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Resident Peritoneal NK Cells

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In this study, we describe a new population of NK cells that reside in the normal, uninfamed peritoneal cavity. Phenotypically, they share some similarities with the small population of CD49b$^-$, CD27$^+$ immature splenic NK cells, as well as liver NK cells, but they differ in their expression of CD62L, TRAIL, and EOMES. Functionally, the peritoneal NK cells resemble the immature splenic NK cells in their production of IFN-γ, GM-CSF, and TNF-α and in the killing of YAC-1 target cells. We also found that the peritoneum induces different behavior in mature and immature splenic NK cells. When transferred i.v. into RAGγc knockout mice, both populations undergo homeostatic proliferation in the spleen, but only the immature splenic NK cells are able to reach the peritoneum. When transferred directly into the peritoneum, the mature NK cells survive but do not divide, whereas the immature NK cells proliferate profusely. These data suggest that the peritoneum is not only home to a new subset of tissue-resident NK cells, but that it differentially regulates the migration and homeostatic proliferation of immature versus mature NK cells.

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The peritoneal cavity is known to be home to a specific type of macrophage (12, 13), a resident population of self-renewing B lymphocytes (14, 15), and a newly described population found in fat-associated lymphoid clusters (16). These three populations play a critical role in innate immunity against helminth infection and in controlling peritoneal inflammation. Not much is known, however, about resident peritoneal NK (peNK) cells. A few studies have looked at NK cells after an inflammatory insult in the peritoneum, for example, during peritonitis (17), after i.p. injection of vaccinia virus (18), or after i.p. placement of tumors (19) or grafts (20). However, the rapid influx of mature spNK cells into the inflamed peritoneum makes the identification of any resident NK cells difficult in these conditions. Although the control panels in these studies show that a small number of NK cells also reside in the normal uninfamed peritoneum, this population of NK cells has largely been ignored. In this study, we show that under steady-state conditions, the peritoneal cavities of both wild-type and RAG knockout (KO) mice harbor a distinctive population of NK cells that are phenotypically different from splenic and other tissue-resident NK cells, although they share some phenotypic and functional features with the small subset of immature NK cells in the spleen. When injected i.v., only this immature subset, and not the mature spNK cell population, is able to enter the peritoneum, even though both subsets engage in homeostatic proliferation in the spleen. When injected i.p., only the immature NK cells engage in homeostatic expansion whereas the mature NK cells survive in the peritoneum without dividing. Thus, it appears that the peritoneal cavity is a highly selective environment that not only contains its own specific populations of macrophages and B cells, but also contains a distinct resident NK cell population closely related to immature spNK cells.

Materials and Methods

Mice

Marilyn mice, which have been described previously (21), C57BL/10RAG KO (with either the CD45.2 or CD45.1 alleles), RAGγc KO, C57BL/6, B10.A, and germ-free B10.A mice were from Taconic Farms. CXCR3 KO mice and their control (C57BL/6) mice were obtained from The Jackson Laboratory. All mice, except germ-free mice, were housed in specific pathogen-free conditions. The National Institutes of Health is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Cell isolation and purification of NK cells

We collected cells from the peritoneal cavity by using three peritoneal washes with 5 ml ice-cold PBS (Invitrogen) containing 10 U/ml heparin (American Pharmaceutical Partners). Before transfer into RAGγc KO mice, NK cells from spleens of RAG KO mice were enriched by negative selection using an NK cell isolation kit (Miltenyi Biotec). Purity of negatively selected NK cells, measured by FACS as the percentage of NK1.1$^+$ cells, was $\geq 90\%$. Whenever stated, NK cells were stained with Abs against NK1.1, CD49b, and CD27, and different subpopulations were sorted to a final purity $\geq 96\%$ using either a FACSAria or FACS Vantage cell sorter (BD Biosciences).

