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CD27 Dissects Mature NK Cells into Two Subsets with Distinct Responsiveness and Migratory Capacity

Yoshihiro Hayakawa and Mark J. Smyth

Lineage differentiation and the formation of heterogeneous mature subsets are crucial for immune cells to maintain a breadth of responsiveness to pathogens while controlling reactivity to self. In this study, we report that CD27 is a key marker of the NK cell lineage, dissecting the mature Mac-1\(^{high}\) NK cell pool into two functionally distinct subsets. The CD27\(^{low}\) NK cell subset possesses a higher threshold to stimulation and appears to be tightly regulated by the expression of NK cell inhibitory receptors. Comparatively, the CD27\(^{high}\) NK cell subset displays a greater effector function, exhibits a distinct tissue distribution and responsiveness to chemokines, and interacts productively with dendritic cells. Importantly, we have verified that CD27\(^{high}\) and CD27\(^{low}\) subsets with distinct cell surface phenotypes also exist in human peripheral blood. These findings clearly reclassify mature NK cells into two distinct subsets and begin to discern their specific role in immune responses. The Journal of Immunology, 2006, 176: 1517–1524.

Although NK cells lack the ability to generate Ag-specific receptors by gene rearrangement, NK cells distinguish abnormal cells by using a repertoire of Ig-like and C-type lectin receptors that deliver a finely tuned balance of inhibitory and activating signals (1–3). A number of these receptors recognize ligands on transformed and virus-infected cells and thus stimulate NK cell cytotoxic production (e.g., IFN-\(\gamma\)) (4), secretion of cytotoxic granules (5), and expression of TNF superfamily death-inducing ligands (6) that effectively control virus infection and tumor initiation and spread (7–9). Furthermore, NK cells can also regulate subsequent components of the adaptive immune system in host protection from tumors or viruses, via their capacity to cross talk with APCs like dendritic cells (DCs)\(^3\) (10–17).

The multiple stages of NK cell development have been proposed on the basis of their function, phenotype, and proliferative capacities (18–21). In the mouse, the earliest lineage-committed precursors are characterized by expression of the IL-2R and IL-15R common \(\beta\) subunit (IL-2/IL-15R\(\beta\) or CD122). At the next stage of maturation, there is sequential acquisition of NK1.1 and CD94/NKG2 receptors and the integrin \(\alpha_\text{I}\beta_2\) subunit. NK cells then express Ly-49 and \(c\)-kit, followed by an NK cell expansion stage that is characterized by an up-regulation of DX5 and down-regulation of the integrin \(\alpha_\text{I}\beta_2\) subunit. During the perceived final stage of NK cell maturation, NK cells up-regulate Mac-1 (CD11\(\beta\)) and CD43 and acquire effector function, producing high levels of IFN-\(\gamma\) and exerting high levels of cytotoxicity (22). We have recently demonstrated that TRAIL identifies immature mouse NK cells during adult life, and it is the dominant cytotoxic effector molecule expressed by NK cells in fetal mice (23). These observations gained from in vivo analysis suggest that mouse NK cell development is much more complicated and sophisticated than the simple picture that in vitro NK cell cultures might predict.

The subdivision of NK cells into functional subsets was originally proposed in the early 1980s by Lanier et al. (24), and then revisited in late 1990s with the discovery that CD56 expression levels distinguished functionally distinct human NK cell subsets (25–27). It has been shown that human CD56\(^{bright}\) and CD56\(^{dim}\) NK cells are distinct in their functions, including cytotoxicity, cytokine production, and migratory capacity (27). However, CD56 is not expressed in rodents, and there has been no similar clear evidence for functionally distinct mature NK cell subsets in mice. Thus, until now it has been very difficult to relate our knowledge of mouse NK cell biology with human NK cell biology and translate this information into clinical practice.

