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Expansion and Contraction of the NK Cell Compartment in Response to Murine Cytomegalovirus Infection

Scott H. Robbins,* Marlowe S. Tessmer,* Toshifumi Mikayama,† and Laurent Brossay2*

NK cells are capable of responding quickly to infectious challenge and contribute to the early defense against a wide variety of pathogens. Although the innate NK cell response to murine CMV (MCMV) has been extensively characterized, its resolution and the fate of the activated NK cell population remains unexplored. Herein, we characterize both the expansion and contraction phases of the NK cell response to MCMV. We demonstrate that NK cell recruitment into the immune response to MCMV infection is restricted to the first 3 days of infection and as the peripheral NK cell compartment expands, NK cells undergo accelerated phenotypic maturation. During the resolution of the immune response, NK cell compartmental contraction is marked by the selective death of responding NK cells. Additionally, throughout the infection, a naive NK cell pool that remains responsive to additional stimuli is actively maintained. These findings illustrate the plasticity of the NK cell compartment in response to pathogens and underscore the homeostatic maintenance of the resting peripheral NK cell pool. The Journal of Immunology, 2004, 173: 259–266.

Much is known about the in vivo kinetics of Ag-specific T cell responses to pathogens. Collaboratively, Ag recognition by the TCR and the simultaneous delivery of costimulatory signals by APCs drives T cells to undergo clonal expansion and maturation from naive to effector cells (1, 2). After a programmed expansion phase (3), responding T cells undergo a contraction phase (4). The result of the contraction phase is the dramatic reduction in the numbers of the responding T cell population. Cells that survive the contraction phase are the source of the stably maintained T cell memory population.

Unlike T cells, NK cells do not possess rearranged Ag-specific receptors and, therefore, the use of MHC class I and/or MHC class II peptide-loaded tetramers cannot be used to distinguish NK cells that are actively participating in an immune response from those that are not. For this reason, the kinetics of the expansion and contraction phases of the NK cell response to pathogens have not been accurately characterized. Specifically, the kinetics of NK cell recruitment into an immune response, the impact immune stimuli have on NK cell maturation, the fate of the responding NK cell population, and the responsiveness of activated NK cells to secondary infection remain important unanswered questions with regard to the in vivo biology of NK cells.

The role of NK cells in defense against murine CMV (MCMV) infection has been clearly demonstrated (5). Experimental depletion of NK cells early during MCMV infection results in unchecked viral replication and increased mortality (6). Early during the course of infection, NK cells serve as direct antiviral effectors by secreting IFN-γ and mediating cytotoxicity (7, 8). These NK cell functions are promoted by the production of the proinflammatory innate cytokines IL-12 and IFN-αβ, respectively (9). Recently, the mouse C57BL/6-derived Ly49H NK cell-activating receptor has been demonstrated to be intimately involved in resistance to MCMV infection (10–14). Ly49H was shown to directly interact with m157, a MCMV-encoded protein expressed on the surface of infected cells (15, 16).

In addition to receiving signals that directly promote effector functions, NK cells also receive signals that stimulate them to proliferate and to accumulate in response to MCMV (5). It has been demonstrated that the NK cell proliferative response during the first 2 days of infection is driven by IFN-αβ (17) and occurs in a nonspecific manner (18). During the later stages of the acute response, days 2–6, selective NK cell proliferation occurs in the Ly49H+ subset (18) in a manner that is dependent on IL-18 and IL-12 and the presence of CD8α+ dendritic cells (19).

The killer cell lectin-like receptor G1 (KLRG1) is a recently described C-type lectin inhibitory receptor that is expressed on ~30% of resting NK cells (20–22) as well as on a subset of CD8+ and CD4+ memory T cells in naive mice (23–25). In this study, we demonstrate that KLRG1 expression is restricted to the most mature NK cell population in naive animals and that the induction of KLRG1 on NK cells during MCMV infection provides a method to visualize and characterize both the expansion and contraction phases of the NK cell response to pathogens. We show that NK cell recruitment into the immune response to MCMV is restricted to the first 3 days of infection and provide evidence that like T cells, NK cells undergo an active maturation process as they participate in an immune response. Our data also indicate that during the resolution of the acute response, the activated NK cells are selectively lost as the NK cell compartment contracts to homeostatic levels. Additionally, we demonstrate that a stable pool of naive peripheral NK cells is maintained throughout the immune response to MCMV and importantly these cells are capable of responding to secondary stimuli.
Materials and Methods