Flow cytometry

Splenocytes, peritoneal exudate, liver cells, or blood cells were incubated for 10 min at room temperature with a blocking mixture comprised of 33%...
that migrated to the peritoneum after i.v. transfer into the RAGc KO mice, expressed very low levels of CD49b, whereas NK cells in the blood and spleen expressed high levels (Fig. 1B). This finding, together with the fact that the NK cells were unable to leave the peritoneum after i.p. transfer, suggested that the peritoneum might harbor a specific set of locally resident NK cells, different from spNK cells. To test this, we first compared the phenotypes of peNK versus spNK cells in both RAG KO and wild-type mice and found that the peNK cells did indeed have a distinct phenotype (Fig. 2A). Gating on NK1.1+ cells in RAG KO mice (Fig. 2A), or on NK1.1+CD3+ cells in normal B6 mice (Supplemental Fig. 1), we saw that most of the peNK cells expressed low levels of CD49b and CD43 and high levels of CD27 and CXCXR3, a phenotype expressed by only 4–11% of the spNK cells. The only markers that both spNK and peNK cells expressed similarly were NK1.1, NKp46, and NKGD2, which are normally expressed by both mature and immature NK cells (7, 26). These differences suggest that in steady-state conditions, peNK cells may be a distinct population from spNK cells.

However, a careful look at the phenotype of spNK cells showed that peNK cells looked very similar to the small subset of CD49b+ CD27+ spNK cells previously identified as “immature” NK cells. Both sets were CD49b+, CD27+, and CXCXR3+ and lacked the expression of CD43 (Fig. 2A).

To further compare the two subsets, we assessed their expression of other markers that discriminate between mature and immature NK cells, such as CD11b (for which the immature CD49b+ CD27+ spNK cells have previously been shown to be negative) (3, 4), Ly49IC/CD, CD62L, and CD127 (IL-7Rα). peNK cells seemed to be similar to the immature spNK cells in the expression of most of these markers (expressing low levels of CD11b and containing subsets expressing Ly49IC ~20% and CD127 ~57%), but they differed in their expression of CD62L (Fig. 2B), for which the peNK cells resembled the mature spNK cells (Fig. 2B, upper panel). From these results we conclude that peNK cells are very similar to the immature spNK, but differ in their expression of CD62L.

A population of liver-specific NK cells has been described that shares the CD49b+/CD27+ phenotype with the peNK and immature spNK cells (27) (Fig. 2A, 3A). The liver NK cells differ from the typical spNK cells in that they are TRAIL+, whereas most spNK cells are negative (27) (Fig. 3). Liver NK cells, however, are similar to neonatal spNK cells (27) and they have been suggested to be of a different origin from typical mature CD49b+ spNK cells (28). To determine whether peNK cells and liver NK cells are perhaps the same cells situated in different tissues, or whether they are instead different subsets of NK cells, we stained liver and peNK cells with TRAIL, as well as with EOMES, a transcription factor that is typically expressed by CD49b+ spNK cells (29). We found that peNK cells are different from both liver NK cells and from immature spNK cells in TRAIL and EOMES expression (Fig. 3A), peNK cells and mature CD49b+ spNK cells were mostly EOMES+ and TRAIL−, whereas liver NK cells and immature spNK cells were split into two groups, with about half the cells being TRAIL− EOMES+ and the other half being the opposite (Fig. 3A). Thus, although the peNK cells resemble the immature spNK cells for CD49b, CD27, and a series of other markers, they differ from them, and from the liver NK cells, in TRAIL and EOMES expression. We also measured the cell division rate of the immature NK cell populations (defined as NK1.1+, CD49b+, EOMES+, TRAIL−) in different tissues under steady-state conditions and found that the immature liver TRAIL− NK population had approximately double the number of cells undergoing cell division than did the peNK cells. Peritoneal and spleen CD49b+/TRAIL− cells had a similar cell division rate (Fig. 3B).
We next compared the functional capabilities of the peNK cells with those of the mature and immature subpopulations of spNK cells (sorted according to CD49b and CD27 expression) when cultured with a physiological stimulus, such as YAC-1 cells, or with the stronger stimulus, PMA plus IO. Twenty-four hours after in vitro stimulation with YAC cells, all of the populations of NK cells produced IFN-γ, but only the peNK cells and the CD49b^2/CD27^+ immature spNK cells produced GM-CSF, and none of the populations produced TNF-α (Fig. 4A). When stimulated with PMA plus IO, all of the populations of NK cells produced higher amounts of IFN-γ and varying amounts of GM-CSF, whereas only the peNK cells and the CD49b^2/CD27^+ immature spNK cells also made TNF-α. None of these NK cells produced any of the other 30 cytokines that we tested (see Materials and Methods). We assessed their phenotype 30 d later. Fig. 5A shows that a small proportion of the NK cells trapped in the peritoneum divided in the month that they were resident, but most of the cells remained quiescent.

