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Neutrophils Exhibit Differential Requirements for Homing Molecules in Their Lymphatic and Blood Trafficking into Draining Lymph Nodes

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Although much is described about the molecules involved in neutrophil migration from circulation into tissues, less is known about the molecular mechanisms that regulate neutrophil entry into lymph nodes (LNs) draining a local inflammatory site. In this study, we investigated neutrophil migration toward LNs in a context of inflammation induced by immunization of BALB/c mice with OVA emulsified in CFA. We demonstrated that neutrophils can enter LNs of OVA/CFA-immunized mice not only via lymphatic vessels but also from blood, across high endothelial venules. By adoptive transfer experiments, we showed that this influx was dependent on an inflammatory-state condition and previous neutrophil stimulation with OVA/anti-OVA immune complexes. Importantly, we have demonstrated that, in the migratory pattern to LNs, neutrophils used L-selectin and P-selectin glycoprotein ligand-1, macrophage-1 Ag and LFA-1 integrins, and CXCR4 to get access across high endothelial venules, whereas macrophage-1 Ag, LFA-1, and CXCR4 were involved in their trafficking through afferent lymphatics. Strikingly, we found that stimulation with immune complexes significantly upregulated the expression of sphingosine-1-phosphate receptor 4 on neutrophils, and that treatment with the sphingosine-1-phosphate agonist FTY720 altered neutrophil LN-homing ability. These findings summarized in this article disclose the molecular pattern that controls neutrophil recruitment to LNs. ***The Journal of Immunology*****, 2014, 193: 1966–1974.**

The recruitment of neutrophils into tissues is a key step during inflammation and involves a sequential series of molecular interactions between the leukocyte and endothelial cells. Low-affinity interactions involving selectins initiate neutrophil rolling along the endothelium. Local chemotactic signals then activate neutrophils to upregulate cell-surface integrins, which interact with endothelial adhesion molecules resulting in firm adhesion, followed by migration into tissues (1). The ability of these cells to mobilize and traffic to where they are needed is central for their functions in promoting immune defense during infection and in driving inflammatory diseases (2).

Entry of immune cells from the bloodstream into lymph nodes (LNs) requires initial rolling of cells on high endothelial venules (HEVs), followed by integrin activation through chemokine signaling, firm adhesion, and diapedesis (3, 4). In addition, an alternative pathway for LN access involves entry into peripheral nonlymphoid tissues through postcapillary venules, with subsequent trafficking to draining LNs (dLNs) through afferent lymphatics (4, 5). Although molecular mechanisms involved in leukocyte trafficking across HEVs are well described, the molecules that control movement of immune cells into LNs through lymphatic vessels are not fully defined. Whereas chemokine receptor CCR7 expression is seen to be critical for migration of T cells and dendritic cells (DCs), integrins seem not to be required for DC trafficking through lymphatic vessels (6–8). In addition to the well-known role of sphingosine-1-phosphate (S1P) for lymphocyte egress from LNs (9), several works have revealed the involvement of S1P receptors (S1PRs) in T cell trafficking to LNs in lymph and blood routes. Indeed, S1PR1 was shown to inhibit T cell entry into dermal lymphatics, leading to T cell retention in peripheral tissues, and to promote their migration across HEVs by facilitating integrin firm arrest (10, 11). The study of how several chemotactic signals are coordinated is therefore a challenge in the field of cell migration to LNs.

Although neutrophils circulate in blood, they are largely excluded from LNs under steady-state conditions. For many years, it was thought that emigration from tissues was only confined to lymphocytes and DCs, but not to granulocytes. Nevertheless, we and others have shown that neutrophils can migrate to LNs in response to different stimuli, such as inflammation (12–14) or infection (15–18), where they shape the immune response by interacting with DCs and hence modulating Ag presentation (19). Indeed, we previously reported that, when OVA Ag is injected into

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C.V.G. and M.C.P.-P. designed research; C.V.G. and R.P.R. performed research; M.F.H., I.A.G., and M.I.C. analyzed data; B.A.M and G.M. interpreted data; and C.V.G. and M.C.P.-P. wrote the paper.

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Abbreviations used in this article: BM, bone marrow; DC, dendritic cell; $DiIC_{(18)}$-DS, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine-5,5′-disulfonic acid; $DiOC_{(18)}$-SP, 3,3′-dioctadecyl-5,5′-di(4-sulfophenyl)oxacarbocyanine, sodium salt; dLN, draining LN; HEV, high endothelial venule; IC, immune complex; LN, lymph node; Mac-1, macrophage-1 Ag; poLN, popliteal LN; PSGL-1, P-selectin glycoprotein ligand-1; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; S1PR, sphingosine-1-phosphate receptor.

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hind footpads of previously OVA/CFA-immunized mice, the main OVA^+ cells in footpads and popliteal LNs (poLNs) are neutrophils (12). Although neutrophil entry into tissues through the vascular endothelium has been studied in detail, the molecular mechanisms of neutrophil migration into LNs are poorly defined. In this study, we investigated neutrophil migration toward LNs in a context of inflammation and revealed some of the molecular basis for neutrophil homing to LNs.

Materials and Methods

Mice and immunization

Female 8- to 12-wk-old BALB/c mice were from the National University of La Plata (Buenos Aires, Argentina). Mice were maintained in our animal facilities, which comply with the terms of the *Guide to the Care and Use of Experimental Animals* (published by the Canadian Council on Animal Care; assurance no. A5802-01) and of our Institutional Experimentation Animal Committee (authorization no. 15-01-44195). Mice were s.c. immunized with 60 μg OVA (Fraction V; Sigma-Aldrich, St. Louis, MO) or PBS, emulsified in CFA (Sigma-Aldrich). Each immunization was administered in flanks and tail on days 0, 15, and 30 (0.5 ml/animal/dose). Ten days after the last immunization, mice were injected in hind footpads with 30 μg OVA labeled with Alexa Fluor 488 or 647 (Invitrogen, Carlsbad, CA), cells, or vehicle alone.

Reagents

Anti–P-selectin (RB40.34), anti–P-selectin glycoprotein ligand-1 (anti–PSGL-1; 4RA10) and anti–LFA-1 (M17/4) were from BD Biosciences, Pharmingen (San Diego, CA). Anti-CD18 (M18/2) was from Biolegend (San Diego, CA). Protein G–purified Abs against macrophage-1 Ag (Mac-1; M1/70) and L-selectin (MEL-14) were obtained from hybridoma supernatants. Isotype control (rat IgG) was obtained from normal rat sera and purified with protein G-Sepharose. FTY720 and FTY720-P were from Cayman Chemical (Ann Arbor, MI). Pertussis toxin (PTX) and AMD3100 were from Sigma-Aldrich.

