The Bone Marrow Functions as the Central Site of Proliferation for Long-Lived NK Cells

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Natural killer cells are innate lymphocytes that provide early protection against viral infection and tumor growth. Their activation is based on the ratio of activating and inhibitory ligands expressed on the target cell surface that are recognized by NK cell receptors (1). Upon activation, NK cells can kill target cells and produce cytokines that tune the immune response (2). Although viral infections often lead to enhanced NK cell numbers at the site of infection, it is not always clear whether this is the result of increased migration, proliferation, or a combination of the two (3). For example, during infection with murine cytomegalovirus (MCMV), NK cells migrate to the liver (4, 5) and there undergo vigorous proliferation (6). In contrast, in vaccinia virus-infected mice, NK cell numbers increase in the peritoneum mainly due to migration, but no major proliferation takes place at this site (7). NK cell proliferation not only leads to increased cell numbers but also can affect NK cell quality if selective proliferation of certain NK cell subsets takes place, such as during MCMV infection (6). Remarkably, although being part of the innate immune system, recent studies indicate that at least a proportion of NK cells is long-lived (8) and acquires a memory-like phenotype (9–13). Thus, Ly49H+ cells that expand during MCMV infection subsequently gain adaptive traits and are able to respond during recall infection (10). It is currently unknown how these long-lived NK cells are maintained.

NK cells are present both in lymphoid and nonlymphoid organs. In mice, especially the lungs contain high proportions of NK cells (14), and several studies have addressed their role during respiratory virus infections (15–19). For example, the group of Mandelboim (20) showed that influenza virus-encoded hemagglutinin was recognized by the NK cell activating receptor Nkp46 leading to killing of influenza virus-infected cells. The mechanisms of NK cell migration and/or proliferation in response to respiratory virus infections, however, remain unknown. In this study, we have determined the kinetics of respiratory virus-induced NK cell expansion, contraction, and NK cell survival. Surprisingly, we found that not the lung or draining lymphoid tissues but the bone marrow (BM) was the primary site of NK cell proliferation during infection. Using an adoptive transfer model, we demonstrated that the BM contained not only immature NK cells but also mature, long-lived NK cells that migrated back from the periphery to undergo both homeostatic and infection-induced proliferation.

Materials and Methods

**Mice, viruses, and infection**

C57BL/6 (B6) mice were purchased from Charles River. B6.SJL (CD45.1) and CD45.1.2 (F1 of B6 × B6.SJL) mice were bred in house under standard conditions. Respiratory syncytial virus (RSV) A2 (a kind gift of A. Easton, University of Warwick, Coventry, U.K.) was expanded on BSC-1 cells; influenza virus (A/HK/x31; H3N2) was expanded on embryonated eggs. Infections were performed using 7- to 17-week-old mice that were anesthetized with isoflurane and then infected intranasally (i.n.) with 5 × 10^5 PFU RSV A2 or 1 × 10^6 50% egg infective dose influenza virus HKx31 (21). All animal experiments were approved by the Committee on Animal Experiments of the University of Utrecht.

**BrdU incorporation**

To measure in vivo proliferation, a BrdU pulse was given to uninfected, infected, or recipient mice by administration of BrdU i.p. (0.8 mg in 200 μl PBS) and i.n. (0.8 mg in 50 μl PBS) after isoflurane anesthesia. The mice were sacrificed 1 h later, and organs were harvested.