When we compared the phenotypes of the divided and undivided cells (Fig. 5B), we saw that those cells that had not divided, or had divided just once, mostly continued to resemble spNK cells (although their expression of CD49b was somewhat lower). In contrast, those that had divided two or more times displayed a CD49b^2/low, CD27^+ phenotype similar to normal resident peNK cells and to immature spNK cells, suggesting that they might have originated from the immature spNK subset. To test this idea, we sorted spNK cells into two populations: mature (CD49b^+/CD27^-) or immature (CD49b^2/CD27^+) spNK cells, labeled them with CFSE, transferred equal numbers i.p. into RAGc KO mice, and analyzed their phenotype 8 d later. We found that the two populations behaved very differently. Whereas the immature NK cells divided extensively, cell division was a rare event among the mature CD27^- NK cells (Fig. 5C), suggesting that the peritoneal cavity preferentially allows expansion of the immature, but not the mature, spNK cells.

The local environment also had an effect on phenotype. The immature spNK cells retained their peNK-like phenotype for the CD49b and CD27 markers, whereas the mature CD49b^-/CD27^- NK cells downregulated their expression of CD49b to become more like peNK cells, although they did not reach the complete CD49b^-/CD27^- phenotype of the true peNK cell (Fig. 5D). Thus,
the peritoneal cavity seems to be a location wherein some NK populations (e.g., immature NK cells), but not all, can undergo homeostatic expansion, and where even nondividing mature NK cells are induced to change their phenotype.

There are two reasons why mature CD49b+/CD27 spNK cells might not proliferate in the peritoneum. They might be fully differentiated end-stage cells that have little capacity to divide (11),...
or the peritoneum might not supply the right signals to support their division. To discriminate between these possibilities, we asked whether they would divide if they reached the spleen instead of the peritoneum. We sorted mature CD49b+/CD27+ spNK cells, labeled them with CFSE, injected them i.v. into RAGc KO mice, and looked for homeostatic proliferation in the periphery 9 d later. To distinguish any cells that have lost CFSE (because of multiple divisions) from the very few endogenous host NK cells found in the RAGc KO recipients (Fig. 1, RAGc KO no transfer control), we used CD45.1 spNK cells and CD45.2 recipients. We found that, in striking contrast to their behavior in the peritoneum, the mature CD49b+/CD27+ spNK cells, and cells in the spleen were analyzed 9 d later. CFSE dilution is shown on gated donor (CD45.1+) cells (left panels) or immature spNK cells (NK1.1+/CD49b+/CD27+) carrying the congenic CD45.1 marker were transferred i.v. into CD45.2 RAGc KO mice, and analyzed 8 d later. CFSE dilution is shown on gated donor (CD45.1+) cells (right column, middle panel). CD27 versus CD45.1 expression is shown on gated NK1.1+ cells (lower panel). A–E, Numbers in CFSE plots indicate percentages of NK1.1+ cells that had not divided or divided just once. Numbers in quadrants represent the percentage of gated cells that fall within that quadrant. For each experiment, data represent a total of four mice in two independent experiments.

**FIGURE 5.** Mature CD49b+/CD27− spNK cells do not undergo homeostatic expansion in the peritoneum. A and B, One million CFSE-labeled spNK cells from RAG KO mice were transferred i.p. into Ragc KO mice. Thirty days later mice were killed and peritoneal cells were stained with NK1.1, CD49b, and CD27. A, CFSE dilution of the transferred NK1.1+ cells. B, CD49b versus CD27 staining within the NK1.1 cells that had divided either two or more times (left panel) or divided once at most (right panel). C and D, Same as in A and B except that we separately transferred either 3.5 × 10^6 sorted mature CD27+ spNK cells (NK1.1+/CD49b+/CD27+) (left panels) or immature spNK cells (NK1.1+/CD49b+/CD27+) (right panels) and analyzed 8 d later. E, Sorted and CFSE-labeled mature CD27− spNK cells (7 × 10^6; NK1.1−/CD49b−/CD27−) carrying the congenic CD45.1 marker were transferred i.v. into CD45.2 RAGc KO mice, and cells in the spleen were analyzed 9 d later. CFSE dilution is shown on gated donor (CD45.1+) cells (right column, middle panel). CD27 versus CD45.1 expression is shown on gated NK1.1+ cells (lower panel). A–E, Numbers in CFSE plots indicate percentages of NK1.1+ cells that had not divided or divided just once. Numbers in quadrants represent the percentage of gated cells that fall within that quadrant. For each experiment, data represent a total of four mice in two independent experiments.

