These results suggest that commensal bacteria might be responsible for generating KLRG1<sup>int+</sup> NK cells in mice lacking T cells and that T cells suppress this process.

To determine the involvement of Nod2 in this system, we transferred CD45.1-congenic WT NK cells into Rag1<sup>−/−</sup>Nod2<sup>−/−</sup>CD45.2<sup>+</sup> mice. Donor WT NK cells showed a decreased frequency of KLRG1<sup>int+</sup> NK cells and less homeostatic proliferation when transferred into Rag1<sup>−/−</sup>Nod2<sup>−/−</sup> mice compared with WT NK cells transferred into Rag1<sup>−/−</sup> mice (Figure 4B). Therefore, Nod2 in the recipient Rag-deficient mice enhances the homeostatic proliferation and generation of KLRG1<sup>int+</sup> NK cells. Collectively, these findings suggest that both T cells and possibly commensal bacteria regulate KLRG1 induction and homeostatic proliferation.

Availability of IL-15 Regulates Generation of KLRG1<sup>int+</sup> NK Cells

Because IL-15 is known to be important in the development and homeostasis of NK cells and IL-15 is also used by T cells to efficiently develop memory T cells, we investigated whether T cells might suppress the induction of KLRG1 on NK cells by competition for IL-15 (Sun et al., 2009b). We found that the few NK cells present in WT (CD45.1<sup>+</sup>) and Nod1<sup>−/−</sup> or Nod2<sup>−/−</sup> (CD45.2<sup>+</sup>) mice were few NK cells derived from WT (CD45.1<sup>+</sup>) and Nod1<sup>−/−</sup> or Nod2<sup>−/−</sup> (CD45.2<sup>+</sup>) mice after MCMV infection.

To address whether IL-15 is also responsible for elevated KLRG1 expression in Rag<sup>−/−</sup> mice, we crossed Il15<sup>−/−</sup> mice with Rag1<sup>−/−</sup> mice. We found that KLRG1<sup>int+</sup> NK cells were unde-
NK cells and less homeostatic proliferation in \( \text{Rag1}^{-/-}\) mice than in \( \text{Rag1}^{-/-}\text{Il15}^{-/-}\) mice (Figure 5C). Note that donor NK cells were undetectable if transferred into \( \text{Tcra}^{-/-}\text{Il15}^{-/-}\) mice (data not shown), consistent with prior studies (Burkett et al., 2004; Sandau et al., 2004). These findings indicate that the availability of IL-15 might regulate the abundance of KLRG1\(^{int+}\) NK cells at steady state.

Although it is not essential for the development of naive T cells, IL-15 can promote survival of both naive and memory T cells (Bernard et al., 2003). We hypothesized that T cells might suppress the differentiation or activation of NK cells by competitively consuming IL-15 rather than a unique subset of T cells actively suppressing the induction of KLRG1\(^{int+}\) NK cells. Consistent with this hypothesis, we found that CD45.1-congenic WT NK cells transferred into \( \text{Tcra}^{-/-} \text{OT-I or Tcra}^{-/-} \text{OT-II} \) TcR-transgenic CD45.2\(^{-}\) mice failed to undergo homeostatic proliferation and induce KLRG1 (Figure 5D). To determine the involvement of regulatory T cells (Treg) cells in the suppression, we transferred CD45.1-congenic WT NK cells into \( \text{Cd28}^{-/-} \text{CD45.2}^{-}\) mice, which have severely impaired Treg development when NK cells were introduced into either WT or \( \text{Cd28}^{-/-} \) recipients (Figure 5E). These results indicate that either monospecific conventional CD\(^{+}\) T cells or CD\(^{8}\) T cells are sufficient for the suppression of homeostatic proliferation and acquisition of KLRG1 by NK cells and that Treg cells are not required.

To directly evaluate the effect of IL-15 and T cells on the induction of KLRG1 by NK cells, we sorted KLRG\(^{1-}\) NK cells and cultured them in the presence of various concentration of IL-15 with or without T cells. After 72 hr in culture with IL-15, KLRG1 was induced in a dose-dependent manner on NK cells (Figure 5F, filled circle) and the addition of T cells suppressed the generation of KLRG1\(^{int+}\) NK cells (Figure 5G, open circle). To determine whether T cells require cell contact with NK cells to inhibit the induction of KLRG1\(^{int+}\) NK cells, we performed transwell experiments. T cells suppressed the IL-15-dependent induction of KLRG1\(^{int+}\) NK cells in the absence of cell-cell contact (Figure 5G). Thus, T cells might competitively consume IL-15 and therefore block NK cell acquisition of KLRG1.

