Adhesive mechanisms governing interferon-producing cell recruitment into lymph nodes

Thomas G. Diacovo,[1] Amanda L. Blasius,[1] Tak W. Mak,[2,3] Marina Cella,[1] and Marco Colonna[1]

[1]Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110
[2]Advanced Medical Discovery Institute, Ontario Cancer Institute, Department of Medical Biophysics and [3]Department of Immunology, University of Toronto, Toronto, Ontario, M5G 2C1, Canada

Natural interferon-producing cells (IPCs) are found in peripheral lymph nodes (PLNs), where they support NK cell, T cell, and B cell responses to pathogens. However, their route of entry and the adhesive mechanisms used to gain access to PLNs remain poorly defined. We report that IPCs can enter PLNs via a hematogenous route, which involves a multistep adhesive process, and that transmigration is enhanced by inflammation. Results indicate that L-selectin on IPCs is required for efficient attachment and rolling on high endothelial venules in vivo in both nonstimulated and inflamed PLNs. IPCs, however, also possess functional ligands for E-selectin that contribute to this process only in the latter case. In conjunction with selectin-mediated adhesion, both β_1- and β_2-integrins participate in IPC attachment to the inflamed vessel wall, whereas chemotaxis relies in part on the chemokine receptor CCR5. Identification of the adhesive machinery required for IPC trafficking into PLNs may provide opportunities to regulate immune responses reliant on the activity of these cells.

CORRESPONDENCE
Thomas G. Diacovo:
td2142@columbia.edu

Abbreviations used: BCECF, 2′7′,-bis-(2-carboxyethyl)-5(and 6) carboxyfluorescein; HEV, high endothelial venule; ICAM, intercellular adhesion molecule; IPC, IFN-producing cell; IVM, intravital microscopy; LFA, lymphocyte function-associated antigen; *Mtb*, *Mycobacterium tuberculosis*; PLN, peripheral LN; PNAd, peripheral node addressin; PTX, pertussis toxin; RANTES, regulated on activation, normal T cell expressed and secreted; TLR, Toll-like receptor; VCAM, vascular endothelial adhesion molecule.

Natural IFN-producing cells (IPCs), also called plasmacytoid DCs, are a rare subset of cells that are found in both circulating blood and secondary lymphoid organs (1, 2). They are believed to be important in host defense against viral infections because of their ability to detect DNA and RNA viruses through Toll-like receptor (TLR) 9 and TLR7 (3) and their capacity to secrete high levels of type I IFN (4–6), IL-12 (5–7), and proinflammatory chemokines (8–11). Collectively, the cytokine responses of IPCs enhance NK cell and $CD8^+$ T cell responses to viral infections (12, 13) and protect DCs from the cytopathic effect of viruses (5, 10, 12). In addition, IPCs express MHC class II molecules and can present antigens, stimulating T cell proliferation and differentiation in vitro and in vivo (1, 2). IPCs also promote the differentiation of B cells into plasma cells by secreting IFN-α and IL-6 (14). Thus, IPCs may considerably contribute to antiviral responses not only through cytokine production but also by promoting T cell and B cell responses to pathogens.

To support the various innate and adaptive responses to viruses, IPCs must accumulate in secondary lymphoid organs, such as peripheral LNs (PLNs), where they can subsequently interact with NK cells, T cells, and B cells. To do so, it would be anticipated that IPCs must undergo a well-orchestrated series of adhesive interactions with high endothelial venules (HEVs), as shown for T lymphocytes. In regards to the latter, L-selectin initiates this process by facilitating their capture by and subsequent rolling on HEVs through interactions with its endothelial ligand known as peripheral node addressin (PNAd), which is a mixture of glycosylated and sulphated sialomucins that are constitutively expressed on the surface of these HEVs (15–17). As a result, T lymphocytes are brought into close apposition with the vessel wall, which permits the engagement of the secondary adhesion receptors such as the β_2-integrin, lymphocyte function-associated antigen (LFA)–1, that binds to intercellular adhesion molecules (ICAMs) expressed on endothelium (18). This results in the cessation of rolling, as LFA-1 is responsible for mediating the firm attachment of T cells. Typically, this β_2-integrin exists in an inactivated state on circulating lymphocytes. Its ability to interact with ICAMs relies on Gαi-linked intracellular signaling events that result from the engagement of chemokine receptors such as CCR7 (19) with its cognate ligands CCL21 (20, 21) and, possibly, CCL19 (22, 23). This results in alterations in conformation and surface distribution

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of LFA-1 that facilitate binding (24). Subsequent transmigration of T lymphocytes also relies, in part, on the interaction between CCR7 and these chemokines. In contrast, CXCR3 and CCR5 are preferentially expressed on T cell subsets that are recruited to sites of inflammation, and ligands for these receptors are known to be produced in PLNs in response to specific antigenic stimuli (25, 26).

