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Natural killer cell trafficking *in vivo* requires a dedicated sphingosine 1-phosphate receptor

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Consistent with their function in immune surveillance, natural killer (NK) cells are distributed throughout lymphoid and nonlymphoid tissues. However, the mechanisms governing the steady-state trafficking of NK cells remain unknown. The lysophospholipid sphingosine 1-phosphate (S1P), by binding to its receptor $S1P_1$, regulates the recirculation of T and B lymphocytes. In contrast, $S1P_5$ is detected in the brain and regulates oligodendrocyte migration and survival *in vitro*. Here we show that $S1P_5$ was also expressed in NK cells in mice and humans and that $S1P_5$-deficient mice had aberrant NK cell homing during steady-state conditions. In addition, we found that $S1P_5$ was required for the mobilization of NK cells to inflamed organs. Our data emphasize distinct mechanisms regulating the circulation of various lymphocyte subsets and raise the possibility that NK cell trafficking may be manipulated by therapies specifically targeting $S1P_5$.

Natural killer (NK) cells are lymphocytes of the innate immune system that are involved in antitumor immune surveillance[1] and in the early control of several viral, bacterial and parasitic infections[2,3]. NK cells are able to kill cells recognized as targets and to produce cytokines such as interferon-γ. NK cell development occurs in the bone marrow[4]. Several lines of evidence suggest that lymph nodes, and possibly to some extent the thymus, also support NK cell development[4,5]. After the initial stages of differentiation, NK cells undergo a maturation process and relocate throughout the body in lymphoid and nonlymphoid organs[6]. Chemokine receptors such as CCR2, CCR5, CXCR3 and CX3CR1 are involved in the rapid NK cell mobilization that occurs in inflammatory conditions[7–17]. In contrast, the mechanisms governing NK cell trafficking in steady-state situations are unclear. No gross abnormality in NK cell distribution is detected in mouse strains lacking chemokine receptors such as CXCR3, CXCR6, CCR1, CCR5 or CX3CR1 (refs. 10,15,16,18), suggesting that individual chemokine receptors are not important in NK cell trafficking in steady-state conditions.

In addition to chemokine receptors, other G protein–coupled receptors, called the sphingosine 1-phosphate (S1P) receptors, influence lymphocyte migration[19–22]. S1P is a secreted lysophospholipid bound to plasma proteins that influences lymphocyte trafficking as well as proliferation, adherence and morphogenesis[22]. Coordinated activities of biosynthetic enzymes (sphingosine kinases) and biodegradative enzymes (sphingosine lyase and sphingosine phosphatases) maintain S1P gradients *in vivo*, with high S1P concentrations in extracellular fluids (100–300 nM) and low S1P concentrations in tissues (3–100 nM)[19,20]. Five S1P receptors have been identified ($S1P_1$–$S1P_5$)[19,20]. $S1P_1$ functions as a chemotactic S1P receptor in T cells and B cells[23–25]. Circulating T cells and B cells are absent from mice lacking $S1P_1$, and $S1P_1$-deficient thymocytes accumulate in the thymus[24]. Intravenously transferred $S1P_1$-deficient thymocytes can enter secondary lymphoid organs, but they are unable to exit[24]. Similarly, $S1P_1$-deficient B cells cannot exit secondary lymphoid organs[24]. Therefore, it has been proposed that a common $S1P_1$-dependent mechanism controls lymphocyte egress from the thymus and secondary lymphoid organs[19]. Several groups have also shown that a pharmacological agonist of S1P receptors, FTY720 (fingolimod), inhibits the egress of T cells and B cells from lymphoid organs[22]. FTY720, which is structurally similar to sphingosine, is phosphorylated *in vivo*; this phosphorylated metabolite acts as an agonist for four of the five known S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$). However, chronic treatment with FTY720 leads to 'functional antagonism', possibly due to the disappearance of S1P receptors from the cell surface. Because of these effects, FTY720 is used as an immunosuppressant, and a phase II clinical trial has suggested that FTY720 may provide an effective treatment for multiple sclerosis[22]. In addition, FTY720 also regulates vascular integrity by binding to $S1P_1$ expressed on endothelial cells; in this way, FTY720 indirectly influences lymphocyte trafficking[21,25].

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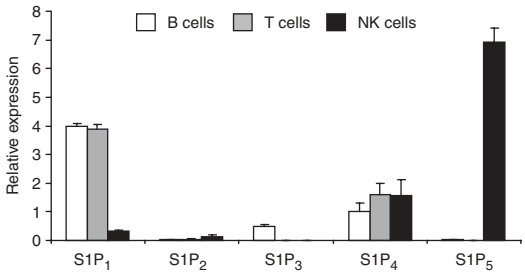

Figure 1 Expression of genes encoding S1P receptors in mouse lymphocytes. Quantitative RT-PCR analysis of the expression of transcripts encoding S1P receptors, relative to the expression of *Hprt1* (hypoxanthine guanine phosphoribosyl transferase) mRNA, in various sorted lymphocyte populations. Results are the mean + s.d. of three independent experiments.

One of the five S1P receptors, $S1P_5$ (also called GPCR NRG-1 or Edg8), is expressed mainly in the white matter tracts of the central nervous system and in particularly large amounts in oligodendrocytes, the myelinating cells of the brain[26,27]. $S1P_5$-deficient immature oligodendrocytes have lower responses to S1P *in vitro*; however, $S1P_5$-deficient mice have no deficits in myelination[28]. Therefore, the precise function of $S1P_5$ in oligodendrocyte function remains to be determined. Here we show that $S1P_5$ is also expressed in the immune system, specifically in NK cells, and that $S1P_5$ regulates NK cell trafficking *in vivo* by a FTY720-resistant mechanism.

RESULTS

Human and mouse NK cells specifically express $S1P_5$

NK cells can be defined across mammalian species as $CD3^-NKp46^+$ lymphocytes[29,30]. To identify other genes selectively expressed in NK cells, we screened human and mouse microarray databases for genes with an expression pattern similar to that of the gene encoding NKp46 (*Ncr1*). Endothelial differentiation gene 8 (*Edg8*), which encodes $S1P_5$, met this criterion (http://symatlas.gnf.org/SymAtlas/ and data not shown). The Edg family is composed of eight G protein–coupled receptors that bind to specific lysophospholipids. Edg receptors are divided into two subgroups according to ligand specificity and sequence. Members of the first subgroup bind S1P and include Edg1 ($S1P_1$), Edg3 ($S1P_3$), Edg5 ($S1P_2$), Edg6 ($S1P_4$) and Edg8 ($S1P_5$). Members of the second subgroup bind lysophosphatidic acid[31]. We found that $S1P_5$ was 'preferentially' expressed on $CD56^{dim}$ human NK cells (**Supplementary Fig. 1** online), which constitute the most mature subset of human NK cells. $S1P_5$ was also specifically expressed in mouse NK cells (**Fig. 1**). We did not detect transcripts encoding $S1P_1$, $S1P_2$ or $S1P_3$ in mouse NK cells, but the gene encoding $S1P_4$ was expressed in similar amounts in all mouse lymphocytes.