Cell preparation and adoptive transfer

Total bone marrow (BM) cells were isolated from BALB/c mice and depleted from erythrocytes using RBC lysing buffer (Sigma-Aldrich). In some experiments, BM neutrophils were purified using anti–Ly-6G–PE (1A8; BD Pharmingen) and then anti-PE microbeads (Miltenyi Biotec, Auburn, CA). Cells were then stimulated for 1 h at 37°C with OVA/anti-OVA immune complexes (ICs; formed by incubation of inactivated polyclonal anti-OVA rabbit sera [1:75; NATOCOR, Córdoba, Argentina] plus OVA [1.6 μg/ml; Sigma-Aldrich] 30 min at 37°C). Cells were then differentially labeled with 3,3′-dioctadecyl-5,5′-di(4-sulfophenyl) oxacarbocyanine, sodium salt ($DiOC_{(18)}$-SP; 8 μg/ml; Invitrogen) or 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine-5,5′-disulfonic acid ($DiIC_{(18)}$-DS; 4 μg/ml; Invitrogen) 5 min at 37°C and then 15 min at 4°C. $DiOC_{(18)}$-SP^+ cells ($8–10 \times 10^6$; 50% of them neutrophils, as determined by flow cytometry) were injected into left footpads (20 μl; right footpads received PBS as control), and $DiIC_{(18)}$-DS^+ cells ($20–35 \times 10^6$ cells, of which 50% were neutrophils) in 150 μl PBS were injected i.v. in each mouse to assess LN migration. Ninety minutes after injection, blood, footpads, and poLNs were harvested and single-cell suspensions were prepared.

Cell preparation and flow cytometry

Mice were sacrificed and blood, skin from hind footpads, and poLNs were removed to obtain single-cell suspensions. Blood samples were treated for 10 min with RBC lysing buffer, and poLNs were mechanically dissociated to form single-cell suspensions. Footpads were incubated for 30 min in PBS containing 0.5% trypsin and 5 mM EDTA, finely cut, and then digested with 0.14 U/ml Liberase-Blendzyme II and 100 μg/ml DNase I (Roche, San Francisco, CA) for 1 h at 37°C. Flow-cytometric staining was conducted as previously described (12). CD45 (30-F11) and CXCR4 (2B11-CXCR4) were from BD Pharmingen; CXCR3 (CXCR3-173), CCR4 (2G12), and CCR7 (4B12) were from Biolegend. CXCR2 (242216) and CCR3 (83101) were from R&D Systems (Minneapolis, MN). Data were acquired on a FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR). In adoptive transfer experiments, a minimum of 300,000 events/sample were acquired.

Confocal microscopy

Footpads and poLNs were fixed in 4% paraformaldehyde, transferred to 30% sucrose, snap frozen in liquid nitrogen, and stored at −70°C. Acetone-fixed 16-μm cryostat sections were first incubated with anti–LYVE-1 (223322; R&D Systems) or MECA-79 (BD Pharmingen) primary Abs, followed by incubation with anti-rat Ig Alexa Fluor 594 (Invitrogen) and next with FITC- or allophycocyanin-conjugated Gr-1 (RB6-8C5; BD Pharmingen). Coverslips were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA). Single confocal sections were obtained using a Carl Zeiss LSM 5 Pascal laser-scanning confocal microscope (Carl Zeiss, Gottingen, Germany) and processed with LSM 5 PASCAL software. In some cases, pseudocolor channels were swapped for visual representation.

Quantitative RT-PCR

Total RNA was isolated from purified BM neutrophils using TRIzol (Invitrogen), treated with RQ1 DNase I (Promega, Madison, WI), and cDNA synthesis was carried out by using Moloney murine leukemia virus reverse transcriptase (Promega). Equivalent amounts of cDNA were used in quantitative RT-PCR on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Forster City, CA) with the following primers (Sigma-Aldrich): S1PR1 forward 5′-TTGAGCGAGGCTGCTGTTT-3′, reverse 5′-TAGAGGGCGAGGTTGAGTGAG-3′; S1PR2 forward 5′-CCAACAGTCTCCAAAACCAACC-3′, reverse 5′-CTCTGAGTATAAGCCGCCCA-3′; S1PR3 forward 5′-GACAAGCCTAGCGGGAGAGA-3′, reverse 5′-TGCGGGAAGAGTGTTGAAAAT-3′; S1PR4 forward 5′-CTTTGTGGTGTGCTGGGGTC-3′, reverse 5′-GCGGAAGGAGTAGATGAGAGGA-3′; S1PR5 forward 5′-TACTCCCCCCAGATTTCCAA-3′, reverse 5′-GAGATGTCGCTGCTGTGTCCT-3′; HPRT forward 5′-AAGCTTGCTGGTGAAAAGGA-3′, reverse 5′-TCCAACAAAGTCTGGCCTGT-3′.

Statistical analysis

Data are shown as means ± SEM. Multiple comparisons were tested using one-way ANOVA, followed by post hoc comparison with control by Dunnett's test or Bonferroni's posttest for adoptive transfer experiments. Single groups were tested using the Mann–Whitney *U* test (GraphPad Prism 4 Software; GraphPad, San Diego, CA). Results with $p \leq 0.05$ were considered significant.

Results

Neutrophil migration to dLNs implies both lymphatic and blood routes

We previously showed that, after OVA challenge in OVA/CFA mice, not only did OVA^+ neutrophils appear at the injection site, but neutrophils also represented the main OVA^+ cells in dLNs. Moreover, OVA^+ neutrophils were localized in the subcapsular space, principally in ipsilateral LNs, suggesting that neutrophils had reached LNs via afferent lymphatics (12). It has been shown that neutrophils can access poLNs from footpads via lymphatics in a CCR7-dependent mechanism (14); however, the contribution of blood migration in neutrophil influx to LNs has not been determined. For this reason, our first objective was to conduct a more comprehensive evaluation of neutrophil migratory routes. We performed immunohistochemistry analysis of OVA/CFA mice after OVA challenge. As expected, we found neutrophils associated with LYVE-1^+ lymphatic vessels in footpads and poLNs (Fig. 1A), indicating that these cells could migrate through lymph. Moreover, we also detected neutrophils in the proximity of MECA-79^+ HEVs of poLNs (Fig. 1B), suggesting the implication of blood migration.