**From Fig. 5B, we knew that the mature CD49b+/CD27− spNK cells do not divide when placed directly into the peritoneum, suggesting that the peritoneum is not a supportive environment for this population. However, from Fig. 1B, we knew that a few spNK cells given i.v. can populate the peritoneal cavity. Because these cells express the CD49b− peritoneal phenotype, we surmised that they are more likely to be derived from immigrating immature spNK cells than from the mature population. To test this, we sorted mature CD49b+/CD27− immature CD49b+/CD27− spNK cells from CD45.1 congenic RAG KO mice, transferred them i.v. into RAGc KO mice, and looked at the spleen and peritoneum 8 d later. Fig. 6 shows that sorted mature CD49b+/CD27− spNK cells did not migrate into the peritoneum, although they did populate the spleen, where they maintained their phenotype. In contrast, the immature CD49b−/CD27+ spNK cells migrated to both the spleen and the peritoneum. In the spleen, most of them gained CD49b expression, and a few lost CD27, implying that they have the ability to reconstitute the spNK cell populations, as previously shown for the CD11b+/CD27+ spNK cells (9, 11). In the peritoneum, however, they remained CD49b−/CD27+, resembling the phenotype of the resident peNK cells. These data show that immature spNK cells have a selective ability to migrate to the peritoneum compared with the mature spNK cells, and that once there, the immature spNK cells do not follow the same maturation process that they would have done in the periphery.

It is currently unknown by which mechanism NK cells migrate from the periphery to tissues under steady-state conditions (30). Immature spNK cells and peNK cells are both positive for the G-coupled chemokine receptor CXCR3 (3) (Fig. 2A). Furthermore, as Prlic et al. (18) have shown that G-coupled receptors are important for NK cell migration into the peritoneum after vaccinia virus infection, we hypothesized that the selective migration of immature, rather than mature, NK cells into the peritoneum might be due to their expression of CXCR3 (Fig. 2A). To test this, we first asked whether the CXCR3 KO mice might lack peNK cells, but found no difference in the number or phenotype of peNK cells with respect to wild-type mice (Supplemental Fig. 2). We also transferred sorted spleen NK cells from wild-type or CXCR3 KO mice i.v. into RAGc KO mice and again found no difference in the number or phenotype of the NK cells that migrated to the peritoneum (Supplemental Fig. 3). These data show that CXCR3 expression in the immature spNK cells is not necessary for their migration and retention in the peritoneum, at least under steady-state conditions.

Because CD62L is involved in the migration of NK cells to the lymph node (31), and because peNK cells are largely CD62L−, this molecule might also have had a role in migration and/or retention of the immature NK cells into the peritoneum. However, 15 d after i.v. transfer of whole spleen NK cells into RAGc KO mice, we
noticed that most of the NK cells found in the peritoneum remained CD62L− (Supplemental Fig. 3). This is the characteristic phenotype of the immature CD49b−/CD27+ spNK cells, suggesting that CD62L expression on NK cells is not required for migration into the peritoneal cavity, nor for their persistence over a period of ~2 wk.

We next asked whether the presence of commensal bacteria was necessary for the migration and/or development of peNK cells. To examine this possibility, we looked at the peritoneal lavage and spleens of germ-free mice and found no differences, either in the immature CD49b−/CD27+ spNK cells or in the peNK cells, between the germ-free and the conventionally raised specific pathogen-free mice (Supplemental Fig. 4). This shows that the presence of the peNK cell population is not dependent on endogenous microflora.

**Discussion**

In this study we show that the peritoneal cavity is home to a previously uncharacterized resident population of NK cells. At first sight, the lack of CD49b expression, together with the expression of CD27, suggested that peNK cells were related to immature spNK cells and/or liver NK cells. Further phenotypic analysis using TRAIL (a characteristic marker of liver NK cells) and the transcription factor EOMES (characteristic of mature spNK cells) showed that the immature CD49b−/CD27+ spNK cells actually consist of two subsets: one subset is TRAIL+/EOMES+, similar to mature CD49b+/CD27+ spNK cells. We found that liver NK cells share their phenotype with the former, whereas peNK cells share their phenotype with the latter (TRAIL−/EOMES−). Thus, although sharing some phenotypic markers with liver NK cells, the peNK cells seem to be a different population of tissue resident NK cells that might be closely related to approximately half of the immature CD49b−/CD27+ spNK cells. The peNK cells were functionally active, as they produced cytokines (IFN-γ, GM-CSF, and TNF-α) and also killed YAC-1 target cells.