Mice

Male C57BL/6 and B6SJL-Ptprc<sup>+/+</sup>/BoAItAc (Taconic Laboratory Animals and Services, Germantown, NY) were purchased for these studies. All mice were maintained in pathogen-free breeding facilities at Brown University (Providence, RI). All mice were males between 5 and 9 wk of age. Experiments were conducted in accordance with institutional guidelines for animal care.

Infection protocols

Stocks of Smith strain MCMV salivary gland extracts were prepared as previously described (26). Infections were initiated by the i.p. delivery of 5 × 10<sup>5</sup> PFU of MCMV. For each of the MCMV time course experiments conducted, the infections were initiated on different days so that each animal in any given experiment was sacrificed on the same day. For α-galactosylceramide (α-GalCer) experiments, mice were treated i.p. with 2 μg of α-GalCer.

In vivo BrdU treatments

In vivo BrdU pulsing was performed by injecting 1 mg of BrdU per MCMV-infected mouse i.p. 1.5 h before sacrifice. Analysis for BrdU incorporation was performed using the BrdU Flow kit (BD Biosciences, San Diego, CA).

Isolation of lymphocytes

To obtain splenic lymphocytes, spleens were minced, passed through nylon mesh (Teijin, Kansas City, MO) and washed once in 3% PBS/serum, and cell suspensions were layered on Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada). Hepatic lymphocytes were prepared by mincing and passage through a 70-μm nylon cell strainer (BD Falcon, Franklin Lakes, NJ). After washing three times in 3% PBS/serum, cell suspensions were layered onto two-step discontinuous Percoll gradients (Pharmacia Fine Chemicals, Piscataway, NJ). Splenocytes and hepatic lymphocytes were collected after centrifugation for 30 min at 900 × g.

NK cell stimulation for IFN-γ production

For in vitro stimulations, splenic lymphocytes were isolated and then incubated with IL-2 (1000 U/ml) and IL-12 (10 ng/ml) for 6 h with brefeldin A (Sigma-Aldrich, St. Louis, MO) added for the last 3 h of culture. Cells were then harvested and analyzed for TCRβ<sup>+</sup>, NK1.1<sup>+</sup>, KLRG1<sup>+</sup>, and intracellular IFN-γ protein by four-color staining followed by flow cytometry. For in vivo stimulations, splenic and hepatic lymphocytes were isolated from mice that had been administered α-GalCer 6 h before sacrifice and analyzed as stated above.

Adoptive transfer

Splenocytes prepared from C57BL/6 mice (CD45.2<sup>+</sup>) were enriched for NK cells using DX5 magnetic beads (Miltenyi Biotec, Auburn, CA), stained for NK1.1 and KLRG1, and then FACS sorted into KLRG1<sup>+</sup> and KLRG1<sup>−</sup> NK cell subsets (purity was ≥96% for both subsets). After sorting, 1 × 10<sup>5</sup>−2 × 10<sup>6</sup> cells of each subset were independently transferred into B6.SJL hosts (CD45.1<sup>+</sup>) via i.v. injection. Depending on the experiment, recipient animals were then simultaneously infected with MCMV, left uninected, or had been previously infected with MCMV. The recipient animals were then sacrificed at the indicated time points and analyzed for KLRG1 expression on TCRβ<sup>+</sup>, NK1.1<sup>+</sup>, and CD45.2<sup>+</sup> lymphocytes isolated from both the spleens and livers of recipient animals.