### Antibiotics Improve Control of MCMV Infection

Our findings suggest that commensal bacteria might drive the generation of KLRG1\(^{int+}\) NK cells, which we have shown are less responsible to MCMV infection than KLRG1\(^{-}\) NK cells. Although Ly49H\(^{+}\) NK cells are responsible for early phase control of MCMV infection, severe combined immunodeficiency (SCID) or Rag-deficient mice are unable to completely control MCMV and die within 1 month after infection. Although mutation in m157, the viral ligand of Ly49H, can be observed in MCMV isolated from SCID or Rag-deficient mice (French et al., 2004), we considered the possibility that the poor proliferation of \( \text{Rag1}^{-/-}\) NK cells due to the increased frequency of KLRG1\(^{int+}\) NK cells might be another reason for the failure to control MCMV in \( \text{Rag1}^{-/-}\) mice. Prior studies have reported that NK cell control of MCMV infection is dependent on robust NK cell proliferation (Orr et al., 2009),...
therefore suggesting that mice bearing a higher frequency of more-responsive KLRG1– NK cells might control infection more efficiently. We found that Abx treatment increased the frequency of KLRG1– NK cells in Rag1–/– mice and Tcra–/– mice (Figure 6A). Moreover, Ly49H+ NK cells isolated from Abx water-treated Tcra–/– and Rag1–/– mice showed improved MCMV-driven proliferation and memory generation compared to those from control water-treated Tcra–/– and Rag1–/– mice (Figures 6B–6E).

We tested whether Abx treatment of Rag1–/– mice would affect their ability to control MCMV infection. Rag1–/– mice were given either control or Abx-treated water before infection (starting at day −21) and continued after MCMV infection (day 0) throughout the experiment. We monitored survival, the percentage of Ly49H+ NK cells, and viral DNA copy numbers in peripheral blood and oral lavage. Ly49H+ NK cells in control Rag1–/– mice expanded but diminished at day 21, whereas those in Abx water-treated Rag1–/– mice increased and persisted at day 21 (Figure 6F). Furthermore, Abx water-treated Rag1–/– mice clearly survived longer than control Rag1–/– mice (median survival days: 45 versus 25; p < 0.0001; Figure 6G). The viral load in blood was readily detectable at day 3, temporarily decreased at day 7, and increased after day 14, but Abx treatment did not reduce the viremia (Figure 6H). Although the viral
load in oral lavage was increased after MCMV infection (reflecting the accumulation of virus in salivary glands), a lower viral load was detected in Abx water-treated \( \text{Rag1}^{-/-} \) mice than in control water-treated \( \text{Rag1}^{-/-} \) mice (Figure 6I). Additionally, to test whether prolonged survival by Abx water treatment is NK-cell-dependent, we injected anti-NK1.1 antibody to control or Abx water-treated \( \text{Rag1}^{-/-} \) mice 1 day before MCMV infection. In the absence of NK cells, both control and Abx water-treated \( \text{Rag1}^{-/-} \) mice died with the same kinetics (Figure S5). These results indicate that Abx treatment allows for a more-robust NK cell response, possibly due to increasing the abundance of the more-responsive KLRG1\(^{+/+}\) NK cell subset, and is beneficial for NK cell control of persistent virus infection.

**DISCUSSION**

Recently, several types of NK cells have been reported to possess immunological memory. Peng et al. (2013) reported that a subset of DX5\(^{+}\) NK cells in liver is responsible for hapten-specific responses. These DX5\(^{+}\) hepatic NK cells (or ILC1 cells) do not express Ly49H (Sojka et al., 2014) and lack Eomes (Daussy et al., 2014), in contrast with conventional NK cells that are Eomes-positive and express DX5 and activating Ly49 receptors. In this report, we demonstrate that memory progenitors can be identified as KLRG1\(^{+/+}\) Ly49H\(^{+}\) NK cells with the capacity for MCMV-specific expansion and memory generation. The KLRG1\(^{+/+}\) Ly49H\(^{+}\) NK cells express a more-mature phenotype.
(CD27− CD11b+ ) than the KLRG1−Ly49H+ NK cells, suggesting that once Ly49H+ NK cells have reached a mature stage of differentiation, they lose their potential for memory generation. It is unlikely the KLRG1 receptor itself is responsible for this lack of responsiveness because memory NK cells express high levels of KLRG1, and we have shown that they are capable of secondary expansion equivalent to naive NK cells when they are rechallenged with MCMV in vivo (Sun et al., 2009a, 2010). Unexpectedly, we found that T cells and possibly commensal bacteria reciprocally regulate the pool of memory NK cell progenitors.