In this study, we show in mice that the mature Mac-1\(^{high}\) NK cell pool can be further dissected into two functionally distinct CD27\(^{high}\) and CD27\(^{low}\) subsets. In concert with distinct patterns of surface receptor expressed by these two NK cell subsets, their prototypic NK cell effector functions, proliferative capacity, tissue organization, interactions with DCs, and response to chemokines can be clearly distinguished. Furthermore, CD27 can dissect human peripheral blood NK cells into similar two subsets that provide a new definition distinct from CD56. Thus, this study provides the first functional dissection of mature NK cell subsets in the mouse and begins to provide a platform from which both mouse and human NK cells can be explored in immune response and disease.

Materials and Methods

Mice

Inbred wild-type C57BL/6 (B6) mice were purchased from The Walter and Eliza Hall Institute of Medical Research. CD45.1 congenic C57BL/6 mice were purchased from the Animal Resources Centre. B6 RAG-1\(^{-/-}\) mice were bred and maintained at the Peter MacCallum Cancer Centre. All experiments were performed according to animal experimental ethics committee guidelines.

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\(^3\) Abbreviations used in this paper: DC, dendritic cell; IP-10, IFN-\(\gamma\)-inducible protein-10; I-TAC, IFN-\(\gamma\)-inducible protein-10; L-N, lymph node.
Reagents

Abs to TCRβ (H5-579), NK1.1 (PK136), pan NK cells (DX5), CD122 (TM-β1), CD11b (M1/70), CD27 (LG.3A10), CD69 (H.2F3), CD45R/ B220 (RA3-6B2), CD94 (18d3), Ly-49C/I (5E6), Ly-49D (4E5), Ly-49G2 (4D11), Ly-49I (YL-190), NKGD2 (CX5), NKGA/C/E (28.65), NKGA/A/B (16a1), killer cell lectin-like receptor G (KLRG1; 2F1), CD43 (S7), CD127 (A7R34), CD62L (MEL-14), c-kit (2B8), CCR3 (228003), CCR5 (C34-3448), CCR4 (2B11/CCCR4), and CD45.1 (A20) were purchased form BD Pharmingen, R&D Systems, and eBioscience. Purified anti-mouse CCR7 was kindly provided by J. Swirner (Gottingen, Germany).

Flow cytometry

Mononuclear cells from the spleen, liver, bone marrow (BM), lymph node (LN), blood, and lung were isolated, as described. For staining NK cells, mononuclear cells were first preincubated with CD16/32 (2.4G2) mAb to avoid the nonspecific binding of Abs to FcγR. Then the cells were incubated with a saturating amount of mAbs. Flow cytometric analysis was performed with an LSR II instrument (BD Biosciences).

BM chimeras

BM cells were isolated from B6 CD45.1 congenic mice and transferred (2 × 10^6) into sublethally irradiated (5 Gy) wild-type B6 mice (CD45.2). Some mice were injected with 500 μg of anti-CD70 (FR70) at days 1, 0, 1, and 3, and then every 3 days. Cells from spleen were harvested at the indicated time points after transfer and stained for CD45.1, NK1.1, TCRβ, Mac-1, and CD27. NK cells from donor BM origin were determined by electronic gating on NK1.1^+ TCRβ^+ CD45.1^+ cells.

In vivo BrdU uptake assay

Mice were injected i.p. with 2 mg of BrdU (Sigma-Aldrich) twice per day (10–12 h apart). On the day of analysis, cells were harvested from BrdU-injected mice, and at least two non-BrdU-treated control mice were included with each experiment as negative control. Cells were stained for NK1.1, TCRβ, Mac-1, and CD27, and then subsequently stained for intracellular incorporation of BrdU (BrdU Flow Kit; BD Biosciences), according to manufacturer’s instruction. Some mice were injected daily with 500 μg of anti-CD70 (FR70) together with BrdU injection. This dose has been shown to neutralize CD70 function in vivo (12).

Cytotoxicity assay

Cytotoxic activity was assessed against Yac-1 or RMA-Rae-1β target cells by a standard 51Cr release assay. Effector cells were isolated from RAG-1^−/− spleen and purified Mac-1^highCD27^high and Mac-1^highCD27^low NK cells by cell sorting (>95% purity). Target cells were labeled with 100 μCi/ml Na_2^51CrO_4 for 60 min at 37°C, and labeled target cells (10^5/well) were incubated in a total volume of 200 μl with effector cells in 96-well U-bottom plates. The plates were centrifuged before incubation, and after 4 h the supernatant was harvested and counted in a gamma counter.