NK cell distribution in $S1P_5$-deficient mice

We studied the function of $S1P_5$ by comparing NK cells from $S1P_5$-deficient and wild-type mice. $S1P_5$-deficient mice were born at the expected mendelian frequencies, developed normally and were fertile[28]. NK cells were present in all organs tested in $S1P_5$-deficient mice, but the tissue distribution of these cells was very different from that in wild-type mice. The percentages of NK cells in the blood, spleen and lungs were much lower in $S1P_5$-deficient than in wild-type mice

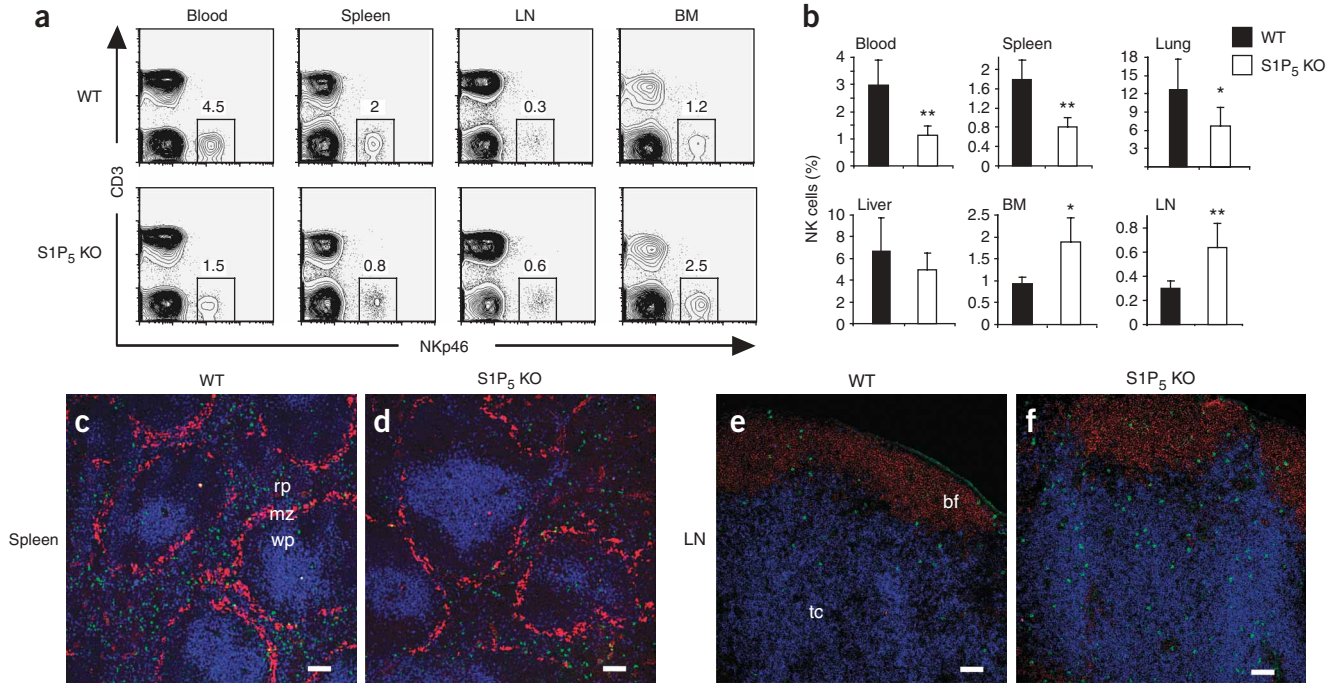

Figure 2 Steady-state tissue distribution of NK cells wild-type and $S1P_5$-deficient mice. (**a**,**b**) Flow cytometry of lymphocyte populations isolated from various organs of $S1P_5$-deficient ($S1P_5$ KO) mice and wild-type (WT) littermates (n = 10–20 mice, depending on the organ) and stained for CD3 and NKp46. LN, lymph node; BM, bone marrow. (**a**) Numbers above outlined areas indicate percent NK cells. Total cell counts were similar for wild-type and $S1P_5$-deficient mice (data not shown). (**b**) *, $P < 0.05$, and **, $P < 0.01$, $S1P_5$-deficient versus wild-type (Student's t-test). (**c**–**f**) Confocal microscopy of spleen and inguinal lymph node sections from wild-type and $S1P_5$-deficient mice, stained for CD3 (blue), NKp46 (green), and SignR1 (red, spleen) or CD19 (red, lymph nodes). Scale bars, 100 μm. wp, white pulp; rp, red pulp; mz, marginal zone; bf, B cell follicle; tc, T cell–rich cortex. Data are representative of six (**a**,**b**) or three (**c**–**f**) independent experiments.

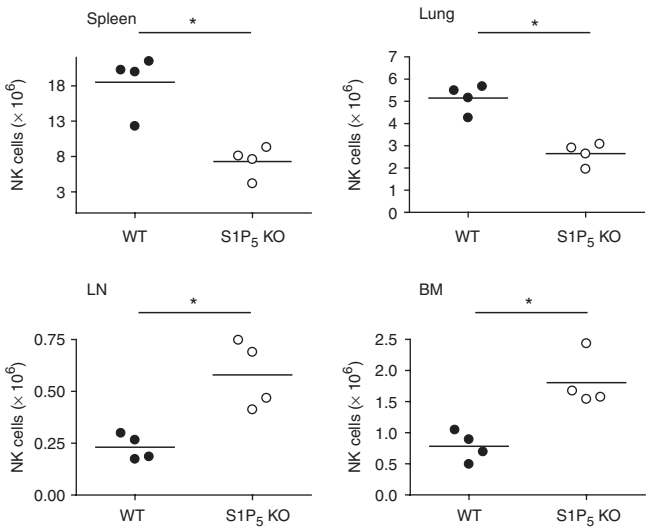

Figure 3 Tissue distribution of NK cells in wild-type and $S1P_5$-deficient mice treated with IL-15. Flow cytometry of NK cells in lymphocyte populations isolated from various organs of wild-type and $S1P_5$-deficient mice injected intravenously with 5 μg RLI on day 0 and day 1 and killed on day 3. Each circle represents an individual mouse ($n = 4$ mice per group). *, $P < 0.05$, $S1P_5$-deficient versus wild-type (Mann-Whitney test). Data are representative of two experiments.