Rag-deficient mice have been used in NK cell studies to exclude the involvement of T and B cells. However, NK cell development and responsiveness in the absence of T and B cells have not been extensively examined. It was reported that Rag plays a cell-intrinsic role in the development of NK cells because accumulation of NK cells in spleen, liver, and bone marrow of Rag-deficient mice is slower than in WT mice during ontogeny (Andrews and Smyth, 2010). Although Rag is not expressed in mature NK cells, a subset of NK cell precursors in bone marrow experience incomplete TcR V(D)J recombination (Pilbeam et al., 2008). Furthermore, Karo et al. (2014) reported that Ly49H+ NK cells derived from precursor cells that had transiently expressed Rag during their early development preferentially expand and generate memory NK cells during MCMV infection, whereas NK cells that arose from precursor cells that failed to express Rag are more prone to apoptosis, possibly due to less-efficient repair of DNA damage. Our current findings, however, suggest that the absence of T cells, in Rag-deficient or TcRζ-deficient mice, impacts the frequency of memory NK cell progenitors in a cell-extrinsic manner, potentially by competing for IL-15. Although IL-15 is necessary for the development and homeostasis of NK cells at steady state, we observed that IL-15 might also drive the generation of KLRG1int+ NK cells at the expense of memory progenitors. Consistent with our observations, it has also been reported that continuous injection of IL-15 causes up-regulation of KLRG1 expression and hyporesponsiveness of NK cells (Elpek et al., 2010). Gasteiger et al. (2013) recently reported that Treg and NK cells compete for IL-2 in the host and that this affects the maturation of NK cells and preserving memory NK cells.

Collectively, these prior observations and our findings in this study suggest a model in which NK cells are regulated both by the microbiota and essential for the induction of IL-15 in intestinal macrophages; however, it remains to be elucidated why Nod, but not Toll-like receptors, apparently plays a role in the induction of IL-15. It is also unclear how IL-15 induced by commensal bacteria in the intestine might affect the frequency of memory progenitors in spleen and blood. However, Yokoyama et al. (2009) reported that enterocyte-specific overexpression of IL-15 results in a significant increase of NK cell number in peripheral blood and enlargement of spleen. Further studies are needed to understand whether NK cells circulate through the intestine to encounter IL-15 or if blood-borne signals, as suggested by Ganal and colleagues, induce IL-15 in myeloid cells located distally. Collectively, these prior observations and our findings in this study suggest a model in which NK cells are regulated both by T cells in the host and the microbiota. We speculate that Nod-dependent sensing of commensal bacteria might regulate the production of IL-15 by myeloid cells. In steady state, T cells and NK cells compete for the limiting amounts of IL-15, thereby restraining the maturation of NK cells and preserving memory NK cell progenitors.

NK cells adoptively transferred into Rag2−−/Il2rg−− mice undergo homeostatic proliferation and acquire the expression of KLRG1 (Sun et al., 2011). Unexpectedly, we observed that WT NK cells also underwent homeostatic proliferation when adoptively transferred into Rag1−− or Tcra−− hosts, despite the presence of endogenous host NK cells, which was dependent on IL-15 and was diminished by Abx treatment. Recently, commensal bacteria have been implicated as a novel regulator of the immune system (Atarashi et al., 2013; Ivanov et al., 2009; Lathrop et al., 2011; Round and Mazmanian, 2010); however, the impact of commensal bacteria on NK cell development, homeostasis, and effector function are poorly understood. Ganal et al. (2012) reported that NK cells in germ-free mice are poorly activated by various pathogen-associated molecular patterns due to impaired cytokine production by nonmucosal myeloid cells, resulting in decreased activation of NK cells and poor clearance of MCMV in spleen at day 3 postinfection compared to specific pathogen-free mice. Similar impairment of myeloid cells was also induced by Abx treatment and resulted in a decreased number and activation of influenza and lymphoctic-choriomeningitis-virus-specific CD8+ T cells (Abt et al., 2012). We observed a similar trend at day 3 postinfection of Rag-deficient mice with MCMV; however, Abx treatment improved control of MCMV infection at later time points and prolonged survival of the mice. French et al. (2004) have reported that m157-mutated MCMV outgrow in C57BL/6-SCID mice by day 28 postinfection. Although both control and Abx-treated Rag-deficient mice succumbed to MCMV infection, we have demonstrated that Abx treatment increased the frequency of memory progenitors and improved expansion of Ly49H+ NK cells that might allow for better control of MCMV and result in prolonged survival.