Cytokine assay

For in vitro NK cell culture, NK cells were isolated from B6 RAG-1^−/− spleen and purified Mac-1^highCD27^high and Mac-1^highCD27^low NK cells by cell sorting (>95% purity). Cells (5 × 10^5/well) were stimulated with IL-12 (100 ng/ml; R&D Systems) and/or IL-18 (100 ng/ml; R&D Systems), or BM-derived DCs (1:1 ratio) in RPMI 1640 medium with human rIL-2 (500 U/ml; Chiron). BM-derived DCs were generated with culture supernatants from GM-CSF- and IL-4-producing cells, as previously described (28). After 24-h incubation, the cell-free supernatants were harvested and subjected to ELISA. The amounts of INF-γ (BD Pharmingen) were quantitated by specific sandwich ELISAs.

Chemotaxis assay

Chemotactic migration activity was performed, as previously described (29). Briefly, RAG-1^−/− splenocytes (10^6) or purified NK cells (10^5) were placed in the upper chamber of Transwell inserts (8-μm pore size; Corning Costar). For assays with purified NK cells, spleen cells were isolated from B6 RAG-1^−/− and purified Mac-1^highCD27^high and Mac-1^highCD27^low NK cells by cell sorting (>95% purity), and then placed in the upper chamber. Inserts were placed in wells containing medium alone (control) or medium containing recombinant chemokine. The chemokines secondary lymphoid tissue chemokine/CCL21, IFN-γ-inducible protein-10 (IP-10)/CXC11, IFN-inducible T cell a chemotactant (I-TAC)/CXC11.1, MIP-1α/CCL3, and stromal cell-derived factor 1/CXCL12 were kindly provided by S. McColl (University of Adelaide, Adelaide, South Australia, Australia). After incubation and harvest, the number of cells migrating to the bottom chamber were counted. With B6 RAG-1^−/− splenocytes, the cells were further subjected to staining with Abs and quantified by flow cytometry.

Statistical analysis

Data were analyzed for statistical significance using the Mann-Whitney U rank sum test. Values of p < 0.05 were considered significant.

Results

CD27 dissects mature NK cells into two major subsets

Generally, lymphocyte differentiation into specific subsets and/or long-lived, Ag-specific effector/memory cells is critical for effective adaptive immunity. In particular, interactions between a variety of TNFR superfamily and TNF superfamily members have been shown to play a key role in promoting lymphocyte survival, and the expression level of TNF receptor superfamily correlates with the maturity of T cells and B cells (30). Interestingly, proportion of naive NK cells constitutively expresses CD27, and we have shown previously that stimulation via CD27 triggers NK cell activation, cytokine production, and induction of subsequent adaptive immune responses (12, 31). We initially investigated the expression of CD27 on naive NK cells in the context of known NK cell maturation and differentiation markers (Fig. 1). Surprisingly, CD27 expression dissected the mature spleen Mac-1^high NK cell population into Mac-1^highCD27^high and Mac-1^highCD27^low NK cells. CD27^low NK cells expressed a mature CD122^+ DX5^high Ly-49s^+ CD43^highMac-1^high NK cell phenotype, clearly demonstrating dissection of the mature NK cell pool by CD27 expression.

Surface phenotype of NK cell subsets

Because it had been previously suggested that NKR expression was tightly linked to the maturation of NK cells (3, 32, 33), we next examined the expression of NKR and other maturation and activation molecules on immature Mac-1^low and mature Mac-1^high NK cell subsets. In this respect, there were clear differences among the NK cell subsets defined as Mac-1^lowCD27^high, Mac-1^highCD27^high, and Mac-1^highCD27^low expression (Fig. 2). The Mac-1^highCD27^low NK cell subset demonstrated higher proportions of cells expressing Ly-49C and I (which recognize self MHC class I molecules in C57BL/6 mice) and KLRG1. By contrast, a reduced proportion of CD94/NKG2^high cells was observed.