(**Fig. 2a**,**b**). Conversely, the percentages of NK cells in bone marrow and lymph nodes were twice as high in $S1P_5$-deficient mice as they were in wild-type mice. In contrast, similar percentages of T lymphocytes and B lymphocytes were present in the organs of $S1P_5$-deficient and wild-type mice (data not shown). Immunohistological analysis confirmed that there were fewer NK cells in the spleen and more NK cells in the lymph nodes in $S1P_5$-deficient mice than in wild-type mice (**Fig. 2c–f**). *In situ* visualization showed that $S1P_5$-deficient NK cells were located in the same splenic regions as were NK cells in wild-type mice (mainly in the red pulp and a few in the marginal zone[6,29]; **Fig. 2c**,**d**). Also like wild-type NK cells, $S1P_5$-deficient NK cells were present mostly in the interfollicular and cortex regions of lymph nodes (**Fig. 2e**,**f**). Although $S1P_5$-deficient NK cells were present deeper in the T cell cortex than were wild-type NK cells (**Fig. 2e**,**f**), this localization may have been due merely to their accumulation in lymph nodes.

Next we tested the function of $S1P_5$ in NK cell distribution in conditions of inflammation. We used interleukin 15 (IL-15) as a proinflammatory stimulus, as IL-15 is critical for NK cell activation in various inflammatory conditions[32]. IL-15 is *trans*-presented by the IL-15 receptor α-chain (IL-15Rα) to lymphocytes displaying surface complexes of IL-2Rβ and the common γ-chain. Soluble IL-15Rα bound or fused to IL-15 can also efficiently activate and trigger proliferation of NK cells[33]. We treated wild-type and $S1P_5$-deficient mice with the hyperagonist 'RLI' fusion protein, composed of IL-15 attached by a flexible linker to an IL-15R sushi domain[33]. Treatment with RLI induced a 10- to 20-fold expansion of NK cell populations in blood, spleen, lymph nodes, bone marrow and liver in both wild-type and $S1P_5$-deficient mice (**Fig. 3** and data not shown). However, spleen and lung NK cell numbers in RLI-treated $S1P_5$-deficient were reduced to one half those in RLI-treated wild-type mice (**Fig. 3** and data not shown). Conversely, the number of NK cells in the bone marrow and lymph nodes was twice as high in RLI-treated $S1P_5$-deficient mice as it was in RLI-treated wild-type mice.

We then tested whether $S1P_5$ functions in a way intrinsic to NK cells. For this, we reconstituted irradiated recipient mice with a 1:1 mixture of wild-type CD45.1$^+$ and $S1P_5$-deficient CD45.1$^-$ bone marrow cells. At 10 weeks after transplantation, the $S1P_5$-deficient/wild-type NK cell ratios ranged from 0.4 to 0.6 in spleen and blood and from 1.4 to 2.3 in lymph nodes and bone marrow (**Fig. 4**). In contrast, we noted approximately equal numbers of $S1P_5$-deficient and wild-type T cells and B cells in all organs examined (**Fig. 4**). These observations indicate that, consistent with its expression pattern, $S1P_5$ has an intrinsic function in NK cell distribution.

$S1P_5$ expression during NK cell maturation

Three subsets of NK cells differing in expression of CD11b and CD27 have been described in the mouse[34] (**Supplementary Fig. 2a** online). Adoptive transfer experiments indicate that these three subsets represent various stages of NK cell maturation: CD11b^{dull} NK cells are the most immature; 'double-positive' CD27^{hi}CD11b^{hi} cells constitute an intermediate population; and CD27^{dull} NK cells represent the most mature subset. Double-positive and CD27^{dull} NK cells had similar capacities to kill target cells and to secrete interferon-γ in a broad range of *in vitro* stimulation conditions (**Supplementary Fig. 2b**,**c** and data not shown). However, these three NK cell subsets differ widely in tissue distribution[34]. CD11b^{dull} NK cells were present mainly in bone marrow and lymph nodes, whereas CD27^{dull} NK cells were more abundant in blood, spleen, lung and liver than in bone marrow and lymph nodes, and double-positive NK cells were more equally distributed throughout all organs examined (**Supplementary Fig. 2d**). $S1P_5$ expression correlated with the pattern of NK cell distribution, as it gradually increased with NK cell maturation and was highest in CD27^{dull} NK cells (**Fig. 5a**). Nevertheless, $S1P_5$ expression in CD11^{dull} NK cells, although low compared with that in CD27^{dull} NK cells, was greater than that in T cells (**Fig. 5a**). The distribution of NK cell subsets in $S1P_5$-deficient mice was very different from that in wild-type mice. CD27^{dull} NK cells (which in wild-type mice have high expression of $S1P_5$) constituted lower percentages of the lymphocyte populations in blood, spleen and lung and higher percentages of the lymphocyte populations in bone marrow and lymph nodes in $S1P_5$-deficient versus wild-type mice (**Fig. 5b** and data not shown). The percentages of CD11b^{dull} and

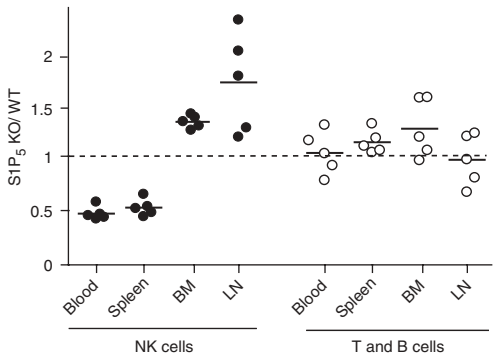

Figure 4 NK cell–intrinsic function for $S1P_5$ in NK cell tissue distribution. Flow cytometry of lymphocyte populations obtained from various organs of irradiated CD45.1$^+$ mice injected 10 weeks before with a 1:1 mixture of wild-type CD45.1$^+$ and S1P5-deficient CD45.2$^+$ bone marrow cells; cells in suspension were counted and NK cells, T cells and B cells were identified by staining for NK1.1, CD3 and CD19. Results are presented as the ratio of the number of $S1P_5$-deficient cells to the number of wild-type cells. Each circle represents an individual mouse ($n = 5$ mice per group). Data are representative of three experiments.