To provide another line of evidence that both routes were involved, we performed adoptive transfer experiments. Our previous results demonstrated that neutrophil influx to LNs requires an Ag-specific humoral response (12). We therefore stimulated neutrophils with OVA/anti-OVA ICs before their transfer. Cells were then differentially labeled to distinguish transferring routes, injected i.v. or into footpads of OVA/CFA mice, and 90 min later we examined their arrival in poLNs. As shown in Fig. 1C, neutrophils (identified by their high expression of the Ly-6G marker) arrived in poLNs via

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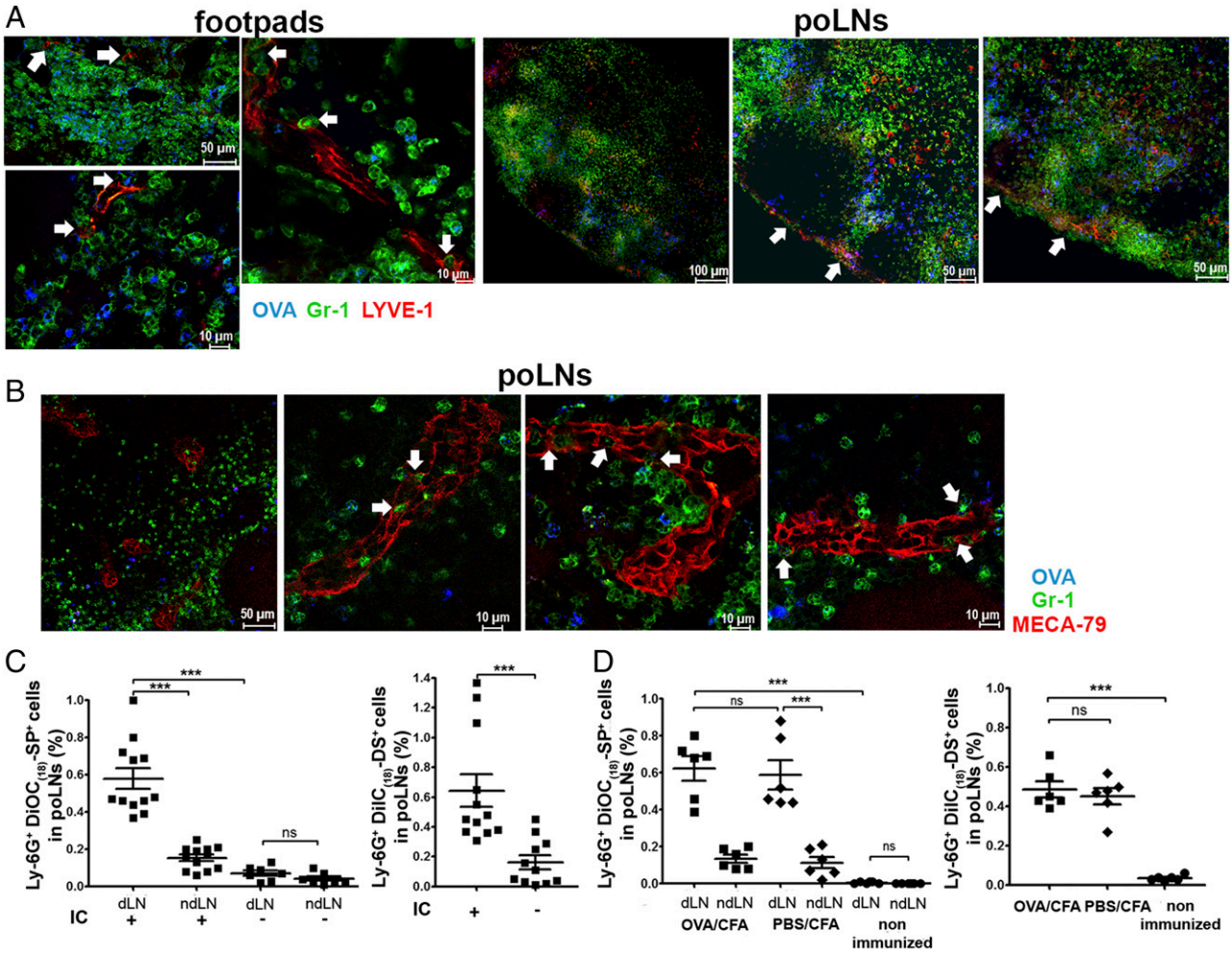

FIGURE 1. Characterization of neutrophil migratory routes to LNs. (**A** and **B**) Representative confocal images of skin of hind footpads and poLNs from OVA/CFA mice 6 h after OVA injection into footpads. The distribution is shown of OVA Ag (blue), Gr-1$^+$ neutrophils (green) in the proximity (marked with white arrows) of LYVE-1$^+$ lymphatic vessels (red; A), or MECA-79$^+$ HEVs (red; B) in footpads and poLNs. Data are representative of six mice in three experiments. (**C** and **D**) Flow-cytometry analysis of lymphatic and blood migration of neutrophils to poLNs 90 min after cell transfer. Total BM cells were incubated with OVA/anti-OVA ICs (*IC*+) or not (*IC*−), stained, and transferred to OVA/CFA mice (■; C and D), PBS/CFA (◆; D), or nonimmunized mice (●; D). Results are expressed as percent of transferred neutrophils (Ly-6G$^+$ DiOC$_{(18)}$-SP$^+$ cells) recovered in left poLN (dLNs) and in right poLN (nondraining LNs [ndLN]) 90 min after left footpad injection or as percent of neutrophils recovered from poLNs 90 min after their i.v. injection (Ly-6G$^+$ DiIC$_{(18)}$-DS$^+$ cells). Data are representative of two to three mice per group in at least three experiments. ***$p \leq 0.001$. ns, not significant.

both lymphatic and blood routes. We also confirmed these migratory pathways in adoptive transfer experiments using purified neutrophils (Supplemental Fig. 1A). Moreover, IC-stimulated neutrophils migrated more efficiently than did nonstimulated cells (Fig. 1C), cells preincubated with OVA alone or only with anti-OVA rabbit sera (Supplemental Fig. 1B). Neutrophils were also transferred to recipients that were injected with CFA alone or that were unprimed. The migratory pattern observed in PBS/CFA was comparable with that found in OVA/CFA mice: only IC-stimulated neutrophils (but not nonstimulated cells; data not shown) were able to reach poLNs (Fig. 1D). These results support the idea that neither cells nor humoral components (specific to OVA Ag and present in OVA/CFA mice) activate neutrophils indirectly in the skin or in blood of OVA/CFA mice. In contrast, no donor cells were found in poLNs of nonimmunized mice, even if neutrophils were previously incubated with ICs (Fig. 1D). Taken together, these results demonstrate that neutrophils can gain access to LNs via both lymph and blood under inflammatory conditions, and that previous stimulation with ICs dictates their trafficking.