![FIGURE 1](http://www.jimmunol.org/)

CD27 dissects the mature Mac-1^high NK cell pool into two subsets. Cells isolated from B6 mice spleen were stained for the NK cell maturation markers (CD122, DX5, Ly-49s: Ly-49A/C/D/G2/I, CD43, Mac-1), as indicated, together with NK1.1, TCRβ, and CD27. The dot plots shown are profiles for a NK maturation marker and CD27 expression on electronically gated NK1.1^+ TCRβ^− cells. Data are representative of at least three experiments.
in the Mac-1<sup>high</sup>CD27<sup>low</sup> NK cell subset (Fig. 2). Furthermore, the activation/differentiation marker expression was also distinct among these NK cell subsets, with Mac-1<sup>high</sup> NK cell subsets displaying higher CD62L expression, and a proportion of CD27<sup>high</sup> NK cells constitutively expressing CD69. Interestingly, Mac-1<sup>low</sup>CD27<sup>high</sup> NK cells specifically expressed IL-7R<sub>H9251</sub> (CD127) and higher level of c-kit<sub>H9252</sub>, confirming that Mac-1<sup>low</sup>CD27<sup>high</sup> NK cells may represent an earlier stage of NK cell development.

**Distinct tissue distribution of mature NK cell subsets**

The tissue distribution of the lymphocyte subsets has been considered diverse and may be important for tissue-resident/specific immune responses in general. Therefore, the tissue distribution of NK cell subsets in vivo may provide clues as to their involvement and role in immune responses in their microenvironment. Thus, we examined the distribution of Mac-1<sup>low</sup>CD27<sup>high</sup> NK cells constitutively expressing CD69. Interestingly, Mac-1<sup>low</sup>CD27<sup>high</sup> NK cells specifically expressed IL-7R<sub>H9251</sub> (CD127) and higher level of c-kit<sub>H9252</sub>, confirming that Mac-1<sup>low</sup>CD27<sup>high</sup> NK cells may represent an earlier stage of NK cell development.

**Delayed appearance of mature Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells**

We next examined the kinetics of the appearance of mature NK cell subsets in a BM chimera assay using B6 Ly-5.2 congenic mice (Fig. 4). Upon congenic BM transfer into sublethally irradiated B6 Ly-5.1 mice, donor-derived Ly-5.2<sup>H11001</sup> NK cells developed from the transferred BM progenitor cells and expressed an immature Mac-1<sup>low</sup>CD27<sup>high</sup> NK cell phenotype 1 wk posttransfer (Fig. 4a). At 2 wk post-BM, the appearance of Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells was observed, and then Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells finally began to appear by 4 wk post-BM transfer. Eight weeks after BM transfer, a dominant proportion of Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells was observed in the spleens of host mice, indicating that the Mac-1<sup>high</sup>CD27<sup>low</sup> NK cell subset appears as a relatively late stage of NK cell differentiation after BM reconstitution. Furthermore, continuous neutralization of CD70 by Ab did not affect NK cell subset formation following BM transfer (Fig. 4b), suggesting that

![Figure 2. Characterization of surface phenotype of NK cell subsets.](image-url)
the CD27-CD70 interaction may not be critical for NK cell subset formation in vivo.

**Mature CD27^{high} NK cells predominantly proliferate in vivo**

A recent study demonstrated that a large proportion of BM NK cells was in cell cycle and was most likely the precursors of the nondividing or slowly dividing spleen NK cells (34, 35). We have conducted an analysis that indicates that mature Mac-1^{high}CD27^{high} NK cells are the predominant population in both the spleen (Fig. 5a) and BM (data not shown) that actively take up BrdU together with immature Mac-1^{low}CD27^{high} NK cells (data not shown) (22). Thus, the CD27^{low} subset has a very limited capacity for proliferation and turnover among NK cell subsets (Fig. 5a). Consistently, Mac-1^{high}CD27^{low} NK cells were the predominant proportion of NK cells that survived upon administration of hydroxyurea, a cell cycle-specific cytotoxic agent (data not shown), implying that Mac-1^{high}CD27^{low} NK cells may represent a long-lived/senescent subset of NK cells. Furthermore, continuous inhibition of CD70 did not affect this NK cell turnover, as determined by in vivo uptake of BrdU (Fig. 5b), indicating that the CD27-CD70 interaction is not involved in maintaining the homeostasis of NK cells in vivo.