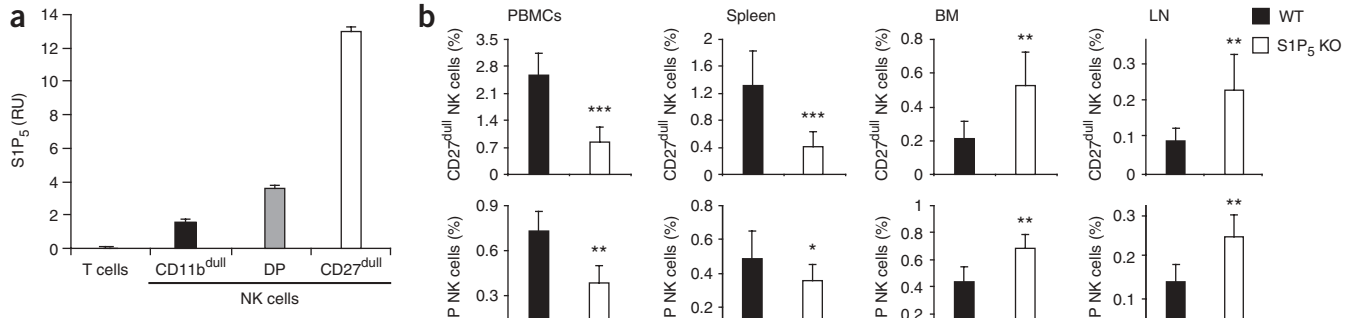

Figure 5 Effect of $S1P_5$ deficiency on the tissue distribution of NK cell subsets. (**a**) Quantitative PCR analysis of expression of $S1P_5$ mRNA in various sorted mouse T cells and NK cell subsets, normalized to the expression of *Hprt1* mRNA. RU, relative units; DP, double-positive. Data are the mean + s.d. of three experiments. (**b**) Flow cytometry of the distribution of NK cell subsets in various organs of $S1P_5$-deficient and wild-type littermates ($n = 5$–10 mice per group, depending on the organ), presented as the percent of each NK cell subset among all cells in each organ. A minimum of 2×10^5 cell events were acquired by flow cytometry. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, $S1P_5$-deficient versus wild-type (Mann-Whitney test). Data are representative of six experiments.

double-positive NK cells in the lymphocyte populations in each organ were less affected by $S1P_5$ deficiency, except in bone marrow and lymph nodes. In bone marrow and lymph nodes, these cells made up higher percentages of the lymphocyte population in $S1P_5$-deficient mice than in wild-type mice; this observation is consistent with functional importance of the low but significant expression of $S1P_5$ on these less mature NK cell subsets (**Fig. 5b**). These findings collectively indicate that the altered NK cell distribution in $S1P_5$-deficient mice is due mainly but not exclusively to the altered distribution of $CD27^{dull}$ NK cells, which constitute the most prominent NK cell subset in blood

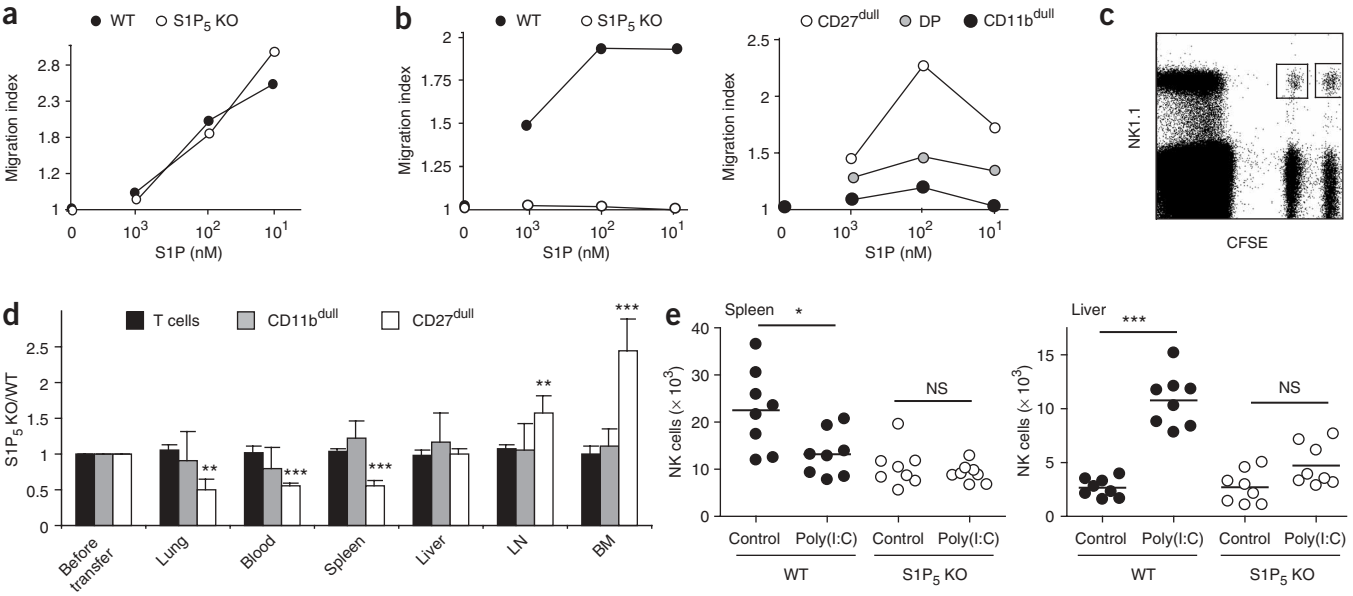

Figure 6 Defective *in vivo* and *in vitro* migration of $S1P_5$-deficient NK cells. (**a**,**b**) Chemotactic responses to S1P of splenic T cells (**a**) and NK cells (**b**) from wild-type and $S1P_5$-deficient mice (**b**, right, wild-type NK cells). The migration index is the ratio of the number of migrated cells in control versus test conditions. Data are representative of three independent experiments. (**c**,**d**) Analysis of $S1P_5$-deficient and wild-type splenocytes labeled with 0.2 μM or 2 μM CFSE, respectively, and transferred intravenously into littermate recipient mice. (**c**) Flow cytometry of $CD3^-$ splenocytes from recipient mice 24 h after transfer. Wild-type and $S1P_5$-deficient donor cells can be distinguished by differential CFSE fluorescence; boxed areas indicate gates used to identify NK cells of each genotype. Additional staining for NK1.1 and CD3 allows comparison of the relative proportion of NK cells of each genotype. (**d**) Cells from various organs stained for NK1.1, CD3, CD11b and CD27. The ratio of $S1P_5$-deficient to wild-type donor NK cells was calculated and is compared before and after transfer. Data are representative of three independent experiments (mean + s.d., **d**). (**e**) Analysis as described in **c**,**d**, but with recipient mice injected intraperitoneally with 150 μg poly(I:C) and analyzed 14 h later. Each circle represents an individual mouse ($n = 8$ mice per group). NS, not significant. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, $S1P_5$-deficient versus wild-type (Mann-Whitney test). Data are representative of three experiments.