**Sample collection and tissue preparation**

Mice were sacrificed by injection of sodium pentobarbital i.p., and spleens, livers, lungs, BM, and bronchoalveolar lavage (BAL) were collected for...
lymphocyte purification. BAL was collected by lavage three times with 1 ml PBS containing 10 μl EDTA and incubated for 1 h at 37°C on a culture dish to remove adherent cells. BM cells were obtained by flushing the femurs and tibiae. Lungs and liver were perfused with PBS before excision. Lungs were minced and incubated in PBS containing collagenase (2.4 mg/ml; Roche Applied Science) and DNase (1 mg/ml; Roche Applied Science) for 30 min at 37°C. Single-cell suspensions were prepared by passage through cell strainers, and lymphocytes were isolated using Lympholyte-M (Cederlane) according to the manufacturer’s instructions. Differences in frequencies of CD11b- and CD27-expressing lung NK cells between DNase- and collagenase-treated and untreated samples were <10% of cells within a specific subset, which is in the range of variation between samples, indicating that this treatment did not lead to a significant loss of CD11b or CD27 expression. Liver lymphocytes were prepared as described (22) with the exception that Lympholyte-M (Cederlane) was used for density separation. Single-cell suspensions of spleens were prepared by passage through cell strainers. RBCs were removed from the spleen and BM by ammonium chloride lysis.

**Abs and flow cytometry**

Cell surface staining with mAbs was performed in the presence of Fc-block (2.4G2) in PBS supplemented with 2% FCS and 0.02% NaN3 for 20–30 min on ice. For intracellular staining of BrdU, cells were fixed with 2% paraformaldehyde for 20 min at room temperature, permeabilized overnight in 0.5% saponin at 4°C, and then stained for 1 h on ice. Fluorochrome-conjugated Abs were purchased from eBioscience (CD49b (DX5), CD11b (M1/70), CD27 (LG.7F9), NKp46 (29A1.4), CD45.2 (104), CD45.1 (A20)), BioLegend (TCRβ (H57-597), NK.1.1 (PK136)), and Molecular Probes [anti-BrdU (PhycoPacific)]. Samples were measured on a FACSCalibur or FACS-CantoII (BD Biosciences) and analyzed with FlowJo software (Tree Star).

**NK cell isolation, cell labeling, and adoptive transfer**

NK cells were enriched from peripheral organs (lung, liver, spleen) and BM of naive mice or mice that had been infected with influenza virus 2–4 wk earlier using an NK cell isolation kit (Miltenyi Biotec). For adoptive transfer, 0.3 × 10^6 to 0.7 × 10^6 purified cells were injected i.v. into congenic mice. In some experiments, prior to transfer, cells were labeled by incubation with 5 μM CFSE (Invitrogen) in PBS for 10 min at room temperature. CFSE was quenched with FCS, and cells were washed twice with PBS before injection.

**Results**

**Influenza virus infection induces NK cell influx into the airways**

To determine the kinetics and phenotype of NK cell responses to respiratory virus infection, we infected B6 mice i.n. with the mouse-adapted influenza virus strain A/HK/x31 (H3N2). Infection induced influx of NK cells into the airways (Fig. 1A–C). Relative proportions of NK cells peaked between days 3 and 5 and then declined (Fig. 1C), and absolute numbers peaked around day 5 (Fig. 1B). To assess the maturation state of NK cells in different organs of influenza virus-infected mice, CD27 and CD11b expression on these cells was measured. NK cells can be divided into distinct subsets, with CD11b^-CD27^- representing the most immature cells, which then progress through the CD11b^-CD27^ and CD11b^-CD27^ stages successively to reach the most mature CD11b^+CD27^ stage (23, 24). In agreement with earlier reports (23, 24), we found that the BM contained mostly immature CD11b^-CD27^- and CD11b^-CD27^- NK cells and the lungs and spleen mostly mature CD11b^-CD27^- NK cells (Fig. 1D). The BAL contained relatively high percentages of both CD11b^-CD27^- and CD11b^-CD27^- NK cells (Fig. 1D). From these data, we infer that influenza virus infection induced the influx of mostly CD11b^-CD27^- and CD11b^-CD27^- NK cells into the airways.