Abs and reagents

TCR-β-FITC and TCR-β-αllophycocyanin (clone H57), NK1.1-PE, and NK1.1-αllophycocyanin (clone PK136), Ly49 C/I/F/H-FITC (clone 1B11), CD94-FE (clone 18d3), CD11b-PE (clone M1/70), CD51-PE (clone RMV-7), CD117-FE (clone 2B8), and isotype controls were purchased from eBioscience (San Diego, CA). In addition, CD45.2-FITC (clone 104), Ly49A/αllophycocyanin (clone 12A8), Ly49G2-FITC (clone 4D11), DX5-PE; CD43-PE (clone 1B11), Bcl-2-PE (clone 3F11), IFN-γ-αllophycocyanin (clone XMGL1.2), biotinylated KLRG1 (clone 2F1), isotype controls, and streptavidin-PerCP were purchased from BD Pharmingen (San Diego, CA). The anti-KLRG1 rat mAb (clone 1F10) was produced in our laboratory as described previously (22, 25). The above-mentioned Abs were used for FACS analysis in this study. Mouse IL-2 and IL-12 were purchased from BD Pharmingen. α-GalCer was synthesized by Kirin Brewery (Gunma, Japan).

Flow cytometric analysis

Cells were suspended in buffer comprised of PBS containing 3% FCS and 0.02% NaN<sub>3</sub>. Cells were first incubated with 2.4G2 mAb for 20 min. Cells were then stained with mAbs specific for cell surface markers or isotype controls for 30 min at 4°C. After two washes, streptavidin-PerCP was added for another 30 min when appropriate. Cells were then washed and fixed in 2% formaldehyde in PBS. Intracellular staining for IFN-γ and Bcl-2 protein was performed using the Cytofix/Cytoperm kit (BD Pharmingen). The TUNEL assay was performed using the In Situ Cell Death Detection kit, fluorescein (Boehringer Mannheim, Indianapolis, IN). Depending on the experiments and the tissue, 2.5 × 10<sup>3</sup>−3 × 10<sup>6</sup>, events were collected on a FACSCalibur. The data were acquired and analyzed using CellQuest software (BD Biosciences).

Results

The expression of KLRG1 is restricted to the most mature NK cells in naive mice

Recently, the in vivo stages of NK cell development have been phenotypically and functionally defined (27, 28). Mature NK cells in the peripheral tissues can be identified by the preferential expression of both DX5 and CD11b and the preferential lack of CD51 and CD117 (c-kit) expression (28). To determine whether KLRG1 could serve as a marker for NK cell maturation status, we phenotyped hepatic NK cells using mAbs specific to the NK cell maturation markers DX5, CD11b, CD43, CD51, and CD117 as well as to the NK cell receptors CD94, Ly49s (A/D, C/I/F/H, and G2), and KLRG1. We demonstrate that >99% of KLRG1<sup>+</sup> NK cells isolated from the liver of naive C57BL/6 mice also express both DX5 and CD11b (Fig. 1). Additionally, >96% of KLRG1<sup>+</sup> NK cells express Ly49 molecules. The KLRG1<sup>+</sup> NK cell population in the liver also preferentially lacks both CD51 and CD117 expression. Similar results were obtained when NK cells isolated from the spleen, bone marrow, and blood were analyzed (data not shown). These findings indicate that, in naive mice, KLRG1 expression is restricted to a unique subset of the most phenotypically mature NK cells.

The kinetic profile of the NK cell response to MCMV infection is marked by the KLRG1 molecule

Previously, we demonstrated that during the immune response to MCMV infection there is a dramatic increase in the percentage of

![FIGURE 1. The expression of KLRG1 is restricted to the most mature NK cells in naive mice. Hepatic lymphocytes were isolated and analyzed for DX5, CD94, Ly49s (A/D, C/I/F/H, and G2), CD11b, CD43, CD51, and CD117 expression. Marker expression on the total NK cell population, the KLRG1<sup>+</sup> NK cell subset, and the KLRG1<sup>−</sup> NK cell subset is shown for one animal per group of three mice. One experiment representative of three is shown.](http://www.jimmunol.org/Downloadedfromwww.jimmunol.org)
NK cells that are positive for the expression of the KLRG1 molecule (22). KLRG1 expression therefore identifies the most mature NK cells in naive animals as well as NK cells that have been activated during the course of MCMV infection. To determine whether KLRG1 expression could be used as a marker for the study of the NK cell compartmental response during both the expansion and contraction phases of the primary immune response to MCMV infection, we examined the total number of NK cells present throughout the course of infection, as well as the relationship the absolute number of NK cells has to the number of NK cells present within both the KLRG1+ and the KLRG1− NK cell subsets.