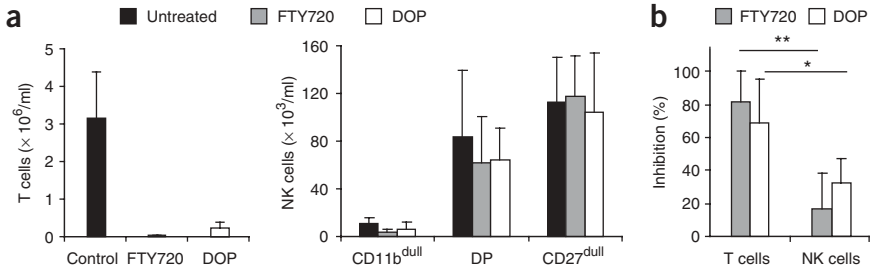

Figure 7 Pharmacological modulation of NK cell trafficking. (**a**) Flow cytometry of peripheral blood mononuclear cells from wild-type C57BL/6 mice left untreated or treated for 7 d with drinking water containing FTY720 or DOP; cells were counted and were stained for NK1.1, CD3, CD11b and CD27, and results are presented as the number of T cells and NK cells per ml of blood (mean + s.d. of five mice per group). Data are representative of three experiments. (**b**) S1P-induced chemotaxis of splenic T cells and NK cells from wild-type C57BL/6 mice left untreated or treated for 7 d with drinking water containing FTY720 or DOP. The migration index is the ratio of migrated cells in control (untreated) and test conditions. Results are presented as the mean + s.d. percent inhibition of chemotaxis ($n = 4$ mice per group). Data are representative of three experiments.

and spleen and in peripheral organs such as lung. Although $S1P_5$ influenced the tissue distribution of $CD27^{dull}$ NK cells, the cell surface phenotype and function of these cells was unaltered by $S1P_5$ deficiency (**Supplementary Fig. 3a–c** online).

$S1P_5$ regulates NK cell trafficking

The lower numbers of blood and splenic $CD27^{dull}$ NK cells in $S1P_5$-deficient mice could be explained by involvement of $S1P_5$ in the survival of $CD27^{dull}$ NK cells, as shown for other S1P receptors and cell types[35,36]. However, several lines of evidence indicated that this was not the case. We did not detect a difference in the viability of NK cells freshly isolated from various organs of wild-type mice and $S1P_5$-deficient mice (**Supplementary Fig. 3d**), and wild-type and $S1P_5$-deficient NK cells showed similar survival after culture in the presence or absence of IL-15 or IL-2 (**Supplementary Fig. 3e**). In addition, S1P treatment *in vitro* did not influence the viability of wild-type NK cells (**Supplementary Fig. 3f**).

Alternatively, like $S1P_1$ in T cells, $S1P_5$ might act as a chemotactic receptor for S1P and thereby regulate NK cell recirculation[24]. We first tested this hypothesis by comparing the migration of wild-type and $S1P_5$-deficient splenocytes in response to S1P gradients *in vitro*. Wild-type T cells and NK cells migrated in response to S1P gradients in a dose-dependent way, as reported before[24,37,38]. Whereas wild-type and $S1P_5$-deficient T cells migrated similarly toward to S1P (**Fig. 6a**), $S1P_5$-deficient NK cells were unresponsive to S1P (**Fig. 6b**). These observations indicate that $S1P_5$ acts as a nonredundant chemotactic receptor for S1P in NK cells. The S1P-induced chemotactic responsiveness of wild-type NK cells increased with maturation, consistent with parallel increases in $S1P_5$ expression (**Fig. 6b**).

Next we tested the function of $S1P_5$ in NK cell trafficking *in vivo*. For this, we labeled splenocytes from wild-type and $S1P_5$-deficient mice with different concentrations of the cytosolic dye CFSE and adoptively transferred these differentially labeled cells at a ratio of 1:1 into littermate recipient mice (**Fig. 6c**). CFSE-labeled NK cells were present in all organs analyzed; thus, transferred NK cells can rapidly traffic through lymphoid and nonlymphoid organs (data not shown). $CD11b^{dull}$ NK cell and T cell populations in recipient mice contained approximately equal proportions of cells derived from wild-type and $S1P_5$-deficient donors (**Fig. 6d**). In contrast, $CD27^{dull}$ populations were enriched for $S1P_5$-deficient donor cells in the lymph nodes and bone marrow but were depleted of these cells in the spleen, blood and lung (**Fig. 6d**). To further test the function of $S1P_5$ in NK cell trafficking *in vivo*, we did similar adoptive transfer experiments but challenged recipient mice with poly(I:C), a compound established as stimulating NK cells and inducing the recruitment of NK cells from spleen to liver[39]. Consistently with published reports, wild-type NK cells accumulated in the liver (four- to fivefold increase) and decreased in the spleen after treatment with poly(I:C) (**Fig. 6e**). In contrast, poly(I:C) treatment failed to elicit significant alterations in NK cell abundance in the spleens or livers of $S1P_5$-deficient mice (**Fig. 6e**).

In vitro studies have shown that FTY720 is an agonist for $S1P_1$ as well as $S1P_3$, $S1P_4$ and $S1P_5$ (ref. 31). To gain insight into the mechanism of action of $S1P_5$ *in vivo*, we treated mice with FTY720 and examined the distribution of various lymphocyte subsets. We noted many fewer T cells in the blood of mice treated with FTY720 than in the blood of control mice, as described before[22,40]. However, FTY720 treatment did not affect the trafficking of NK cell subsets (**Fig. 7a**). In addition to being assessed with FTY720, S1P-dependent function can also be assessed with 4′-deoxypyridoxine (DOP), a vitamin B6 antagonist that irreversibly inhibits S1P lyase[23]. DOP abrogates the endogenous S1P gradient between the blood and lymphoid organs. Thus, DOP treatment inhibits the function of S1P chemotactic receptors, such as $S1P_1$, and results in the disappearance of T cells from the blood[23]. DOP treatment induced substantial decreases in the number of T cells in the blood but left NK cell numbers unchanged (**Fig. 7a**). We also measured the *in vitro* migration of NK cells and T cells toward S1P gradients after *in vivo* FTY720 and DOP treatment. As reported before, FTY720 and DOP induced substantial inhibition of $S1P_1$-dependent chemotaxis of T cells *in vitro*. In contrast, these pharmacological agents exerted less effect on $S1P_5$-dependent NK cell chemotaxis (**Fig. 7b**). Thus, $S1P_5$ regulates the trafficking and the tissue distribution of NK cells *in vivo* by a mechanism that is different from the mechanism for $S1P_1$ in T cells and B cells and that is resistant to effects of FTY720 and DOP.

DISCUSSION

Published reports have demonstrated the functions of chemokine receptors, including CCR2, CCR5, CXCR3 and CX3CR1, in the recruitment of NK cells after inflammation[7–16]. However, except for work demonstrating that the entry of NK cells into lymph nodes from blood is dependent on $CD62L$[41], data on the mechanisms regulating steady-state NK cell recirculation are lacking. Here we have shown that $S1P_5$ is involved in NK cell trafficking in steady-state and inflammatory situations. More specifically, we found that homing of NK cells to blood, spleen, lung and inflamed liver was defective in $S1P_5$-deficient mice. Notably, NK cells were not completely absent from these organs, suggesting the existence of other mechanisms that compensate, at least in part, for the lack of $S1P_5$. This phenomenon is reminiscent of published observations indicating that recruitment of NK cells to inflamed sites in mice lacking various chemokine receptors is diminished but not completely abrogated[7,8,10,16]. Thus, alternative NK cell recruitment mechanisms coexist with $S1P_5$, and NK cell trafficking relies on several pathways.