Although Ganal and colleagues did not address how commensal bacteria influence myeloid cells in distal sites, it is possible that systemically circulating Nod ligands (Clarke et al., 2010) deliver instructive signals to peripheral myeloid cells. Nod1 and Nod2 are intracellular pattern-recognition receptors sensing D-glutamyl-meso-diaminopimelic acid and muramyl dipeptide, respectively, which are unique motifs within bacterial peptidoglycans. Nod1 is expressed ubiquitously, whereas Nod2 is limited to myeloid subsets and intestinal epithelial cells. Once ligand binds, Nod proteins phosphorylate Rip2 and activate NF-κB, resulting in the expression of a variety of proinflammatory cytokines. The II15 promoter contains a NF-κB-binding element that is necessary for the efficient transcription of IL-15 (Azimi et al., 2000; Washizu et al., 1998). Recently, Jiang et al. (2013) demonstrated that Nod2 is important for sensing gut microbiota and essential for the induction of IL-15 in intestinal macrophages; however, it remains to be elucidated why Nod, but not Toll-like receptors, apparently plays a role in the induction of IL-15. It is also unclear how IL-15 induced by commensal bacteria in the intestine might affect the frequency of memory progenitors in spleen and blood. However, Yokoyama et al. (2009) reported that enterocyte-specific overexpression of IL-15 results in a significant increase of NK cell number in peripheral blood and enlargement of spleen. Further studies are needed to understand whether NK cells circulate through the intestine to encounter IL-15 or if blood-borne signals, as suggested by Ganal and colleagues, induce IL-15 in myeloid cells located distally. Collectively, these prior observations and our findings in this study suggest a model in which NK cells are regulated both by T cells in the host and the microbiota. We speculate that Nod-dependent sensing of commensal bacteria might regulate the production of IL-15 by myeloid cells. In steady state, T cells and NK cells compete for the limiting amounts of IL-15, thereby restraining the maturation of NK cells and preserving memory NK cell progenitors.
Nod receptors on host nonhematopoietic cells sense commensal bacteria and enhance IL-15 production in myeloid cells. Competitive consumption of IL-15 by T cells inhibits the induction of KLRG1+ NK cells and preserves progenitor KLRG1− NK cells that preferentially develop into KLRG1+ memory NK cells. Cell competition, the availability of excess IL-15 drives terminal maturation of NK cells in the host. Controlling the balance of effector NK cells versus memory progenitor NK cells might be beneficial for optimal responsiveness of NK cells to microbial infections.

**EXPERIMENTAL PROCEDURES**

**Mice and Infections**

CD45.1-congenic C57BL/6 mice were purchased from the National Cancer Institute and bred at the University of California, San Francisco (UCSF). Rag1−/−, Tcra−/−, μmt, Rag2−/−Il2rg−/−, I15−/−, Rag1−/−Nod2−/−, Rag1−/−I15+, Rag1−/−I15−, Rag1−/−Nod2−/−, Rag1−/−I15+, Rag1−/−Nod2−/−, CD28−/−, Tcra−/− OT-I TCR transgenic, Tcra−/− OT-II TCR transgenic, and KIR8−/− (Ly49H-deficient) (Fodi-Cornu et al., 2008) mice on a C57BL/6 background were bred at UCSF. All mice were maintained in accordance with guidelines of the Institutional Animal Care and Use Committee. Mixed bone marrow chimERIC mice were generated as described previously (Sun et al., 2009a). Briefly, recipient (CD45.1) mice were irradiated at 550 rad twice with a 4-hr interval. WT (CD45.1) and mutant (CD45.2) bone marrow cells were mixed (1:1) and adaptively transferred (total 2 × 10^6 cells/mouse) into recipient mice after irradiation. One tablet of SCIDS MD’s (6 mg sulfamethoxazole and 1.2 mg trimethoprim; Bio-Serve) was added to each cage 1 day before irradiation. Mice were rested for 8 weeks and then used for experiments. For MCMV infection, 50 μl of heparinized blood was lysed by ACK buffer and stained with anti-TCRβ (H57-597; BioLegend), -NK1.1 (PK136; BioLegend), -Ly49H (3D10; eBioscience), -CD45.1 (A20; BioLegend), -CD45.2 (104; Tonbo Biosciences), and -KLRG1 (2F1; BioLegend). Cells were analyzed on a BD LSR II flow cytometer using FlowJo software (Tree Star).