As shown in Fig. 2, there is a decrease in the total number of NK cells present in the spleen of MCMV-infected animals at day 1.5 postinfection while there is a modest increase in total NK cell numbers in the liver at this time point, in accordance with previous observations (29). After day 1.5, there is a dramatic increase in total NK as well as KLRG1+ NK cell numbers in both the spleen and the liver. The peak in NK cell numbers in both compartments, day 7 for the spleen and day 5 for the liver, corresponds to the peak in prevalence of the KLRG1− subset. Additionally, the decrease in NK cell numbers during the contraction phase of the NK cell response to MCMV infection is marked by a selective decrease in KLRG1+ NK cells. Importantly, there is constant maintenance, in terms of number, of KLRG1− NK cells throughout the course of the infection.

**KLRG1 expression is induced on responding NK cells during the course of MCMV infection**

The expansion of the KLRG1+ NK cell subset in response to MCMV infection could be explained by: 1) the selective proliferation of KLRG1+ NK cells or 2) the direct induction of the KLRG1 molecule on KLRG1− NK cells. To address this issue, we first analyzed the total, the KLRG1+, and the KLRG1− NK cell populations for acute BrdU incorporation (injected i.p. 1.5 h before sacrifice) at day 1.5 after MCMV infection. A comparison of the above-mentioned NK cell subsets showed that there was not a significant difference in the percentage of NK cells within each subset that is positive for BrdU incorporation (Fig. 3, a and b). Thus, the expansion of the KLRG1+ NK cell subset early during MCMV infection does not result from the selective proliferation of KLRG1+ NK cells.

To demonstrate the direct induction of the KLRG1 molecule on KLRG1− NK cells, we took advantage of congenic mice that express allele variants of the leukocyte common Ag CD45. Using this method, donor CD45.2+ splenocytes were first enriched for NK cells using DX5 magnetic beads and then FACs sorted into KLRG1+ and KLRG1− NK cell subsets (Fig. 3c). After sorting, the KLRG1+ and KLRG1− NK cell subsets were transferred independently into CD45.1+ hosts via i.v. injection. Recipient animals were then infected with MCMV or left uninfected and subsequently analyzed as described in the legend for Fig. 3.

As shown in Fig. 3c, the KLRG1− NK cell population remained positive in both MCMV-infected and uninfected animals. In contrast, the KLRG1+ NK cell population became KLRG1+ when transferred into mice that were either MCMV infected or left uninfected. Importantly, the kinetics of the transition of KLRG1− NK cells into KLRG1+ NK cells was significantly accelerated in the context of MCMV infection (day 3 for MCMV infected vs day 14 for uninfected). These data demonstrate that KLRG1 expression is directly induced on NK cells during MCMV infection and that KLRG1 expression can also be acquired during homeostatic NK cell maturation. In addition, these data indicate that the acquisition of KLRG1 expression is an irreversible process.

**Kinetics of NK cell recruitment into the immune response to MCMV infection**

The kinetics of NK cell recruitment into the immune response to MCMV infection is unknown. To explore this dynamic process, we used the strategy of transferring KLRG1− NK cells into mice that had been previously infected with MCMV for varying numbers of days and then monitoring the transition of the donor KLRG1− NK cells into the KLRG1+ NK cell population. We analyzed the transferred NK population for KLRG1 expression on day 3 post-transfer because data from our previous experiments, described in Fig. 3, indicated that transferred KLRG1− NK cells would not transition into the KLRG1+ subset by day 3 posttransfer unless they receive immune system stimulation. Using this strategy, we demonstrate that KLRG1− NK cells become KLRG1+ if they are transferred into mice that have been previously infected with MCMV for 1 or 3 days (Fig. 4). However, when transferred on or after day 4 post-MCMV infection, the KLRG1− donor NK cells remain KLRG1−. Thus, the recruitment of NK cells into the immune response to MCMV infection appears to be restricted to the first 3 days of infection.