**Functionally distinct NK cell subsets**

Because self MHC-recognizing inhibitory Ly-49 receptors are thought to prevent autoaggression by NK cells, a Mac-1^{high}CD27^{low} NK cell subset that expresses a more abundant level of self MHC-recognizing Ly-49s (Ly-49 C and I) might be expected to react distinctly with self vs nonself cells. Indeed, after purifying the Mac-1^{high}CD27^{high} and Mac-1^{high}CD27^{low} subsets by cell sorting, the Mac-1^{high}CD27^{high} NK cell subset demonstrated its distinct cytotoxic capacity against MHC class I, NKG2D ligand-expressing RMA-Rae-1B target tumor cells (Fig. 5c). By contrast, the CD27^{low} subset displayed only background levels of cytotoxicity against RMA-Rae-1B cells. The cytotoxic capacity of Mac-1^{high}CD27^{low} NK cells against the class I MHC-mismatched NK cell-sensitive (NKG2D ligand-expressing) Yac-1 target cells was relatively lower (2-fold) than that mediated by Mac-1^{high}CD27^{high} NK cells (Fig. 5d), while NKG2D expression was similar between the populations (Fig. 2). Interestingly, Mac-1^{high}CD27^{high} NK cells displayed non-NKG2D-dependent cytotoxicity against Yac-1 target cells, suggesting that Mac-1^{high}CD27^{high} NK cells can kill target cells by an activation pathway distinct from NKG2D (Fig. 5d).

Early cytokine production by NK cells in response to a variety of APC (e.g., DC)-derived factors, such as IL-12 and IL-18, is known to be critical for effective early NK cell control of infection and the induction of subsequent adaptive immune responses. Therefore, we next examined the potential of mature NK cell subsets to produce cytokine in response to IL-12 and IL-18 in vitro. Surprisingly, only Mac-1^{high}CD27^{high} NK cells produced detectable amounts of IFN-γ in response to either IL-12 or IL-18 alone (Fig. 5e). Although Mac-1^{high}CD27^{low} NK cells produced IFN-γ...
Upon stimulation with both IL-12 and IL-18, considerably higher amounts of IFN-γ were produced by Mac-1highCD27high NK cells (Fig. 5e). These results clearly indicated that Mac-1highCD27high NK cells possessed a greater ability to produce IFN-γ, compared with Mac-1highCD27low NK cells, in response to cytokine stimulation. Recently, human and mouse NK cells have been shown to engage in a productive cross talk with DCs, resulting in NK cell IFN-γ secretion (15, 16). Importantly, Mac-1high CD27high NK cells also displayed a far greater ability to produce IFN-γ, compared with CD27low NK cells, when they were cocultured with DCs (Fig. 5e), strongly suggesting that Mac-1highCD27high NK cells may play a predominant role in NK:DC cross talk in lymphoid organs, where they are the predominant NK cell population (Fig. 3).