Interestingly, the peritoneal cavity not only has its own characteristic NK cell population but it also influences NK cell behavior, in that it supports the immigration and homeostatic proliferation of the immature spNK cells, but not of mature splenic CD49b+/CD27− NK cells, suggesting that the peritoneal cavity can specifically regulate the entry and division of its resident NK cells. Mature CD49b+/CD27− NK cells did not proliferate when placed directly in the peritoneum, although they did not disappear, as we were able to find the undivided population even 1 mo after transfer. The same cells, when injected i.v., followed homeostatic expansion in the spleen. This type of selective regulation is not only seen under steady-state conditions, as in this study, but also in inflammatory states. For example, Prlic et al. (18) transferred RAG KO spleen NK cells i.v. with or without an i.p. injection of vaccinia virus, and they found that large numbers of NK cells entered the peritoneum under the inflammatory conditions, but that they did not divide there as much as those that had migrated to the spleen. Furthermore, in our experiments, mature spNK cells changed their phenotype when placed directly into the peritoneum, losing some of their markers (CD49b) and approaching a more peritoneal phenotype, although they did not divide and never quite attained the fully peritoneal phenotype. Our data, paired with the previous studies, suggest that the peritoneum differentially regulates different NK cell subsets, both at the levels of migration and of proliferation.

Our findings also suggest that experiments based on adoptive transfer of NK cells into NK-empty mice should carefully choose the NK marker used for the sorting or purification of the NK cells, as well as the route of injection. The latter is usually not a problem as long as researchers keep using the i.v. route. However, some of the purification protocols being used today are based on DX5, which recognizes CD49b on NK cells. Transfer of CD49b− NK cells might not fully reconstitute all populations of tissue-resident NK cells, for example, in liver, as shown by Takeda et al. (27) when transferring TRAIL− mature spNK cells, or the peritoneal cavity of the host mice. This could have important consequences for the experimental results. The use of a common marker for both mature and immature spNK cells (such as NK1.1 or NKP46) for NK cell purification before the transfer would allow for a more complete reconstitution of tissue-resident NK cells. Additionally, there are likely to be tissue-resident NK cells in tissues that have not yet been characterized. The results of NK cell transfer experiments should take into account that these cells may be missing.

Peritoneal regulation of NK cell populations might have consequences for peritoneal-related pathologies. For example, NK cells found in the peritoneum of patients with endometriosis have been found to have lower levels of lytic activity than those of control patients (32). Although this is an inflammatory condition, our finding of a phenotypically new subset of NK cells in the peritoneum suggests that a similar population may also exist in humans. It has been suggested that human peNK cells help to clear endometrial bodies during the normal phases of menstruation, and the low lytic function may explain why endometrial bodies produced during the menstruation cycle are not cleared from the peritoneal cavity (33). It is possible, therefore, that the human peritoneum also harbors a unique population of NK cells, and that the understanding of their origin and function could lead to better prevention or treatment of endometriosis.

To assess the factors that govern the selective migration of immature NK cells into the peritoneum, we started with the che-
mokine receptor CXCR3. Both immature CD49b+/CD27+ spleen NK cells and peNK cells express this chemokine receptor, whereas most of the mature CD49b+/CD27- spNK cells do not. Furthermore, CXCR3 and its ligand CXCL9 have been identified as factors that increase the recruitment of T and B cells into the peritoneal cavity of mice lacking Mertk (34). We therefore analyzed the peritoneal populations in CXCR3 KO mice and tested the homing ability of CXCR3 KO NK cells. These studies showed that expression of CXCR3 is not necessary for the recruitment or the persistence of NK cells in the peritoneum under steady-state conditions, although other chemokine receptors might have possible compensatory effects. Additionally, the CXCR3 KO NK cells that migrated to the peritoneum were negative for CD62L, suggesting that L-selectin is also not necessary. Other authors have found that the production of CXCL13 by specific cells of the peritoneal cavity (such as peritoneal macrophages and omental cells) is responsible for the recruitment of B1 B cells into the peritoneum through ligation to its receptor CXCR5, which is expressed by peritoneal B1 B cells (35). However, neither the immature CD49b+/CD27+ spNK cells nor the peNK cells express CXCR5 (data not shown), and thus it is not likely to be a major recruiting factor. To test the role of other potential chemokine receptors, we analyzed the splenic and peNK cell populations of CX3CR1 KO, CXCR6 KO, and CCR1 KO mice. None of these mice differed from WT mice in their populations of immature spleen NK cells and peNK cells (data not shown), showing that none of these receptors has a prominent role in NK cell migration into the peritoneum under steady-state conditions. Finally, the presence of the resident peNK cells does not depend on the presence of microflora, as germ-free mice also harbor the same peNK cell populations as conventional mice, suggesting that the signals involved in the migration and differentiation of the resident peNK cell populations are intrinsic to the tissue.