**NK cells undergo accelerated phenotypic maturation in response to MCMV infection**

As a result of our findings that KLRG1 expression is restricted to the most phenotypically mature NK cells in naive mice, we hypothesized that MCMV infection may not simply lead to KLRG1 expression on the overwhelming majority of NK cells but it may also drive an accelerated maturation of the entire responding NK cell population. To investigate this possibility, we examined the expression of DX5, CD11b, CD51, CD117, and KLRG1 on NK cells on days 0, 3, 7, 9, 12, and 15 after MCMV infection. On day
3 after MCMV infection, KLRG1 expression remains restricted to the DX5<sup>−</sup> and CD11b<sup>−</sup> NK cell populations (Fig. 5). Similar finding were observed at earlier time points, days 1 and 1.5 postinfection (data not shown). However, on day 3 after MCMV infection, KLRG1 expression can be seen on a significant percentage of both CD51<sup>+</sup> and CD117<sup>+</sup> NK cells. This demonstrates that the loss of CD51 and CD117 surface expression is not an absolute prerequisite for NK cell KLRG1 expression. By day 7 after MCMV infection, when ~90% of the NK cells express KLRG1, the NK cells of the liver are overwhelmingly DX5<sup>−</sup>, CD11b<sup>−</sup>, CD51<sup>−</sup>, and CD117<sup>−</sup>. Interestingly, by day 9 after MCMV infection, the heightened prevalence of immature NK cells in the liver can be visualized by the presence of an increased percentage of NK cells that lack the expression of DX5 and CD11b as well as KLRG1 (Fig. 5). Similar results were obtained when splenic NK cells were analyzed (data not shown).

**NK cell compartmental contraction is marked by the selective death of responding NK cells**

Because of our findings that KLRG1 induction on NK cells is an irreversible process, it seemed likely that the decrease in the KLRG1<sup>+</sup> NK cell subset after day 7 was due to the selective death of KLRG1<sup>+</sup> NK cells. To test this hypothesis, we isolated hepatic lymphocytes on days 0, 3, 7, 9, 12, and 15 after MCMV infection and analyzed the KLRG1<sup>+</sup> and KLRG1<sup>+</sup> NK cell populations for intracellular expression of the antiapoptotic molecule Bcl-2. It has been demonstrated that high Bcl-2 expression is important for IL-15-mediated survival of peripheral NK cells (30, 31). Additionally, the loss of Bcl-2 expression has been shown to occur in Ag-specific CD8<sup>+</sup> T cells just before death during the T cell contraction phase (32). As shown in Fig. 6, both KLRG1<sup>+</sup> and KLRG1<sup>+</sup> NK cells isolated from the liver of naive animals express high levels of Bcl-2. However, during the course of MCMV infection, Bcl-2 expression is selectively lost in the KLRG1<sup>+</sup> NK cell subset. Similar results were obtained when splenic NK cells were analyzed (data not shown).

**FIGURE 3.** KLRG1 expression is induced on responding NK cells during the course of MCMV infection. a, Splenic and hepatic lymphocytes were isolated from MCMV-infected C57BL/6 mice that had been pulsed with BrdU via i.p injection 1.5 h before sacrifice on day 1.5 postinfection. BrdU incorporation in the total, the KLRG1<sup>+</sup>, and the KLRG1<sup>+</sup> NK cell populations is shown for one representative animal per group of three mice. b, BrdU incorporation in the NK cell populations from the experiment described in a. Results are expressed as mean ± SD of three mice per group. One experiment representative of three is shown. c, Splenic lymphocytes were isolated from naive CD45.2<sup>+</sup> mice, enriched for DX5<sup>+</sup> cells, sorted into KLRG1<sup>+</sup> and KLRG1<sup>+</sup> populations, and injected i.v. into CD45.1<sup>+</sup> hosts that were subsequently infected with MCMV or left uninfected. Splenic lymphocytes were then isolated on day 3 from MCMV-infected animals and on day 3 or day 14 from uninfected animals and analyzed for TCR, NK1.1, KLRG1, and CD45.2. Percent donor KLRG1<sup>+</sup>CD45.2<sup>+</sup> NK cells from one animal per group of two mice, representative of at least two experiments for each condition, is shown.