**Distinct chemokine sensitivity of NK cell subsets**

A recent study has demonstrated the importance of chemokines for NK cell recruitment to LN, and this NK cell recruitment appears to be involved in subsequent Th1 immune responses through NK cell IFN-γ production (17). In this context, we comprehensively examined the expression of chemokine receptors on NK cell subsets as determined by Mac-1 and CD27 expression. Notably, we found distinct chemokine receptor expression on mature NK cell subsets (Fig. 6a). Importantly, Mac-1high CD27high NK cells constitutively express CXCR3, whereas there was no detectable CXCR3 on Mac-1highCD27low NK cells among mature NK cell pool. Both of the mature NK cell subsets demonstrated a very broad level of expression of CXCR4, while neither CCR5 nor CCR7 expression was detected (Fig. 6a). Furthermore, we have also confirmed functional evidence for a distinct chemokine sensitivity of mature NK cell subsets using an in vitro chemotaxis assay. In response to CXCR3 ligands (IP-10, I-TAC), Mac-1highCD27high NK cells showed specific chemotactic activity, whereas Mac-1highCD27low NK cells did not (Fig. 6b). Both of the mature NK cell subsets responded to CXCR4 ligand (stromal cell-derived factor 1), although there were slight differences in their responsiveness (Fig. 6b). Thus, NK cell subsets may be functionally divided based on their distinct migratory capacity and their chemokine receptor expression. Specific chemotaxis of the NK cell subsets was confirmed by assays using IP-10 or secondary lymphoid tissue chemokine and purified Mac-1highCD27high and Mac-1highCD27low NK cells (Fig. 6c).

**Discussion**

NK cells are well recognized as one of the first lines of host defense against pathogens and tumors (1). Recent evidence strongly suggested that NK cells are not only effector cells, but also important in regulating the adaptive immune response through their interactions with APCs, particularly DCs (14, 15). Despite a recent expansion in our knowledge defining unique developmental stages of the NK cell lineage (3, 18), mature NK cell differentiation is very poorly understood.

The concept of dissecting mature NK cells into functionally distinct subpopulations has previously been pursued in humans. Human NK cells can be subclassified into two functionally distinct subsets, CD56bright and CD56dim NK cells (20, 27). Human CD56bright NK cells are considered to be poorly cytotoxic, but potent cytokine producers, whereas CD56dim NK cells express higher levels of cytotoxicity, but are poor cytokine producers. It has also been shown that CD56bright NK cells express distinct levels of chemokine receptors and adhesion molecules (26) and may accumulate within inflammatory sites (36). Because a functional homologue of the human CD56 molecule does not exist in the mouse, it has been impossible to discern the relationships between these human NK cell subsets and mature NK cells studied in the mouse. Consequently, the mouse studies have been limited in their impact on the clinical monitoring of human NK cell function and the design of improved therapeutics for human disease.

Our analysis has now clearly dissected the mature mouse NK cell pool into at least two major subsets, Mac-1highCD27high and Mac-1highCD27low NK cells. Most importantly, each of these NK cell populations displayed a distinct NKR expression profile that correlated with their functions. In particular, it was striking that Mac-1highCD27low NK cells displayed skewed expression of inhibitory Ly-49 receptors (C and I isoforms) that recognize self MHC class I molecule on the B6 background (H-2b). Comparatively, the Mac-1highCD27low NK cell population contained a greater proportion of Ly-49 C- and I-expressing NK cells, whereas Ly-49G2-expressing NK cells were similarly distributed in both Mac-1highCD27high and Mac-1highCD27low NK cell subsets. Similar distinctions in killer Ig-related receptor expression were noted on CD27high and CD27low human NK cell subsets (data not shown). There was also a significant difference in the constitutive
expression of the KLRG1 between the mouse NK cell subsets, with only Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells expressing abundant levels of KLRG1. Interestingly, it has been reported that following virus-induced proliferation/expansion of NK cells and virus-specific CD8<sup>+</sup> T cells in vivo, the responsiveness of effector cells is impaired and such senescent effector cells expressed higher levels of KLRG1 (37, 38). We have demonstrated that the KLRG1-expressing, Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells can be distinguished from the NK cells incorporating BrdU in vivo. Consistently, Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells were resistant to a cell cycle-sensitive cytotoxic agent; therefore, they may represent long-lived/senescent NK cells.