**Cell Preparation**

NK cells were enriched with an NK Cell Isolation kit (Miltenyi Biotec) or a method where spleen cells were incubated with purified rat antibodies against mouse CD4, CD5, CD8, CD19, Gr-1, and Ter119, followed by anti-rat immuno-globulin G conjugated to magnetic beads (QIAGEN). Purity of enriched NK cells was analyzed by staining with anti-TCRβ, NK1.1, and Ly49H. After enrichment, TCRβ+ NK1.1− cells were typically 50%−70% and half expressed Ly49H. One hundred thousand Ly49H+ NK cells were injected intravenously into Ly49H-deficient recipients 1 day before viral infection. In some experiments, NK cells were labeled with 1 μM CellTrace Violet (Invitrogen), according to the manufacturer’s instructions, and 5 × 10^6 cells were injected into recipients via intravenous injection. In some experiments, enriched NK cells were further sorted as TCRβ+ KLRG1+ or TCRβ+ KLRG1− cells using a BD Aria III flow cytometer.

**Commensal Bacteria Depletion**

Mice were given broad-spectrum Abx (ampicillin 1 g/ml, Sigma; neomycin sulfate 1 g/ml, Sigma; metronidazole 1 g/ml, Sigma; and vancomycin 0.5 g/ml, Goldbio) in drinking water for 3 weeks. Depletion of bacteria was confirmed by culturing of feces and determining colony-forming units on BHI culture plates (NEOGEN).

**Cohousing Experiment**

Female WT C57BL/6 mice purchased from the National Cancer Institute (which served as “recipients”) were cohoused with female Rag1−/− (which served as “donors”) for 3 weeks. Three WT recipients and two Rag1−/− donors were housed per cage. The frequency of KLRG1+ NK cells in splenocytes isolated from the recipients was analyzed.

**Quantitation of MCMV**

MCMV viral loads were monitored sequentially by real-time quantitative PCR (qPCR) as previously described (Kamimura and Lanier, 2014). For oral lavage collection, the mouse sublingual cavity was extensively washed with 20 μl of sterile saline under anesthesia. Five to ten microliters of lavage fluid was collected, and viral load was determined by qPCR analysis. To measure viral load in peripheral blood, 10 μl of peripheral blood was processed with a GenElute Blood Genomic DNA kit (Sigma), eluted with 20 μl of sterile nuclease-free water, and analyzed by qPCR. Primers use MCMV-IE1 Forward: 5′-AGCCACCAACATTGACCACGCAC-3′ and MCMV-IE1 Reverse: 5′-GCC CCAACGAGCACACCACTC-3′. To establish a standard curve for quantitation of viral load, full-length MCMV-IE1 DNA was amplified by primers IE1-full Forward: 5′-TGTCGGCAAAAGATCCCTCGC-3′ and IE1-full Reverse: 5′-TCC TGCTCTGCTGTCTT-3′ and inserted into the TOPO-PCR2.1 vector (Invitrogen).

**Statistical Method**

The unpaired t test was used to compare two groups. The Newman-Keuls test was used to compare three or more groups. The log rank test was used to compare survival between groups of mice. p values provided in figures are reported as *p < 0.05, **p < 0.001, and ***p < 0.0001, respectively.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.12.025.

**AUTHOR CONTRIBUTIONS**

Y.K. planned and performed experiments and wrote the manuscript, and L.L.L. contributed to experimental design, data evaluation, and writing of the manuscript.
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