L-selectin and PSGL-1 were implicated in neutrophil migration to LNs through blood, but not through lymph

Having determined the implication of both routes, we next sought to characterize the molecules involved. Because little is known about the signals that direct neutrophil homing to LNs, we first focused on the role of selectins. It is well-known that L-selectin on lymphocytes interacts with ligands on HEVs to initiate lymphocyte rolling along the luminal surface (3, 20). In contrast, PSGL-1, through its interaction with P- and E-selectin, is an alternate mechanism for leukocyte rolling (21). Resting LNs do not express either E-selectin or P-selectin; however, it has been demonstrated that reactive LNs induced the expression of P-selectin on HEVs after CFA injection (22). Considering this, we examined neutrophil migration in OVA/CFA mice after anti–L-selectin and anti–P-selectin treatment. In vivo L-selectin blockade reduced neutrophil influx to poLNs and skin of footpads 9- and 2-fold, respectively (Fig. 2A, 2B). Similarly, anti–P-selectin treatment significantly decreased the percentage of neutrophils in poLNs, as well as in footpads, almost 2-fold (Fig. 2A, 2B). To further determine the specific contribution of L-selectin and P-selectin ligand

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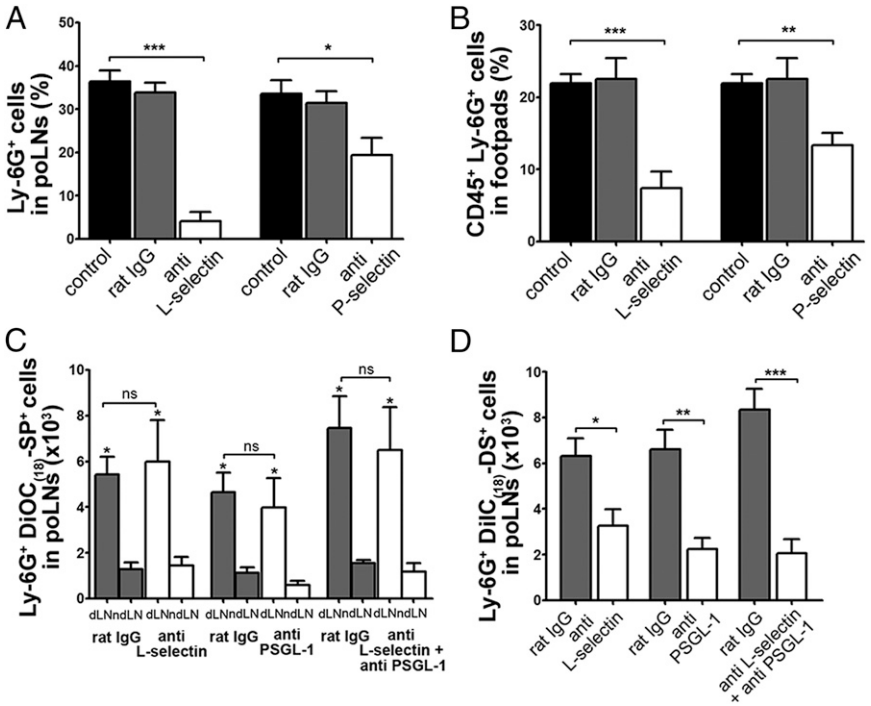

FIGURE 2. L-selectin and PSGL-1 only participate in neutrophil entrance into LNs through blood. (**A** and **B**) Flow-cytometry analysis of neutrophil influx to poLNs (A) and footpads (B) after anti–L-selectin or –P-selectin treatment. OVA/CFA mice were challenged with OVA in their footpads and 1 h later injected i.v. with saline (control), isotype control (rat IgG), or anti–L-selectin (250 μg/mouse) or anti–P-selectin (60 μg/mouse) Abs. Results are shown as percentage of neutrophils in poLNs (Ly-6G$^+$ cells) or footpads (CD45$^+$ Ly-6G$^+$ cells) 5 h after Ab treatment. (**C** and **D**) Flow-cytometry analysis of LN homing through lymph (C) and blood (D) of neutrophils treated with anti–L-selectin, anti–PSGL-1, or anti–L-selectin + anti–PSGL-1 Abs. Total BM cells were incubated with ICs, treated with anti–L-selectin (50 μg/ml), anti–PSGL-1 (10 μg/ml), anti–L-selectin + anti–PSGL-1, or control rat IgG for 20 min at 4°C, stained, and transferred to OVA/CFA mice. Results are expressed as the number of transferred neutrophils recovered into left poLN (dLNs) and into right poLN (nondraining LNs [ndLN]) 90 min after left footpad injection (Ly-6G$^+$ DiOC$_{(18)}$-SP$^+$ cells) or i.v. injection (Ly-6G$^+$ DiIC$_{(18)}$-DS$^+$ cells). Data from three experiments corresponding to two to three mice per group per experiment are plotted. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. ns, not significant.

PSGL-1, we performed adoptive transfer experiments in which the neutrophils administered were previously incubated in vitro with anti–L-selectin or anti–PSGL-1 Abs. As would be predicted, L-selectin blockade reduced neutrophil entry into poLNs from the bloodstream (Fig. 2D); nevertheless, anti–L-selectin treatment did not inhibit lymphatic neutrophil trafficking (Fig. 2C). Similarly, anti–PSGL-1 treatment significantly reduced homing to poLNs across HEVs (Fig. 2D), whereas neutrophil lymphatic migration was not affected (Fig. 2C). When both selectins were simultaneously blocked on transferred cells, neutrophil migration from blood showed a slight decrease relative to the effect observed by each blocking Ab alone (Fig. 2D). Taken together, these findings collectively showed that both L-selectin and PSGL-1 regulate the influx to LNs, enabling neutrophils to extravasate through HEVs.