To assess a role for SIP receptors on the differential egress of CD4 cells and NK cells from the peritoneum, we treated大理石 splenocytes (containing both CD4 and NK cells) with their inhibitor, FTY720, injected them i.p., and assessed their ability to exit from the peritoneum. Both FTY720-treated and untreated cells behaved in the same way. The CD4 T cells migrated to the spleen whereas the NK cells did not (data not shown). The possibility still remains that the SIP receptor family is involved in the trapping of the remaining NK cells in the peritoneum, because others have suggested that migration of T and NK cells is regulated by different mechanisms (26). NK cells use mainly the S1P1 receptor, and some of the effects triggered by this receptor are not inhibited by FTY720 (26).

In summary, our data reveal a new population of peNK cells, suggesting that the peritoneal cavity is home to at least three specific subsets of leukocytes: the B1 B cells (14, 15), the newly described fat-associated lymphoid cluster cells (16), and the new population of peNK cells described in this study. The next task will be to understand the possible relationship between these populations, as well as their role in regulating the health of the peritoneal tissue. Furthermore, it is known that the peritoneal B1 B cells are a self-renewing population that can also contribute to B cell populations in the spleen. Perhaps the peNK cells serve a similar role as a reservoir for the immature CD27+ spNK cells.

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Disclosures

The authors have no financial conflicts of interest.

References


Figure S1. Phenotypic comparison between splenic and peritoneal NK cells in wild type mice. Spleen (upper row) and peritoneal (lower row) cells from C57Bl/6 mice were stained for NK1.1, CD3, CD49b, CD27, and CXCR3. Density plots were gated on NK1.1⁺CD3⁻ cells. Numbers represent the percentage of NK cells that fall within each quadrant. Each marker was analyzed for two to four mice.
**Figure S2.** CXCR3KO mice do not lack peNK cells. Peritoneal cells were collected from either C57Bl/6 (left column) or CXCR3KO mice (right column) and percentage of NK1.1+/TCRαβ- cells determined (upper row), as well as their expression of CD49b and CD27 (lower row, using R2 gate). Numbers in the upper row plots represent percentages of NK1.1+/TCRαβ- cells. Numbers in each quadrant of the lower row plots represent the percentage of gated cells that fall within that quadrant. Data represent one mouse out of two tested in two individual experiments.
Figure S3. CXCR3 is not necessary for migration of immature NK cells into the peritoneum and CD62L is not necessary for their persistence in the peritoneum under steady state conditions. 7x10^5 sorted splenic NK1.1+/CD3- cells from either wild type (left column) or CXCR3KO (middle column) mice were transferred separately i.v. into RagγcKO mice. Fifteen days later, peritoneal (upper and middle row) and spleen (lower row) cells were collected and their percentage of NK1.1+ and expression CD49b analyzed (upper row), as well as their expression of CD27 and CD62L (middle and lower row, gated on NK1.1+ cells). A RAGKO mouse (right column) was stained in same way for comparison purposes. Numbers in the upper row plots represent percentages of NK1.1+ cells. Numbers in each quadrant of the middle and lower row plots represent the percentage of gated cells that fall within that quadrant. Data represent one mouse out of two tested.
**Figure S4.** peNK cells are not dependent on endogenous microflora. Spleen and peritoneal cells were collected from conventionally raised (Specific Pathogen Free: left column) or Germ free (right column) B10.A mice. Cells were stained with antibodies for NK1.1, CD3ε, CD49b and CD27. Numbers in the lower right quadrants of the density plots represent the percentage of CD49b-/CD27+ cells within the NK1.1+/CD3ε- gate. Data represent two mice out of four tested.