Functionally, Mac-1<sup>high</sup>CD27<sup>high</sup> and Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells displayed distinct cytotoxicity and cytokine production. Mac-1<sup>high</sup>CD27<sup>high</sup> NK cells showed much greater responsiveness to activatory ligand expressed on tumor cells (NKG2D and non-NKG2D pathway), and demonstrated effective cytotoxicity against such tumor target cells even in the presence of MHC class I expression. Moreover, Mac-1<sup>high</sup>CD27<sup>high</sup> NK cells responded to IL-12 and IL-18, key cytokines derived from professional APCs, by rapidly producing IFN-γ. Conversely, Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells displayed very low or no responsiveness to the same cellular (NKG2D ligand) and cytokine (IL-12/IL-18) stimulation. Surprisingly, Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells were not able to override inhibitory signals from their Ly-49 receptors even when they recognized ligands to NKG2D. Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells could be stimulated to make IFN-γ with a combination of IL-12 and IL-18; however, the amount of IFN-γ produced was still far lower than that of Mac-1<sup>high</sup>CD27<sup>high</sup> NK cells, suggesting that in a competitive environment the Mac-1<sup>high</sup>CD27<sup>low</sup> cells would be relatively nonresponsive. In addition, Mac-1<sup>high</sup>CD27<sup>high</sup> NK cells produced detectable amounts of GM-CSF upon IL-12 and IL-18 stimulation, whereas GM-CSF production was not detectable from Mac-1<sup>high</sup>CD27<sup>low</sup> cells (data not shown). Neither TNF-α, IL-10, nor IL-13 was detectable upon IL-12/IL-18 stimulation of mature NK cell subsets, although such stimulation may not be optimal to induce these cytokines from NK cells (data not shown). It now remains to examine the production of other NK cell-derived cytokines under the appropriate stimulatory conditions. Furthermore, Mac-1<sup>high</sup>CD27<sup>high</sup> NK cells showed greater responsiveness to DCs. Mac-1<sup>high</sup>CD27<sup>high</sup> NK cells produced higher amount of IFN-γ upon in vitro coculture with BM-derived DCs, compared with Mac-1<sup>high</sup>CD27<sup>low</sup> cells. A similar observation has been reported for the human CD56<sup>bright</sup> NK cell subset (39). Considering the preferential distribution of Mac-1<sup>high</sup>CD27<sup>high</sup> NK cells in lymphoid organs, this particular NK cell subset may have a predominant role in cross talk with other immune cells, particularly DCs. It remains unclear which type of cells might interact with CD27<sup>low</sup> NK cells, but their expression of MHC class I and NKG2D ligands will be an important determinant of the outcome.

**FIGURE 5.** Different homeostatic proliferation and functions of mature NK cell subsets. a, B6 mice were injected i.p. with 2 mg of BrdU twice per day (10–12 h apart) for the indicated time period. Cells were harvested from BrdU-injected B6 mice and stained for NK1.1, TCRβ, and CD27, and then subsequently stained for intracellular incorporation of BrdU. b, Some mice were injected daily with 500 μg of anti-CD70 (FR70) together with BrdU injection. This dose has been shown to neutralize CD70 function in vivo (12). Cells were harvested from BrdU-injected B6 mice and stained for NK1.1, TCRβ, Mac-1, and CD27, and then subsequently stained for intracellular incorporation of BrdU. Data are representative of two experiments. c and d, Mac-1<sup>high</sup>CD27<sup>high</sup> and Mac-1<sup>high</sup>CD27<sup>low</sup> NK cells were sorted from B6 RAG<sup>-/-</sup> spleen cells. Cytotoxicity was determined against RMA-Rae-1β (c) or Yac-1 (d) or target cells in standard 4-h <sup>51</sup>Cr release assay. To inhibit NKG2D function, anti-NKG2D (C7) (30 μg/ml) was added to the assay. Data are representative of two experiments. e, Sorted NK cell subsets (5 x 10<sup>4</sup> cells/well) were stimulated with IL-12 (100 ng/ml), IL-18 (100 ng/ml), IL-12/IL-18 (100 ng/ml), or BM-derived DCs (1:1 ratio) in the presence of IL-2 (500 U/ml), and cell-free culture supernatants were subjected to IFN-γ ELISA. Data are representative of two experiments.
It also remains to be determined whether distinct monokine responsiveness was simply due to their distinct receptor expression or to distinct responsiveness, such as intracellular signaling pathway, of each subset to those monokines.