Blockade of LFA-1 and Mac-1 integrins resulted in impaired neutrophil homing to poLNs

After selectin-mediated rolling, integrin activation is a critical requirement for leukocyte arrest on vessel walls and subsequent transmigration. Two main β2 integrins, LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), were reported to be used by neutrophils in the multistep recruitment process and in models of inflammation (23, 24). Moreover, in lymphocyte homing to LNs, LFA-1 is the dominant integrin involved in firm arrest in HEVs (25). These data prompted us to delineate the importance of these two integrins in neutrophil migration. We therefore analyzed in vivo treatment with anti–Mac-1 or anti–LFA-1 in OVA/CFA mice after OVA challenge. Blockade of LFA-1 and Mac-1 attenuated nearly 3-fold the promotion of neutrophil trafficking into LNs (Fig. 3A). In addition, anti–LFA-1 and Mac-1 reduced the percentage of neutrophils in footpads by 36 and 69%, respectively (Fig. 3B). We next tested the effect of mAbs against LFA-1 or Mac-1 of adaptively transferred neutrophils. Anti–LFA-1 and anti–Mac-1 treatment significantly reduced the number of neutrophils that migrated through lymph (3- and 2-fold, respectively; Fig. 3C) and also from blood (2- and 3-fold, respectively; Fig. 3D) compared with the number of isotype control-treated neutrophils. Similar results were observed when CD18 was blocked on transferred cells (Fig. 3C, 3D). In conclusion, these results suggest that Mac-1 and LFA-1 cooperate in neutrophil movement from blood and lymph to LNs.

Chemokine receptor CXCR4 controlled both neutrophil lymphatic and blood migration

Chemokine receptors are crucial for migration to LNs, and multiple chemokines are known to stimulate neutrophil trafficking (4, 26). Therefore, we initially tested whether treatment with PTX, which blocks signaling from G protein–coupled receptors, would affect neutrophil influx in our experimental model. PTX treatment dramatically blocked LN neutrophil buildup and also altered their trafficking to skin (Fig. 4A). Further, results from ELISA assays of LN homogenates from immunized mice showed that an Ag challenge triggered the modulation of the expression of multiple chemokines in OVA/CFA mice (Supplemental Fig. 2A). Therefore, we next studied the expression of their specific receptors on peripheral-blood neutrophils. Cell-surface analysis revealed that CXCR4 was the only chemokine receptor significantly upregu-

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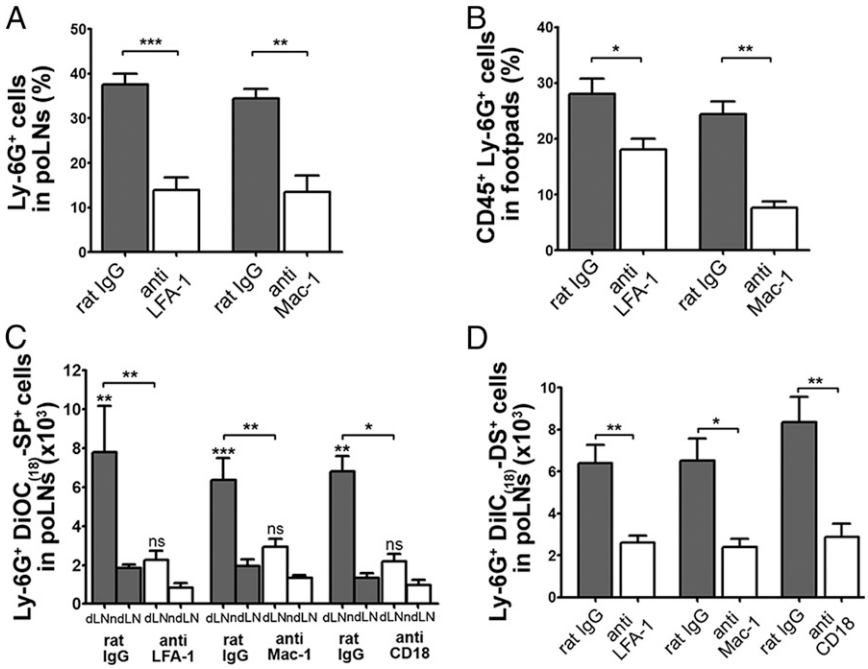

FIGURE 3. Mac-1 and LFA-1 are required to mediate neutrophil migration to LNs via both lymph and blood. (**A** and **B**) Effect of anti–Mac-1 or –LFA-1 treatment on neutrophil recruitment. OVA/CFA mice were injected with OVA into footpads and 1 h later treated with rat IgG, anti–Mac-1 (250 μg/mouse), or anti–LFA-1 (50 μg/mouse). Results are shown as percentage of neutrophils into poLNs (Ly-6G$^+$ cells; A) or into footpads (CD45$^+$ Ly-6G$^+$ cells; B) 5 h after Ab blockade. (B and **C**) Quantification of transferred neutrophils treated in vitro with anti–Mac-1, anti–LFA-1, or anti-CD18 Abs by flow cytometry. Total BM cells were incubated with ICs, treated with anti–Mac-1 (50 μg/ml), anti–LFA-1 (10 μg/ml), anti-CD18 (10 μg/ml), or control rat IgG for 20 min at 4˚C, stained, and transferred to OVA/CFA mice. Results are expressed as number of transferred neutrophils recovered into left poLN (dLN) and into right poLN (nondraining LNs [ndLN]) 90 min after left footpad injection (Ly-6G$^+$ DiOC$_{(18)}$-SP$^+$ cells; C) or i.v. injection (Ly-6G$^+$ DiIC$_{(18)}$-DS$^+$ cells; **D**). Data are representative of two to three independent experiments, with each condition performed in triplicate. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. ns, not significant.

lated on blood neutrophils from OVA/CFA mice after OVA injection (Fig. 4B). Moreover, CXCR4 was found in most of the poLN-infiltrating neutrophils and also in neutrophils from footpads (Fig. 4C). It was previously reported that neutrophil migration from footpads to poLNs was dependent on CCR7 expression (14). Although a fraction of neutrophils from footpads expressed CCR7, we detected a low percentage of neutrophils in poLNs expressing CCR7 in our experimental model (Fig. 4C). We also found higher intracellular levels of CXCR4 on neutrophils from blood, poLNs, and footpads compared with CCR7 levels (Supplemental Fig. 2B). To specifically demonstrate the involvement of CXCR4 in neutrophil migration from lymph and blood, we performed adoptive transfer experiments to OVA/CFA mice that were treated with the CXCR4 inhibitor, AMD3100. Neutrophils displayed diminished migration from footpads and also from blood into poLNs of AMD3100-treated mice (Fig. 4D), confirming the role of CXCR4 in neutrophil trafficking to dLNs in both lymphatic and blood routes.