Our results here have also shown that $S1P_5$ expression increased with NK cell maturation. Accordingly, immature $CD11b^{dull}$ NK cells were less affected by $S1P_5$ deficiency than were double-positive and $CD27^{dull}$ NK cells. Consequently, $S1P_5$-deficient $CD11b^{dull}$ NK cells may seed peripheral organs, where they can complete their maturation. This phenomenon could explain why the NK cell defect in $S1P_5$-deficient mice was less severe than the T cell defect in $S1P_1$-deficient mice[24]. Indeed, unlike NK cells, which complete maturation in various organs throughout the body, T cells originate from the thymus.

How does $S1P_5$ promote the homing of NK cells to the blood, spleen and lung? Given the previously described function of $S1P_1$ in T cells, it is possible that $S1P_5$ operates to facilitate egress of NK cells from the bone marrow and lymph nodes in response to S1P gradients. Indeed, NK cells accumulated in the bone marrow and lymph nodes and withdrew from the blood, spleen and lungs of $S1P_5$-deficient mice. Moreover, $S1P_5$ functioned as a chemotactic receptor for S1P *in vitro*. However, our data showed that FTY720 and DOP had no effect on NK cell trafficking *in vivo* and thus suggest that $S1P_1$ and $S1P_5$ operate through distinct molecular mechanisms. We envisage three different yet not mutually exclusive possible explanations for these observations.

First, the distinct effects of FTY720 and DOP on T cell and NK cell trafficking might be due to different localization of NK cells and T cells in lymphoid organs. Indeed, spleen NK cells are present mostly in red pulp sinuses, whereas T cells are located in the white pulp. Similarly, lymph nodes NK cells are located mainly in lymphatic sinuses of the medulla, whereas T cells are found in the cortex[6,29,42]. The precise concentration of S1P in these different compartments is not known and is difficult to assess but could be differentially affected by DOP. Similarly, FTY720 may differentially access the various compartments of lymphoid organs.

Second, $S1P_1$ and $S1P_5$ may trigger the activation of different intracellular signaling pathways. Indeed, $S1P_1$ couples mainly with $G\alpha_i$-$G\alpha_o$ heterotrimeric G proteins, whereas $S1P_5$ can couple with both $G\alpha_i$-$G\alpha_o$ and $G\alpha_{12}$-$G\alpha_{13}$ proteins[22]. $G\alpha_{12}$-$G\alpha_{13}$ proteins are different from the $G\alpha_i$-$G\alpha_o$ variety, as they specifically interact with and regulate RGS-Rho guanine nucleotide–exchange factors[43]. Thus, RGS-Rho guanine nucleotide–exchange factors relay signals from heterotrimeric $G\alpha_{12}$-$G\alpha_{13}$ protein–coupled receptors to Rho GTPases. Studies have indicated that $S1P_5$ receptors engage this $G\alpha_{12}$-$G\alpha_{13}$ protein–coupled Rho-ROCK signaling pathway to impede oligodendrocyte precursor cell migration[44]. In this scheme, coupling of $S1P_5$ to $G\alpha_{12}$-$G\alpha_{13}$ and Rho activation might be less susceptible than $S1P_1$ to FTY720 and DOP.

Third, $S1P_5$ might not control the egress of NK cells from bone marrow and lymph nodes but instead may prevent the exit of NK cells from blood vessels. Such a possibility would indicate that S1P gradients are not required for the normal function of $S1P_5$ and would explain the absence of an effect of treatment with FTY720 and DOP on NK cell migration. The development of new pharmacological tools such as $S1P_5$-specific agonists[45] and antagonists should facilitate delineation of the $S1P_5$ mechanism of action.

Our results presented here have also distinguished bone marrow and lymph nodes from other organs in terms of NK cell recirculation. $S1P_5$-deficient NK cells accumulated in lymph nodes and bone marrow, whereas blood, spleen and lung were depleted of these cells. These data indicate that bone marrow and lymph nodes may act as a source of all NK cell subsets and are consistent with published data showing involvement of lymph nodes in NK cell production[46]. Indeed, although there is general consensus that NK cell development occurs mainly in bone marrow, human NK cell precursors are present in lymph nodes[46].

Notably, the function of $S1P_5$ in NK cell migration is probably similar in mice and humans. Consistent with the data obtained with mice, $S1P_5$ was 'preferentially' expressed by the most mature $CD56^{dim}$ human NK cells. This differential $S1P_5$ expression correlates with the tissue distribution of human NK cell subsets, as $CD56^{dim}$ NK cells prevail in the blood and spleen and $CD56^{bright}$ NK cells are predominant in lymph nodes[47]. In addition, the absence of an effect of FTY720 on NK cell distribution in mouse correlates with the absence of an effect of FTY720 on human NK cells during clinical trials[48]. Thus, our results collectively indicate that NK cell trafficking is dependent on a dedicated S1P receptor with an apparently unique pharmacology that could potentially be used to specifically manipulate the activity of these cells for therapeutic purposes[49].

METHODS

Mice, adoptive transfer and treatments. All inbred mice (C57BL/6 $CD45.1^+$ and $CD45.2^+$) were from Charles River Laboratories. The generation of $S1P_5$-deficient mice (backcrossed five generations to C57BL/6 mice) has been described[28]. In all experiments, mice were 6–8 weeks old and control mice were littermates. Mixed–bone marrow chimeras were generated as described[50]. For adoptive transfer experiments, 20×10^6 splenocytes from $S1P_5$-deficient and wild-type mice were labeled with 0.2 μM or 2 μM CFSE (5-(and 6-)-carboxyfluorescein diacetate succinimidyl ester; Invitrogen), were mixed at a 1:1 ratio and were injected intravenously into littermate recipient mice. The presence of transferred cells into various organs was analyzed 24 h after transfer. DOP (Sigma) and FTY720 were provided in the drinking water (30 μg/ml and 5 μg/ml, respectively) supplemented with glucose. The RLI fusion protein was administered intravenously (5 μg per injection); poly(I:C) (Invivogen) was injected intraperitoneally (150 μg per mouse). Experiments were done in accordance with institutional guidelines for animal care and use.

Flow cytometry and lymphocyte preparation. Lung and liver lymphocytes were prepared as described[29]. A FACSCanto (Becton-Dickinson) was used for flow cytometry. NK cells were identified as $CD3^-NKp46^+$ or $CD3^-NK1.1^+$. NKp46 expression was detected with the rat monoclonal antibody 29A1.4 (ref. 29). Antibody to CD3 (anti-CD3; 2C11) and anti-NK1.1 (PK136) were from BD Biosciences.

Cell viability. Spleen cells were cultured in AIM V medium supplemented with fatty acid–free bovine albumin (4 mg/ml). S1P (various concentrations; Sigma) and cytokines (50 ng/ml; R&D Systems) were prepared in the same medium. Cell viability was evaluated by flow cytometry by staining for NK cells and with an amine-reactive fluorescent dye (Invitrogen).