**FIGURE 4.** NK cell recruitment during MCMV infection. KLRG1<sup>−</sup> NK cells from CD45.2<sup>+</sup> mice were isolated as in Fig. 3 and transferred i.v. into CD45.1<sup>+</sup> hosts that had been previously infected with MCMV for 1, 3, 4, or 6 days. Splenic lymphocytes were then isolated 3 days after transfer and analyzed for TCR, NK1.1, KLRG1, and CD45.2. Percent donor KLRG1<sup>+</sup>CD45.2<sup>+</sup> NK cells from one animal per group of two mice representative of two experiments (for transfers on days 1 and 6 after MCMV infection) or from four experiments (for transfers on days 3 and 4 after MCMV infection) is shown.

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To directly demonstrate the selective death of KLRG1⁺ NK cells during MCMV infection, we analyzed the KLRG1⁺ and KLRG1⁻ NK cell populations for cell death using the TUNEL assay (33). In both uninfected and MCMV-infected animals, the overwhelming majority of TUNEL⁺ NK cells are present within the KLRG1⁺ NK cell subset. Additionally, the prevalence of TUNEL⁺ NK cells increases during the NK cell contraction phase. Collectively, these data indicate that the selective death of the KLRG1⁺ NK cell subset is the mechanism which leads to the decline in total NK cell numbers after day 7 post-MCMV infection.

FIGURE 5. NK cells undergo accelerated phenotypic maturation in response to MCMV infection. Hepatic lymphocytes were isolated and analyzed for DX5, CD11b, CD51, CD117, and KLRG1 expression, as in Fig. 1, on days 0, 3, 7, 9, 12, and 15 after MCMV infection. One representative animal from a group of three mice for each time point is shown. One experiment representative of three is shown.

To address this issue, we isolated splenocytes from uninfected mice and mice that had been infected with MCMV for 5, 7, 9, or 12 days and restimulated them in vitro with IL-2 and IL-12. As shown in Fig. 7a, under these conditions, both the KLRG1⁺ and the KLRG1⁻ NK cell populations isolated from the spleens of naive animals produced IFN-γ in an equivalent manner. When splenocytes isolated from day 5 MCMV-infected mice are restimulated, the KLRG1⁺ NK cell population is hyperresponsive for IFN-γ production. However, the heightened responsiveness of the KLRG1⁺ NK cell population to restimulation wanes by day 7 after MCMV infection. When splenocytes isolated from mice that had been previously infected with MCMV for 9 or 12 days are restimulated, the KLRG1⁺ NK cell population is languid for IFN-γ production when compared with the KLRG1⁻ NK cell subset.

FIGURE 6. NK cell compartmental contraction is marked by the selective death of responding NK cells. Hepatic lymphocytes were isolated and analyzed for TCRβ, NK1.1, KLRG1, and either Bcl-2 expression or for TUNEL staining on days 0, 3, 7, 9, 12, and 15 after MCMV infection. One representative animal from a group of three mice for each time point is shown. One experiment representative of three is shown.

The naive NK cell pool retains the ability to respond to secondary immune system stimuli during the course of MCMV infection

As a result of our observation that a KLRG1⁻ NK cell population was constantly maintained throughout the course of primary MCMV infection, we hypothesized that this subset could represent a pool of peripheral “naive” NK cells that have the ability to respond to secondary immune system stimuli in that they are not taking part in the current ongoing immune response to MCMV. To address this issue, we isolated splenocytes from uninfected mice and mice that had been infected with MCMV for 5, 7, 9, or 12 days and restimulated them in vitro with IL-2 and IL-12. As shown in Fig. 7a, under these conditions, both the KLRG1⁺ and the KLRG1⁻ NK cell populations isolated from the spleens of naive animals produced IFN-γ in an equivalent manner. When splenocytes isolated from day 5 MCMV-infected mice are restimulated, the KLRG1⁺ NK cell population is hyperresponsive for IFN-γ production. However, the heightened responsiveness of the KLRG1⁺ NK cell population to restimulation wanes by day 7 after MCMV infection. When splenocytes isolated from mice that had been previously infected with MCMV for 9 or 12 days are restimulated, the KLRG1⁺ NK cell population is languid for IFN-γ production when compared with the KLRG1⁻ NK cell subset.
Importantly, at all time points examined, the KLRG1⁺ NK cell population remained equally responsive to stimulation when compared with their naive counterparts.