FTY720 treatment caused altered homing to poLNs

S1P plays an important role in the regulation of immune-cell trafficking, serving as chemoattractant for hematopoietic cells and binding to its five cell-surface receptors. The immunosuppressant drug fingolimod (FTY720; Gilenya), by means of its phosphorylated form FTY720-P, induces lymphocyte sequestration by inhibition of cell emigration from LNs. Particularly, FTY720-P is a high-affinity agonist for S1PRs (with the exception of S1PR2) and blocks S1P-mediated signaling by inducing internalization and degradation of these receptors (27). Although much is described about the role of S1P in lymphocyte homing, little is known about S1P participation in neutrophil trafficking. For this reason, we first determined the pattern of S1PR mRNA expressed by neutrophils. The results from quantitative RT-PCR analysis demonstrated that BM neutrophils expressed considerable levels of S1PR4, and that only S1PR4 was significantly upregulated after IC stimulation in vitro (Fig. 5A). To further confirm S1PR relevance in neutrophil trafficking toward LNs, we treated OVA/CFA mice with FTY720. Although neutrophil influx to poLNs was impaired by 82%, neutrophil migration toward the inflammatory site was not affected (Fig. 5B). In addition, neither neutropenia nor decreased neutrophil expression of adhesion molecules was observed in FTY720-treated mice (Supplemental Fig. 3), discarding the possibility that a reduced number of neutrophils or an altered expression of adhesion molecules caused a reduced influx to LNs (28).

FTY720 treatment affected neutrophil migration across HEVs and through lymph by direct action on neutrophil S1PRs

We next sought to characterize the routes by which S1P could participate. Immunohistochemical analysis revealed that, although there were no main differences in neutrophil distribution in footpads of FTY720-treated mice compared with control mice, fewer neutrophils entered poLNs across HEVs in the FTY720-treated group (Supplemental Fig. 4A). We also performed cell-tracking experiments to measure the influx of transferred neutrophils. FTY720 treatment reduced the recruitment of transferred neutrophils to poLNs from lymph and blood by 51 and 66%, respectively (Fig. 6A). However, it remains possible that the effects of FTY720 on neutrophil trafficking are mediated not only by S1PRs expressed solely on neutrophils, but also by S1PRs expressed on endothelial cells or other S1PR-expressing cells (29). To examine this, we performed homing assays, this time transferring neutrophils that

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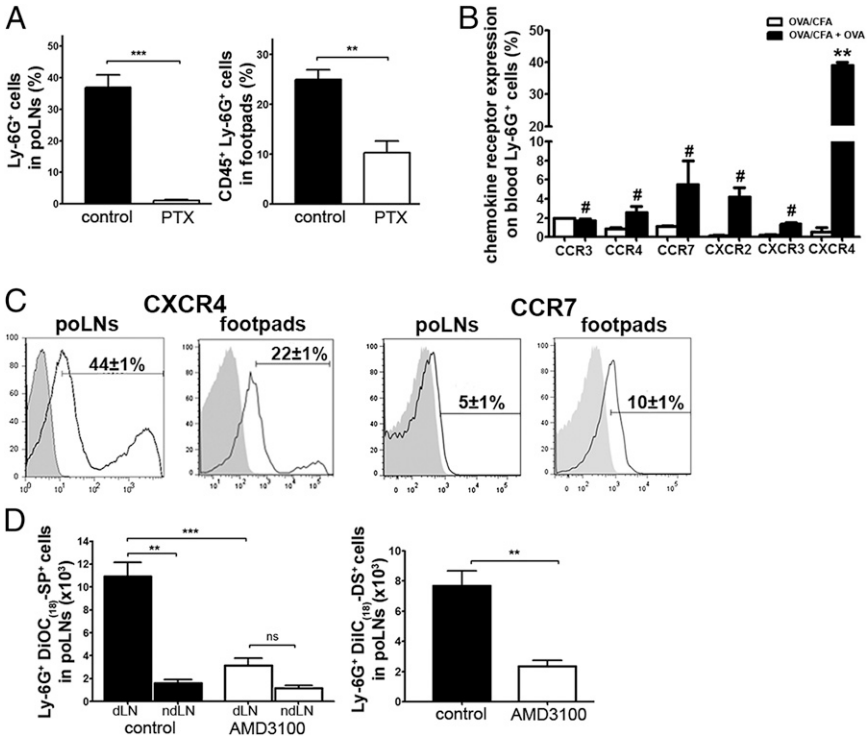

FIGURE 4. CXCR4 participates in neutrophil lymphatic and blood migration to LNs. (**A**) Effect of PTX treatment in neutrophil influx. OVA/CFA mice were injected i.v. with saline (*control*) or PTX (200 μg/kg) 12 h before OVA injection. Bars represent percentage of neutrophils into poLNs (Ly-6G$^+$ cells) or into footpads (CD45$^+$ Ly-6G$^+$ cells) 6 h after OVA challenging. (**B**) Analysis of chemokine receptor expression on blood neutrophils after OVA challenge by flow cytometry. Bars represent the percentage of peripheral blood neutrophils that express CCR3, CCR4, CCR7, CXCR2, CXCR3, or CXCR4 from OVA/CFA mice that were injected into footpads 6 h before with saline (□) or OVA (■). (**C**) Expression of CXCR4 and CCR7 on neutrophils from poLNs and footpads of OVA/CFA mice 6 h after OVA challenge. Representative histograms show chemokine receptor staining on Ly-6G^{high} cells (solid line) and the respective isotype control (shaded gray area); numbers indicate percentage of CXCR4$^+$ or CCR7$^+$ neutrophils. (**D**) Flow cytometry analysis of neutrophil lymphatic and blood migration after AMD3100 treatment. MACS-sorted Ly-6G$^+$ cells were incubated with ICs, stained, and transferred to OVA/CFA mice that were treated i.p. with saline (control; ■) or AMD3100 (5 mg/kg; □) 180 min before cell transference. Results are expressed as number of transferred neutrophils recovered into left dLNs and into nondraining LNs (ndLN) 90 min after footpad injection (Ly-6G$^+$ DiOC$_{(18)}$-SP$^+$ cells) or i.v. injection (Ly-6G$^+$ DiIC$_{(18)}$-DS$^+$ cells). Representative data derived from three to four mice per group in three independent experiments. ns, not significant. $^{\#}p > 0.1$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

were treated with the FTY720 phosphorylated form, FTY720-P. Neutrophils displayed diminished migration from footpads and blood into poLNs (by 4- and almost 5-fold, respectively), confirming the direct effect of FTY720 on neutrophil S1PRs (Fig. 6B). Loss of S1PRs on cells by pharmacological means could result in decreased cell viability. However, we were unable to detect apoptotic responses in neutrophils after FTY720-P in vitro treatment (Supplemental Fig. 4B). Taken together, these findings further indicate that S1P participates in neutrophil trafficking from tissues through lymph and also from blood into LNs by direct action on S1PRs expressed on neutrophils.