Immunofluorescence. Serial frozen sections 10 μm in thickness were analyzed by immunofluorescence. Sections were fixed with acetone and were stained with allophycocyanin-conjugated anti-CD3 (2C11), phycoerythrin-conjugated anti-CD19 (1D3), anti-SignR1 (ERTR9) and anti-NKp46 (goat antibody; R&D Systems), followed staining with an Alexa Fluor 488–coupled secondary antibody (Invitrogen). Slides were analyzed by confocal microscopy (Zeiss LSM 510).

Migration assays. Splenocytes prepared from untreated mice and from mice treated with FTY720 or DOP were suspended in RPMI 1640 medium supplemented with fatty acid–free bovine albumin (4 mg/ml; Sigma). The same medium was used for the preparation of S1P (Sigma). Spleen cell migration was analyzed after 3 h in Transwell chambers (Costar) with polycarbonate filters with a pore width of 5 μm. Migrated cells were stained for CD3 and NK1.1 and were counted by flow cytometry as described[51].

NK cell stimulation assay. Splenic NK cells were enriched by a negative selection procedure. $I\text{-}A^+$, $I\text{-}E^+$, $CD5^+$ and $Ly76^+$ cells were labeled with rat antibodies (53-7.3 culture supernatants, or anti-I-A–I-E (2G9) and anti-Ly76

(Ter119) from BD Biosciences) and were removed with goat anti-rat IgG magnetic beads (Qiagen). Anti-NK1.1 (PK136; 'home-made'), anti-NKp46 (29A1.4; 'home-made') and anti-Ly49D (4E5; BD Biosciences) were allowed to bind to plastic 96-well plates (Thermo Scientific) overnight in carbonate buffer. NK cells were stimulated for 4 h in the presence of fluorescein isothiocyanate–coupled anti-CD107a and GolgiStop (BD-Biosciences). Samples were incubated variously with IL-2 (3,000 U/ml; Peprotech), IL-12 (20 ng/ml; R&D Systems), IL-18 (5 ng/ml; R&D Systems), phorbol myristate acetate (50 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma). Some samples were incubated with YAC1 mouse lymphoma cells at an effector/target ratio of 1:1. At the end of the stimulation, cells were stained with anti-NK1.1 and anti-CD3 and intracellular interferon-γ was measured with the Cytofix/Cytoperm kit (BD Biosciences). Cells were then analyzed by flow cytometry. After stimulation, the effector functions of NK cell subsets were analyzed by six-color staining with Alexa Fluor 700–conjugated anti-CD3 (2C11), allophycocyanin-conjugated anti-NK1.1 (PK136), phycoerythrin-indotricarbocyanine–conjugated anti-interferon-γ (XMG1.2), peridinin chlorophyll protein–conjugated anti-CD11b (M1/70), phycoerythrin-conjugated anti-CD27 (LG.3A10) and fluorescein isothiocyanate–conjugated anti-CD107a (1D4B; all from BD Biosciences).

Quantitative RT-PCR. Lymphocyte subsets stained with the appropriate antibodies were sorted with a FACSVantage (Becton Dickinson). RNA was extracted with the RNeasy micro kit (Qiagen), which includes treatment with DNase. Superscript II reverse transcriptase (Invitrogen) was used to generate cDNA for RT-PCR. A SybrGreen-based kit (Qiagen) was used for PCR. Primers are in **Supplementary Table 1** online.

Statistics. Unpaired two-tailed Student's *t*-tests were used for statistical analysis. The Mann-Whitney test was used for some experiments, where indicated.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

T.W. and E.V. designed the experiments and wrote the paper; T.W. did experiments for **Figures 2–7**; L.C. did experiments for **Figures 2** and **6** and **Supplementary Figure 3**; J.C. did experiments for **Figures 1** and **5**; A.C. provided $S1P_5$-deficient mice, Y.J. and L.G.-A. provided RLI; E.T. did experiments for **Supplementary Figure 2**; M.B. contributed to analysis of the results; and C.C. did experiments not shown and contributed to analysis of the results.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/natureimmunology/.