To determine whether the KLRG1⁺ NK cell subset preferentially responds to a secondary in vivo stimulus during the contraction phase of MCMV infection, we used the glycolipid α-GalCer. α-GalCer administration rapidly activates NK cells in a Vα14I NKT cell-dependent manner (34, 35). When splenic NK cells were analyzed, both the KLRG1⁺ and the KLRG1⁻ NK cell populations isolated from previously unstimulated mice produce IFN-γ in response to α-GalCer in an equivalent manner (Fig. 7b). In contrast, when hepatic NK cells were isolated from mice that had only received α-GalCer stimulation, the KLRG1⁺ NK cells produce less IFN-γ when compared with the hepatic KLRG1⁻ NK cells. However, the KLRG1⁻ NK cell subset from both the spleen and the liver preferentially responded to α-GalCer administration in mice that had been previously infected with MCMV for 12 or 15 days. Collectively, this in vitro and in vivo data indicate that the KLRG1⁻ NK cell population represents a pool of peripheral NK cells that retain the ability to efficiently respond to new immune stimulus irrespective of concurrent MCMV infection and in a manner that is comparable to the naive condition. This data also provides evidence that there are important tissue-specific differences which act to regulate the functional responsiveness of the KLRG1⁺ NK cell subset in vivo.

Discussion

Although the innate NK cell response to MCMV has been extensively characterized (5), its resolution and the fate of the activated NK cell population remains unexplored. Additionally, the ability of the NK cell compartment to respond to secondary stimulation during the course of primary viral infection is unknown. In this report, we demonstrate that the expression of the NK cell receptor KLRG1, as both an activation and maturation marker on NK cells, can be exploited to dissect the kinetics of NK cell compartmental expansion and contraction during the primary response to MCMV infection.

Although initiated earlier during infection, our data demonstrate that the kinetic profile of the NK cell response to MCMV infection echoes that of the prototypical T cell response to viral pathogens. Previous studies have demonstrated that the expansion of the T cell compartment often peaks after a significant reduction in pathogen burden has occurred (36). Interestingly, the height of NK cell expansion occurs well after the peak of the biological NK cell effector response (days 1.5–2 postinfection) (5). The number of NK cells present in the naive condition are therefore sufficient for the
NK cell effector phase. The significant expansion in NK cell numbers after day 2 postinfection does, however, correlate with the reported time points when NK cells are active in maintaining CD8α+ dendritic cells (19) and regulating both CD8+ and CD4+ T cell responses (37). It remains to be determined whether an increase in NK cell numbers is required for these functions to be effectively provided by the activated NK cell population.

Interestingly, the recruitment of NK cells into the immune response to MCMV infection occurs only during the first 3 days of infection. Thus, the activation of NK cells by the in vivo environment created by the innate immune response to MCMV appears to be transient and is terminated before viral clearance (38). In this respect, NK cell recruitment into the immune response to MCMV infection is analogous to reports documenting the temporal restriction of in vivo T cell priming (39, 40). Although multiple mechanisms can initiate in vivo NK cell activation (5), the window of NK cell recruitment during MCMV infection corresponds to high systemic levels of both IFN-αβ and IL-12 (9). However, the absence of IFN-αβ or IL-12 does not affect the induction of KLRG1 on NK cells during the early phase of MCMV infection (data not shown) nor does the absence of these cytokines abolish all NK cell functions (17).