Collectively, the specific functional characteristics and tissue distribution displayed prompts us to propose a novel classification of mature NK cell subpopulations. We contend there are two mature Mac-1high NK cell subsets: 1) CD27high NK cells that have a lower activation threshold and are predominantly involved in NK:DC cross-talk, and 2) CD27low NK cells that have a greater restriction by self MHC, are long-lived, and preferentially reside among nonlymphoid tissues.

The functional distinctions between the CD27high and CD27low NK cell subsets also correlated with very distinct chemokine responsiveness and in vivo distribution of these NK cell subsets. Surprisingly, we have now shown that Mac-1highCD27low NK cells are normally excluded from the LN and lack CXCR3 expression (Fig. 6) (17). By contrast, CD27high NK cells were the predominant proportion of NK cells resident among the LN NK cells and expressed high constitutive levels of CXCR3. CD27high NK cells displayed active chemotaxis toward CXCR3 ligands (CXCL10; IP-10, CXCL11; I-TAC) that are generally induced by IFN-γ (40), implying that the CXCR3-CXCR3 ligand pathway may be a feedback regulatory loop for NK cell IFN-γ production, and further recruit this effector type NK cells into sites of IFN-γ production. Interestingly, we demonstrated that Mac-1highCD27low NK cells were the predominant NK cell subset in lung and peripheral blood. Considering Mac-1highCD27low NK cells are the predominant proportion among NK cell subsets in peripheral blood, the NK cell distribution in lung tissue may largely depend on vascular supply. Therefore, if the Mac-1highCD27low NK cell subset is distributed in all nonlymphoid tissues, it is possible this subset plays a distinct surveillance/patrolling role outside of the lymphoid tissue environment.

It has been recognized that there is clear, but lineage distinct, correlation of CD27 expression with lymphocyte differentiation (30, 41, 42). Furthermore, it has been demonstrated recently that the interaction between CD27 and CD70 plays an important role in hemopoiesis (43). Although our data suggest that the CD27-CD70 interaction does not appear to be critical for the appearance of mature NK cells from BM precursors, it will be crucial to determine the molecular factors that control formation and differentiation of mature NK cell subset in vivo.

Importantly, we have verified that CD27high and CD27low NK cell subsets also correlate with very distinct chemokine responsiveness and in vivo distribution of these NK cell subsets. Collectively, the specific functional characteristics and tissue distribution displayed prompts us to propose a novel classification of mature NK cell subpopulations. We contend there are two mature Mac-1high NK cell subsets: 1) CD27high NK cells that have a lower activation threshold and are predominantly involved in NK:DC cross-talk, and 2) CD27low NK cells that have a greater restriction by self MHC, are long-lived, and preferentially reside among nonlymphoid tissues.

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Importantly, we have verified that CD27high and CD27low NK cell subsets with distinct cell surface phenotypes also exist in human peripheral blood (data not shown), and therefore, CD27 is also a novel mature subset marker in the human NK cell lineage. In agreement with observations in the mouse, we detected in a number of healthy donors two distinct CD27high and CD27low subpopulations among human peripheral blood CD3−CD16/CD56bright NK cells. Evidently, like human CD56bright NK cells, mouse CD27high NK cells are effective producers of IFN-γ. In the mouse, we have demonstrated that both monokines IL-12 and IL-18, and DCs themselves, induce more IFN-γ from the CD27high NK cell subset. What is more perplexing is that, in contrast to mouse CD27high NK cells, human CD56bright NK cells are described as a poorly cytotoxic subset. Further studies will now be required to determine all the functional distinctions between human CD27high and...
CD56\textsuperscript{bright} NK cell subsets. Nevertheless, by discovering CD27 as a common marker for human and mouse NK cell subsets, our study provides a very important platform on which to study mature NK cells in immune responses in experimental animals. It is very likely this may provide a rapid conduit of information from the mouse that can be potentially applied in the study of human NK cells following vaccination and in a variety of human infectious diseases, autoimmunity, and cancer.

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Disclosures
The authors have no financial conflict of interest.

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