**The BM is the primary site of NK cell proliferation during respiratory virus infection**

To determine whether the influx of NK cells into the airways might be due to enhanced proliferation or enhanced recruitment from other organs, we measured NK cell proliferation in response to respiratory virus infection. We performed a kinetic experiment in which influenza virus-infected and control mice received BrdU i.p. and i.n. for 1 h and then were sacrificed. Strikingly, 3 d postinfection, hardly any BrdU incorporation was detected in NK cells recovered from the lungs and the BAL, whereas a considerable percentage of NK cells in the BM and a smaller percentage of NK cells in the spleen were BrdU^+ (Fig. 2A). Similar results were obtained when measuring expression levels of the proliferation marker Ki-67 (data not shown). The lack of BrdU incorporation in NK cells in the respiratory tissues was unlikely to be due to a lack of recovery of proliferating NK cells or inaccessibility of the cells to BrdU, as T cells in these organs had readily incorporated BrdU in their DNA (data not shown). To examine the maturation status of dividing cells, we measured CD27 and CD11b expression on proliferating NK cells in the BM and spleen. NK cells in all maturation stages had incorporated BrdU with the distribution of BrdU^+ NK cells resembling the distribution of all NK cells over the different CD11b/CD27 defined subsets (Fig. 2B). Thus, not only immature but also mature NK cells had proliferated.

Takten together, our data indicate that respiratory virus infection induces the proliferation of both mature and immature NK cells, primarily in the BM and to a much smaller extent (Fig. 2A) also in the spleen.

**Transferred, long-lived NK cells proliferate homeostatically in the BM**

The BM is a well-known place for NK cell development; however, our data so far suggested that also NK cells with a mature phenotype proliferate in the BM. To determine further where mature NK cells proliferate, we transferred NK cells purified from the mouse periphery (lung, liver, spleen) into naive congenic mice. These NK cells were readily recovered from recipient mice in all organs analyzed (lung, liver, spleen, and BM) between the 4th and 5th week after transfer, indicating that some of these cells were long-lived (Fig. 3A). Although NK cells preferentially homed back to their site of origin (Fig. 3B and data not shown), part of the transferred peripheral NK cells migrated to the BM (Fig. 3B).
Respiratory virus infection induces NK cell proliferation in the BM. (A) C57BL/6 mice were infected i.n. with influenza virus, and BrdU was administered i.n. and i.p. 1 h before being sacrificed at the indicated days postinfection. Shown are the percentages of BrdU⁺ NK cells (TCR⁺ NK1.1⁺) in the indicated organs. (B) Graphs show the percentage of total NK cells or BrdU⁺ NK cells in the four indicated subsets in the BM (left plot) and spleen (right plot) isolated from influenza virus-infected mice 4.5 d postinfection. BrdU was administered as in (A). Results are shown as mean ± SEM with two to five mice per group, and BrdU staining was performed at least three times showing similar results.

A higher proportion of recovered transferred NK cells than endogenous NK cells were CD11b⁺CD27⁻ in all organs analyzed, indicating that the population of transferred cells was more mature than the population of endogenous NK cells present in these organs (Fig. 3C, 3D).

To assess whether transferred NK cells underwent homeostatic proliferation, we transferred CFSE-labeled peripheral NK cells from the liver, spleen, and lungs into congenic recipient mice. Analysis of their CFSE contents 4–5 wk after transfer showed that only a small percentage of NK cells recovered from the peripheral organs of the acceptor mice (i.e., lung, liver, and spleen) had undergone division (Fig. 4A, 4B), and of those that had divided, most had undergone not more than one division. In contrast, most of the transferred NK cells recovered from the BM had undergone multiple divisions (Fig. 4A). To confirm that long-lived NK cells proliferate in the BM and not only preferentially home back there after division, we transferred peripheral NK cells into congenic recipients that received a 1-h BrdU pulse 3 wk later. When comparing BrdU incorporation in transferred NK cells in different organs, we exclusively detected BrdU⁺ NK cells in the BM (Fig. 4C) indicating that long-lived NK cells proliferate homeostatically in the BM. To verify further that mature peripheral NK cells can migrate to the BM to proliferate there, we adoptively transferred CFSE-labeled, FACS-sorted, mature (CD11b⁺CD27⁻) NK cells into naive congenic mice. Analysis of CFSE contents of transferred NK cells in the acceptor mice 2 wk later showed that the transferred, mature NK cells recovered from the BM had divided significantly more than had the transferred NK cells recovered from the spleen (Fig. 4D; for gating strategy, see Supplemental Fig. 1). Taken together, these data lead us to infer that mature, adoptively transferred long-lived NK cells undergo homeostatic proliferation in the BM.