Discussion

Many reports attest to the fact that neutrophils can gain access to peripheral LNs in response to different stimuli. In these lymphoid organs, neutrophils appear to exert an immunoregulatory role, interacting with DCs to modulate Ag presentation. Although neutrophil migration to tissues has been well studied, mechanisms mediating recruitment to LNs remain elusive. In this work, to our knowledge, we report the first study to identify the adhesion molecules and receptors that mediate neutrophil lymphatic and blood migration to dLNs in vivo. In this article, we have provided evidence that neutrophils entered inflamed poLNs via both lymphatic and blood routes, and the induction of this influx could be driven by OVA/anti-OVA IC stimulation. Importantly, we have demonstrated that in the migratory pattern to LNs, neutrophils used L-selectin and PSGL-1, Mac-1 and LFA-1 integrins, and the chemokine receptor CXCR4 to get access across HEVs, whereas Mac-1, LFA-1, and CXCR4 were involved in lymphatic trafficking. In addition, the immunosuppressant drug FTY720 was seen to alter neutrophil influx to LNs, probably because of its action on S1PR4 expressed on neutrophils.

A previous report from our group showed that neutrophil influx to LNs of OVA/CFA mice is present only when an Ag-specific humoral response is occurring (12). In this study, we demonstrated that only IC-stimulated neutrophils migrate to dLNs of OVA/CFA mice. However, if we transferred these stimulated cells to nonimmunized mice, neutrophils would not be able to reach LNs. Therefore, we concluded that: 1) previous stimulation with ICs determines neutrophil trafficking toward LNs, and 2) neutrophil migration occurs only under inflammatory conditions. The fact that transferred neutrophils cannot access poLNs in the absence of an inflammatory state matches our previous observations in which neutrophil influx was absent in nonimmunized mice (12). This is well in line with the fact that neutrophils are largely excluded from LNs in steady-state conditions, and that chronic inflammation induced by vigorous immunization causes LN hypertrophy and enhancement of leukocyte trafficking (30). It was demonstrated that CFA immunization provokes the expansion of the lymphatic network within and around LNs, and that during LN activation, HEVs undergo a process of growth and remodeling, thus permitting increased cell mobilization from blood and from the tissue that drains to those LNs (31, 32).

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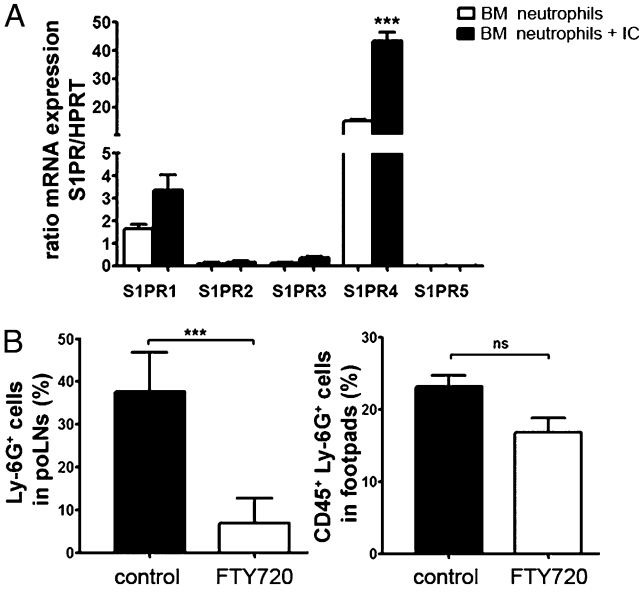

FIGURE 5. FTY720 treatment alters neutrophil influx to LNs. (**A**) Relative S1PR mRNA levels in BM neutrophils before and after in vitro IC stimulation. MACS-sorted Ly-6G$^+$ cells from BM of BALB/c mice were stimulated with ICs (■) or not (□) and 1 h later used to prepare mRNA for quantitative RT-PCR. Each value was normalized to sample's HPRT levels. (**B**) Flow-cytometric analysis of neutrophil migration after saline (control; ■) or FTY720 (1 mg/kg; □) i.p. injection 2 h before OVA challenge. Results are shown as percent of neutrophils into LNs (Ly-6G$^+$ cells) or into footpads (CD45$^+$ Ly-6G$^+$ cells) of OVA/CFA mice 6 h after OVA injection. Data are pooled of three mice per group per experiment in two to three experiments. ns, not significant. ***$p \leq 0.001$.

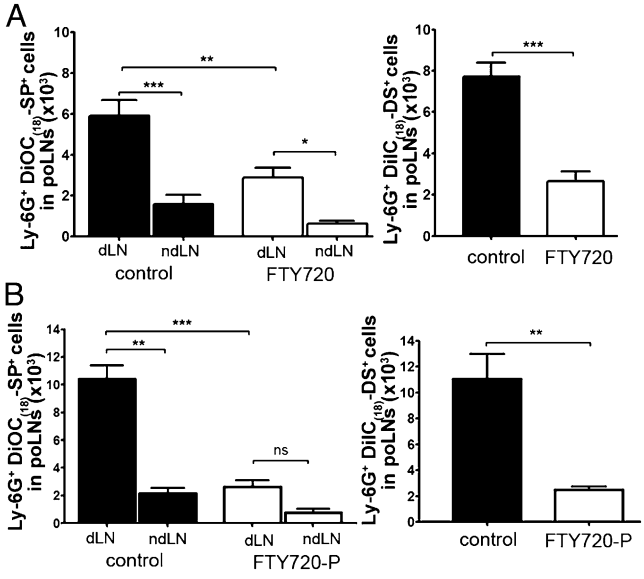

FIGURE 6. FTY720 regulates neutrophil entry to LNs via HEVs and lymph. (**A**) Flow-cytometric analysis of neutrophil homing in FTY720-treated OVA/CFA mice. BM cells were incubated with ICs, stained, and transferred to OVA/CFA mice (control; ■) or OVA/CFA mice that were i.p. injected with 1 mg/kg FTY720 8 h before adoptive transfer (□). (**B**) Homing assay of FTY720-P–treated neutrophils to LNs. MACS-sorted Ly-6G$^+$ cells were incubated with ICs, treated with FTY720-P (1 μg/ml; □) for 30 min at 37°C or not (control; ■), stained, and transferred to OVA/CFA mice. (A and B) Results were expressed as number of transferred neutrophils recovered (Ly-6G$^+$ DiOC$_{(18)}$-SP$^+$ cells) into dLNs and into non-draining LNs (ndLN) 90 min later or as number of neutrophils injected i.v. (Ly-6G$^+$ DiIC$_{(18)}$-DS$^+$ cells) that arrived 90 min later into poLNs. Data from three experiments corresponding to two to three mice per group per experiment are plotted. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. ns, not significant.