Our results demonstrate that the recruitment of NK cells into the immune response to MCMV is followed by an accelerated phenotypic maturation of the entire responding NK cell population. Although significantly accelerated in the context of MCMV infection, this NK cell maturation process appears to be similar to the NK cell maturation process that occurs under normal homeostatic conditions (28). Interestingly, the restriction of KLRG1 expression to DX5+CD11b+ NK cells is maintained as NK cells progress from being naive to becoming activated in response to infection. During the resolution of acute MCMV infection, as the responding KLRG1+ NK cells are selectively lost during NK cell compartmental contraction, this restriction is also conserved. Similar results were observed during the acute NK cell response to primary α-GalCer administration and during the course of lymphocytic choriomeningitis virus and Toxoplasma gondii infections (data not shown). These data indicate that the accelerated phenotypic maturation of the responding NK cell population during MCMV infection is not a pathogen-specific phenomenon.

Importantl, we demonstrate that during both the expansion and contraction phases of the immune response to MCMV infection, a subset of naive NK cells that remains responsive to immune stimulation is actively maintained. The number of NK cells present within this naive pool remain relatively constant throughout the course of infection. As previously noted, the number of NK cells present during the biological NK cell effector phase to primary MCMV infection is not dramatically increased over the naive condition. Given the ability of the naive NK cell pool to remain consistently responsive to stimulation, the numbers of cells present within this NK cell population at any given time point during the course of primary MCMV infection provides a peripheral NK cell population to the host that is competent in its ability to deliver an appropriate innate NK cell effector response.

Additionally, we provide evidence that there are important compartmental differences that exist, with respect to the spleen and the liver, which act to regulate the functional responsiveness of the KLRG1+ NK cell population in vivo. It has been previously demonstrated that NK cell killing and IFN-γ production are differentially required in the spleen and the liver for protection against MCMV infection (8). Tissue-specific microenvironments may therefore play a critical role in determining the functional outcome of NK cell activation. Although splenic KLRG1+ NK cells are able to produce IFN-γ in response to primary in vitro and in vivo stimulation in a manner similar to KLRG1+ NK cells in the context of the experimental conditions described here, KLRG1+ NK cells do not respond as well as KLRG1+ NK cells during the primary biological IFN-γ response to MCMV infection (22). With respect to this observation, it is important to note that temporal changes in the splenic environment initiated by immune stimulus could subsequently contribute, either directly or indirectly, to the differential responsiveness of KLRG1+ NK cells.

From the data presented here, it is clear that the mature DX5+CD11b+ NK cell population of naive animals can be further divided into KLRG1+ and KLRG1− subsets. As KLRG1+ NK cells can be found outside of the DX5+CD11b+ NK cell population in naive and MCMV-infected animals, it follows that the DX5+CD11b+ KLRG1− NK subset is generated from the DX5+CD11b+ KLRG1+ NK population. These two subsets of DX5+CD11b+ NK cells could, therefore, represent temporally distinct NK cell populations within the context of a linear homeostatic progression of NK cell maturation. It is tempting to speculate that the acquisition of KLRG1 expression by NK cells, whether under homeostatic conditions or during an immune response, marks those NK cells that are approaching the end of their natural life span. In support of this hypothesis, we demonstrate here that apoptotic NK cells are most prevalent within the KLRG1+ NK cell subset. Additionally, we demonstrate that “immune experienced” KLRG1+ NK cells appear functionally distinct from their “immune inexperienced” KLRG1− counterparts. It is therefore conceivable that in the context of immune stimulation, KLRG1+ NK cells undergo further, yet currently indistinguishable, phenotypic changes that alter their responsiveness to further stimulation and initiate a molecular program leading to NK cell death.

Collectively, the data presented here illustrate that the in vivo dynamics of the NK cell compartmental response to infection is elegantly orchestrated in that it provides both immediate protection against infection and the maintenance of a naive NK cell population. Our results demonstrate that, as with T cells, the in vivo kinetics of NK cell activation in response to infection is temporally restricted and that during activation, NK cells undergo a maturation process. This activation-induced NK cell maturation process, although accelerated, resembles that which occurs under homeostatic conditions. Additionally, our data indicate that the selective death of the responding NK cell population provides a mechanism by which NK cell compartmental homeostasis is restored during the resolution of the acute immune response. Our findings also provide clues regarding the natural progression of NK cell maturation within the peripheral tissues.

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References


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