**Respiratory virus infection induces proliferation of long-lived NK cells in the BM**

To determine whether long-lived NK cells proliferate in response to respiratory virus infection, we transferred CFSE-labeled peripheral NK cells into congenic recipients that were infected with influenza virus 10 d later. Analyses of CFSE profiles of transferred cells 7 d after infection of recipient mice showed a dramatically increased proportion of divided transferred NK cells in the BM of infected compared with uninfected recipients (Fig. 5A). Infection of recipient mice with RSV caused a similar proliferative response of transferred NK cells in the BM as infection with influenza virus (Fig. 5B). From these data, we infer that a proportion of transferred, mature NK cells migrated to the BM where they were maintained and able to proliferate in response to infection.

**Discussion**

Although it is well established that NK cells play an important role in immune protection against viral infection, relatively little is known about the kinetics of NK cell responses to most viral pathogens. In the current study, we investigated the response of NK cells to respiratory viral infections. We found that upon infection, frequencies of NK cells increased in the airways; however, NK cells did not detectably proliferate there. Instead, proliferation occurred preferentially in the BM. We furthermore used an adoptive transfer model to generate mature long-lived NK cells from respiratory virus-infected donor mice and found that a proportion of long-lived NK cells migrated to the BM and there underwent both homeostatic and respiratory virus infection-induced proliferation. Thus, although the BM harbors high amounts of immature, developing NK
cells, our data indicate that it is also the central site of proliferation for long-lived NK cells.

Previous studies on interactions between NK cells and influenza virus-infected cells by the group of Mandelboim (20) showed that NK cells recognized influenza virus hemagglutinin through the activating receptor Nkp46, which led to target cell killing. Mice that lacked Nkp46 died more readily of influenza virus infection than wild-type mice, despite similar increases in NK cell numbers in the lungs (16). These data suggested that Nkp46 ligation leads to activation but not proliferation. Our finding that NK cells barely proliferated in the lungs upon intranasal influenza virus infection further confirms this proposition. In contrast, stimulation of the activating receptor Ly49H through MCMV m157 led to selective activation but not proliferation. Our finding that NK cells barely proliferated in the lungs upon intranasal influenza virus infection further confirms this proposition. In contrast, stimulation of the activating receptor Ly49H through MCMV m157 led to selective

As our results showed that upon influenza virus infection, NK cells proliferated mostly in the BM and not at the site of infection, we conclude that the increased NK cell numbers in the airways (Fig. 1B), at least in part, were the result of migration. A similar situation is seen during Listeria monocytogenes infections. There, monocytes proliferate in the BM and emigrate CCR2-dependently into the blood (27). Notably, in addition to monocytes, NK cells localized in the BM also express CCR2 (28). Whether NK cells migrate CCR2-dependently from the BM to the site of infection during respiratory virus infection will be the subject of further investigation.

Recent reports have shown that NK cells can mount recall responses up to several months after sensitization (9, 10, 12, 13). This adaptive trait requires the preservation of Ag-specific NK cells for a long period of time. In the current study, we did not directly address the role of specific cytokines involved in the maintenance of mature NK cells in the BM; however, NK cell homeostatic proliferation has been assessed on NK cells isolated from RAG−/− mice that were adaptively transferred to RAG−/− mice back-crossed on an IL-7−/− or IL-15−/− background (29). In addition to having a role in survival of naive NK cells (30), IL-15 played a dominant role in survival of transferred NK cells (29). Notably, when transferred into RAG−/− mice, NK cell proliferation was reduced 3-fold (29). Thus, both IL-15 and IL-7 might play an important role in the maintenance of long-lived NK cells.