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1. Bottino, C., Moretta, L. & Moretta, A. NK cell activating receptors and tumor recognition in humans. *Curr. Top. Microbiol. Immunol.* **298**, 175–182 (2006).
2. Newman, K.C. & Riley, E.M. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nat. Rev. Immunol.* **7**, 279–291 (2007).
3. Lodoen, M.B. & Lanier, L.L. Natural killer cells as an initial defense against pathogens. *Curr. Opin. Immunol.* **18**, 391–398 (2006).
4. Huntington, N.D., Vosshenrich, C.A. & Di Santo, J.P. Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat. Rev. Immunol.* **7**, 703–714 (2007).
5. Stewart, C.A. *et al.* Germ-line and rearranged *Tcrd* transcription distinguish bona fide NK cells and NK-like γδ T cells. *Eur. J. Immunol.* **37**, 1442–1452 (2007).
6. Gregoire, C. *et al.* The trafficking of natural killer cells. *Immunol. Rev.* (in the press).
7. Hokeness, K.L., Kuziel, W.A., Biron, C.A. & Salazar-Mather, T.P. Monocyte chemoattractant protein-1 and CCR2 interactions are required for IFN-α/β-induced inflammatory responses and antiviral defense in liver. *J. Immunol.* **174**, 1549–1556 (2005).
8. Thapa, M., Kuziel, W.A. & Carr, D.J. Susceptibility of CCR5-deficient mice to genital herpes simplex virus type 2 is linked to NK cell mobilization. *J. Virol.* **81**, 3704–3713 (2007).
9. Ajuebor, M.N. *et al.* CCR5 deficiency drives enhanced natural killer cell trafficking to and activation within the liver in murine T cell-mediated hepatitis. *Am. J. Pathol.* **170**, 1975–1988 (2007).
10. Khan, I.A. *et al.* CCR5 is essential for NK cell trafficking and host survival following *Toxoplasma gondii* infection. *PLoS Pathog* **2**, e49 (2006).
11. Martin-Fontecha, A. *et al.* Induced recruitment of NK cells to lymph nodes provides IFN-γ for T_H1 priming. *Nat. Immunol.* **5**, 1260–1265 (2004).
12. Jiang, D. *et al.* Regulation of pulmonary fibrosis by chemokine receptor CXCR3. *J. Clin. Invest.* **114**, 291–299 (2004).
13. Huang, D. *et al.* The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. *FASEB J.* **20**, 896–905 (2006).
14. Yu, Y.R. *et al.* Defective antitumor responses in CX3CR1-deficient mice. *Int. J. Cancer* **121**, 316–322 (2007).
15. Lavergne, E. *et al.* Fractalkine mediates natural killer-dependent antitumor responses *in vivo*. *Cancer Res.* **63**, 7468–7474 (2003).
16. Wald, O. *et al.* IFN-γ acts on T cells to induce NK cell mobilization and accumulation in target organs. *J. Immunol.* **176**, 4716–4729 (2006).
17. Inngjerdingen, M., Damaj, B. & Maghazachi, A.A. Expression and regulation of chemokine receptors in human natural killer cells. *Blood* **97**, 367–375 (2001).
18. Geissmann, F. *et al.* Intravascular immune surveillance by $CXCR6^+$ NKT cells patrolling liver sinusoids. *PLoS Biol.* **3**, e113 (2005).
19. Cyster, J.G. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu. Rev. Immunol.* **23**, 127–159 (2005).
20. Rosen, H. & Goetzl, E.J. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. *Nat. Rev. Immunol.* **5**, 560–570 (2005).
21. Rosen, H., Sanna, M.G., Cahalan, S.M. & Gonzalez-Cabrera, P.J. Tipping the gatekeeper: S1P regulation of endothelial barrier function. *Trends Immunol.* **28**, 102–107 (2007).
22. Brinkmann, V. Sphingosine 1-phosphate receptors in health and disease: Mechanistic insights from gene deletion studies and reverse pharmacology. *Pharmacol. Ther.* **115**, 84–105 (2007).
23. Schwab, S.R. *et al.* Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science* **309**, 1735–1739 (2005).
24. Matloubian, M. *et al.* Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* **427**, 355–360 (2004).
25. Wei, S.H. *et al.* Sphingosine 1-phosphate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. *Nat. Immunol.* **6**, 1228–1235 (2005).
26. Terai, K. *et al.* Edg-8 receptors are preferentially expressed in oligodendrocyte lineage cells of the rat CNS. *Neuroscience* **116**, 1053–1062 (2003).
27. Im, D.S. *et al.* Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. *J. Biol. Chem.* **275**, 14281–14286 (2000).
28. Jaillard, C. *et al.* Edg8/S1P5: an oligodendroglial receptor with dual function on process retraction and cell survival. *J. Neurosci.* **25**, 1459–1469 (2005).
29. Walzer, T. *et al.* Identification, activation, and selective *in vivo* ablation of mouse NK cells via NKp46. *Proc. Natl. Acad. Sci. USA* **104**, 3384–3389 (2007).
30. Walzer, T., Jaeger, S., Chaix, J. & Vivier, E. Natural killer cells: from $CD3^-NKp46^+$ to post-genomics meta-analyses. *Curr. Opin. Immunol.* **19**, 365–372 (2007).
31. Goetzl, E.J. & Rosen, H. Regulation of immunity by lysosphingolipids and their G protein-coupled receptors. *J. Clin. Invest.* **114**, 1531–1537 (2004).
32. Lucas, M., Schachterle, W., Oberle, K., Aichele, P. & Diefenbach, A. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* **26**, 503–517 (2007).
33. Mortier, E. *et al.* Soluble interleukin-15 receptor α (IL-15R α)-sushi as a selective and potent agonist of IL-15 action through IL-15Rβ/γ. Hyperagonist IL-15 x IL-15Rα fusion proteins. *J. Biol. Chem.* **281**, 1612–1619 (2006).
34. Hayakawa, Y. & Smyth, M.J. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J. Immunol.* **176**, 1517–1524 (2006).
35. Goetzl, E.J., Kong, Y. & Mei, B. Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. *J. Immunol.* **162**, 2049–2056 (1999).
36. Wang, W., Graeler, M.H. & Goetzl, E.J. Physiological sphingosine 1-phosphate requirement for optimal activity of mouse $CD4^+$ regulatory T Cells. *FASEB J.* **18**, 1043–1045 (2004).
37. Graeler, M. & Goetzl, E.J. Activation-regulated expression and chemotactic function of sphingosine 1-phosphate receptors in mouse splenic T cells. *FASEB J.* **16**, 1874–1878 (2002).
38. Kveberg, L., Bryceson, Y., Inngjerdingen, M., Rolstad, B. & Maghazachi, A.A. Sphingosine 1 phosphate induces the chemotaxis of human natural killer cells. Role for heterotrimeric G proteins and phosphoinositide 3 kinases. *Eur. J. Immunol.* **32**, 1856–1864 (2002).
39. Wang, J., Xu, J.W., Zhang, W.C., Wei, H.M. & Tian, Z.G. TLR3 ligand-induced accumulation of activated splenic natural killer cells into liver. *Cell. Mol. Immunol.* **2**, 449–453 (2005).

40. Mandala, S. *et al.* Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* **296**, 346–349 (2002).
41. Chen, S., Kawashima, H., Lowe, J.B., Lanier, L.L. & Fukuda, M. Suppression of tumor formation in lymph nodes by L-selectin-mediated natural killer cell recruitment. *J. Exp. Med.* **202**, 1679–1689 (2005).
42. Bajenoff, M. *et al.* Natural killer cell behavior in lymph nodes revealed by static and real-time imaging. *J. Exp. Med.* **203**, 619–631 (2006).
43. Kelly, P., Casey, P.J. & Meigs, T.E. Biologic functions of the G12 subfamily of heterotrimeric G proteins: growth, migration, and metastasis. *Biochemistry* **46**, 6677–6687 (2007).
44. Novgorodov, A.S., El-Alwani, M., Bielawski, J., Obeid, L.M. & Gudz, T.I. Activation of sphingosine-1-phosphate receptor $S1P_5$ inhibits oligodendrocyte progenitor migration. *FASEB J.* **21**, 1503–1514 (2007).
45. Hanessian, S., Charron, G., Billich, A. & Guerini, D. Constrained azacyclic analogues of the immunomodulatory agent FTY720 as molecular probes for sphingosine 1-phosphate receptors. *Bioorg. Med. Chem. Lett.* **17**, 491–494 (2007).
46. Freud, A.G. & Caligiuri, M.A. Human natural killer cell development. *Immunol. Rev.* **214**, 56–72 (2006).
47. Ferlazzo, G. *et al.* The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. *J. Immunol.* **172**, 1455–1462 (2004).
48. Vaessen, L.M., van Besouw, N.M., Mol, W.M., Ijzermans, J.N. & Weimar, W. FTY720 treatment of kidney transplant patients: a differential effect on B cells, naive T cells, memory T cells and NK cells. *Transpl. Immunol.* **15**, 281–288 (2006).
49. Ljunggren, H.G. & Malmberg, K.J. Prospects for the use of NK cells in immunotherapy of human cancer. *Nat. Rev. Immunol.* **7**, 329–339 (2007).
50. Chiesa, S. *et al.* Multiplicity and plasticity of natural killer cell signaling pathways. *Blood* **107**, 2364–2372 (2005).
51. Walzer, T., Arpin, C., Beloeil, L. & Marvel, J. Differential in vivo persistence of two subsets of memory phenotype CD8 T cells defined by CD44 and CD122 expression levels. *J. Immunol.* **168**, 2704–2711 (2002).