It is not clear whether neutrophil arrival via blood or via lymphatics has a consequence on their function at LNs. Several reports showed that LN-infiltrating neutrophils can shape immune responses, especially events related to Ag presentation. It has been shown that neutrophils suppress not only DC activation and Ag uptake/processing, but also migration to LNs by reducing CCR7 expression (33). It has also been demonstrated that neutrophils affected presentation by both DCs and macrophages already having peptide–MHC complexes on LNs (13). Therefore, we hypothesize that neutrophils may regulate the level of Ag available at the inflammatory site, as well as at the cortical sinus of LNs after their lymphatic migration. Regarding blood migration, it was reported that the immunosuppressive effect of neutrophils on LNs was on the DC–T cell interaction. Despite no physical contact with them, neutrophils limit DC activation and consequent T cell immunity. Considering these results, we hypothesize that LN-infiltrating neutrophils from blood may serve as regulators of T cell responses by inhibiting DCs.

Selectins expressed by both leukocytes and endothelial cells regulate leukocyte trafficking into tissues and also control tethering and rolling in HEVs. Naive T cells, which express L-selectin at high levels, are the major population to migrate across HEVs, whereas effector memory T cells and plasmacytoid DCs migrate into LNs across HEVs that express P-selectin under inflammatory conditions. In this study, we demonstrate that neutrophils migrate into LNs from the bloodstream across HEVs using L-selectin and PSGL-1. PSGL-1 is a major selectin ligand on leukocytes and can bind to P-, E- and L-selectin under flow conditions. We observed that anti–PSGL-1 treatment resulted in a greater decrease in neutrophil migration across HEVs than that seen in anti–L-selectin–treated cells. This may be explained by the fact that PSGL-1 can mediate leukocyte–leukocyte interactions and be responsible for L-selectin–dependent leukocyte rolling on inflamed venules (34). In addition, blocking both selectins decreased more relative to the effect observed by each blocking Ab alone, confirming their interaction in neutrophil trafficking. We also observed that neutrophil lymphatic migration was not affected by anti–L-selectin and anti–PSGL-1 treatments (even when both were blocked), demonstrating that these molecules are not involved, or are redundant, in lymphatic trafficking.

The integrins most relevant to leukocyte arrest belong to β1- and β2-integrin subfamilies. It has been demonstrated that LFA-1 is implicated in T cell transendothelial migration from blood into LNs. We showed that not only LFA-1 but also Mac-1 Ab blockade resulted in neutrophil inability to gain access to LNs from bloodstream and lymph. It has been reported that LFA-1 and Mac-1 exert two different molecular mechanisms during in vivo neutrophil recruitment within inflamed blood vessels: whereas LFA-1 mediates neutrophil adhesion, Mac-1 facilitates later crawling (23). Although it is tempting to conclude that this could also happen in lymphatic migration or across HEVs, further studies are needed to directly dissect the role of these molecules in different migratory steps. Contrary to our observations, it was shown that DCs can migrate into lymphatic vessels of normal dermal ear explants in an integrin-independent manner (8). This discrepancy could be explained by differences in cell types and in requirement for adhesion molecules between steady-state and inflammation. It was shown that integrin ligands are induced on lymphatic endothelial cells only under inflammation, and that blockade of both Mac-1 and LFA-1 reduced DC crawling on activated lymphatic endothelium (35, 36).

Under steady-state conditions, the ability to tether and roll within HEVs is not specific to lymphocytes, but is shared by other leukocytes, including neutrophils. However, noninflamed LNs support

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adhesion but not transmigration of neutrophils, because of their lack of specific receptor expression that can bind to those chemokines constitutively expressed by HEVs. We have demonstrated in this article that neutrophils significantly upregulated the expression of CXCR4 after Ag challenge, allowing them to enter LNs. This chemokine receptor has been associated with B cell trafficking across HEVs and with lymphatic migration of cutaneous DCs (3, 37). However, it is well- known that CCR7 has a dominant role in lymphatic and blood migration of most LN-infiltrating leukocytes. A study from Beauvillain et al. (14) showed that CCR7 was involved in neutrophil-lymphatic migration to LNs. Nevertheless, we could not detect a significant expression of CCR7 on neutrophils. This discrepancy could be explained not only by the different experimental procedures, but also by the stimuli used. Whereas in Beauvillain et al.'s report (14), neutrophils reached LNs after priming with GM-CSF and/or IL-17 (14), in our study, neutrophils stimulated with ICs were able to access LNs. A notable aspect that has come to light from both sets of experiments is that neutrophils have to be stimulated to acquire the capacity to reach LNs.

It is becoming increasingly clear that S1P plays an important role in the regulation of leukocyte trafficking, serving as a chemoattractant for immune cells and controlling their migration to LNs. As far as we know, this is the first work to demonstrate that stimulation with ICs significantly upregulated the expression of S1PR S1PR4 on neutrophils. We also showed that FTY720 was an effective drug in altering neutrophil influx to LNs via both lymphatic and blood routes. It has been demonstrated that FTY720 caused leukopenia. Indeed, we observed that FTY720-treated mice displayed neutropenia; however, after Ag challenge, peripheral blood neutrophil counts were similar to those observed in control mice. This apparent neutrophilia could be caused by impairment to access to LNs via the bloodstream, thus provoking neutrophil accumulation in peripheral blood of FTY720-treated mice. In contrast, we did not find abnormalities on neutrophil migration to inflammatory sites, in agreement with a recent report (38), but in contrast with what was previously reported in S1P lyase-null mice (28). In that study, neutrophils displayed a reduced entry from blood into inflamed tissues of S1P lyase-null mice, in which S1P degradation was blocked, because of decreased expression of adhesion molecules on neutrophils (28). Nevertheless, in our experimental model, we did not find altered expression of these molecules after FYT720 treatment.

In conclusion, although the model presented in this article might represent an oversimplification of a more complex network, these results delineate a previously unknown molecular pathway of neutrophil access to dLNs. By advancing the knowledge of neutrophil trafficking, it may be possible to gain the necessary information to provide new strategies for therapy and for better understanding of disease pathogenesis.

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Disclosures

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