Our finding that mature NK cells undergo homeostatic proliferation in the BM dovetails well with the maintenance of immunological memory that has extensively been studied for T and B cells and, in the absence of Ag, is believed to depend on survival signals, homeostatic proliferation, or a combination of the two (31). The BM is known to play a key role in preservation of immunological memory by being a niche for memory T cells and plasma cells (32–34) and by producing the cytokines needed for survival (33). Memory CD4+ T cells have shown to be in close contact with IL-7−expressing stroma cells, where they are maintained in a low proliferative state and receive IL-7 to survive (33). Memory CD8+ T cells rely both on IL-7 and IL-15 for homeostatic proliferation (30, 35–38). Thus, like other “classic” cells of the adaptive im-

FIGURE 4. Long-lived NK cells proliferate homeostatically in the BM. (A and B) NK cells enriched from peripheral organs (lung, liver, spleen) of influenza virus-infected CD45.2 mice, 2 wk postinfection, were labeled with CFSE and transferred into CD45.1.2 mice. CFSE profiles of transferred NK cells were determined 4–5 wk later. (A) Representative CFSE profile of transferred NK cells in the BM (dotted line) and lung (solid line). (B) Percentages of divided NK cells in the organs indicated. (C) Four to five weeks after adoptive transfer of peripheral NK cells, BrdU was administered to recipient mice i.p. and i.n., and mice were sacrificed 1 h later. Depicted are percentages of BrdU+ transferred NK cells in the indicated organs of individual recipient mice. (D) CFSE-labeled, mature (CD11b+ CD27−) NK cells (DX5+TCRβ−) from the peripheral organs (lung, liver, and spleen) of CD45.1 mice were transferred into congenic CD45.1.2 mice.Recipient mice were sacrificed 16 d later, and percentages of divided NK cells (i.e., that had diluted CFSE) were measured in the BM and spleen. Results are representative of at least two independent experiments (A–C) or one experiment (D). Statistical analysis was performed using a Mann–Whitney U test. *p < 0.05, **p < 0.01.

FIGURE 5. Infection-induced proliferation of long-lived NK cells in the BM. NK cells enriched from peripheral organs (lung, liver, spleen) of influenza virus-infected CD45.2 mice, 3–4 wk postinfection, were labeled with CFSE and transferred into CD45.1.2 mice. Recipient mice were left uninfected or infected 10–13 d later with influenza virus (A, B) or RSV (B) and sacrificed at day 7 (A) or 4 (B) postinfection. CFSE profiles of transferred NK cells in different organs were determined. Depicted are percentages of divided, transferred NK cells in the indicated organs (A) or in the BM (B) of individual recipient mice. Results are representative of two independent experiments (A) or represent one experiment with four to five mice per group. Statistical analysis was performed using a Mann–Whitney U test. *p < 0.05, **p < 0.01.
mune system, we show in this study that a proportion of NK cells behaves in a similar way by migrating back to the BM to undergo homeostatic proliferation.

In addition to the cytokines necessary for homeostatic proliferation, a recent study showed that IL-12 is indispensable for the generation of memory NK cells. Compared to their cotransferred wild-type counterparts, adoptively transferred IL-12 receptor-deficient NK cells could not be recovered 2 wk after MCMV infection (39). It is currently unknown which cells provide IL-12 for NK cell survival and at what locations.

Taken together, on the basis of our data we propose that after virus entry and inflammation in the lungs, NK cells migrate from remote storage sites to the site of infection while the remaining NK cells proliferate at these sites probably to effect their replenishment. After becoming activated, NK cells in the airways produce cytokines and participate in viral clearance. A proportion of NK cells becomes long-lived and can migrate back to the BM where they undergo homeostatic proliferation and rapidly proliferate after re-infection.

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Disclosures

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