IPCs express several adhesion molecules and chemokine receptors on their surface that could promote interactions with HEVs and support their emigration from the blood into PLNs. For instance, L-selectin is constitutively expressed on these cells and has been indirectly implicated in supporting IPC trafficking into secondary lymphoid organs. This is suggested by the considerable reduction in number of these cells in noninflamed LNs obtained from L-selectin–deficient mice (27). However, the overall cellularity of L-selectin$^{-/-}$ PLNs is dramatically reduced, which may affect chemokine production and, thus, IPC recruitment (28, 29). Moreover, this observation is contradicted in part by in vivo experiments in which antibody blockade of L-selectin function only inhibited mobilization of DC precursors into the circulation of mice and not the trans-HEV migration of these cells (30). In fact, minimal L-selectin–dependent adhesion of IPCs to noninflamed HEVs was reported. In regards to firm adhesion, IPCs do express β_1- and β_2-integrins, but no in vivo information exists regarding the specific integrin receptors that are required for this process. Migration of these cells into PLNs, which has been previously evaluated under inflammatory conditions, is believed to solely involve CXCR3 (30). However, a role for other chemokine receptors known to participate in cell trafficking to sites of inflammation such as CCR5, which is also expressed on IPCs (30), remains to be determined. The multiplicity of chemokine receptors involved in IPC migration is also underscored by a recent study showing that engagement of TLR7 and TLR9 and production of IFN-α induce IPC responsiveness to CCR7 ligands (31).

To establish whether IPC accumulation in PLNs can occur via a hematogenous route and to elucidate the potential

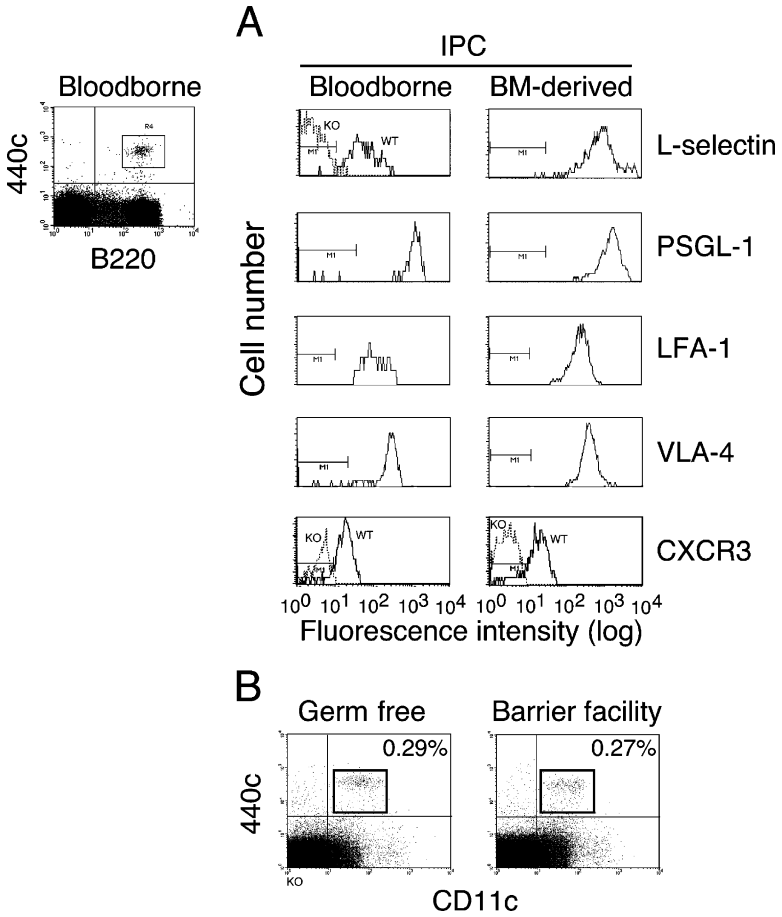

Figure 1. Surface expression of homing molecules on bloodborne- and BM-derived murine IPCs. IPCs were identified in either heparinized whole blood or BM cultured with Flt3 ligand by gating on B220$^+$/440c$^+$ cells. Note that these cells were also CD11c$^+$ and CD11b$^{low/-}$ (not depicted). Both bloodborne and culture-derived IPCs express L-selectin, PSGL-1, LFA-1, VLA-4, and CXCR3. KO controls are also shown for L-selectin and CXCR3 for comparison. (B) Representative dot plot demonstrating the presence of IPCs in PLNs of germ-free mice as compared with those housed solely in a barrier facility. The percentage of cells in each gate is indicated.

adhesive mechanisms involved in this process, we used intravital microscopy (IVM) to study the behavior of murine IPCs in the microvasculature of subiliac LNs in the absence or presence of an inflammatory stimulus. The results of this study provide the first direct evidence that IPCs can emigrate from the blood into PLNs by interacting with HEVs through a coordinated multistep process and delineate important differences from previous studies regarding the molecular requirements for IPC trafficking.

RESULTS

Expression of adhesion molecules on BM-derived and bloodborne IPCs

After developing in the BM, IPCs reach the bloodstream and presumably continue to circulate until constitutive and/or inflammatory signals guide them into secondary lymphoid organs. Given this, bloodborne IPCs would be most appropriate for investigating the molecular mechanisms by which these cells are recruited to LNs. However, as the number of circulating IPCs in the blood are extremely low, representing ~0.1–0.2% of total peripheral blood leukocytes, it is virtually impossible to purify sufficient quantities to directly study their behavior in HEVs of PLNs by IVM. Likewise, isolating IPCs from spleen and LNs is not only hampered by the very small number of such cells in secondary lymphoid organs (32), but expression and/or activation of adhesion molecules may differ among blood, spleen, and LN IPCs. A reasonable alternative to bloodborne IPCs are those derived from mouse BM (BM-IPCs). Substantial quantities of these cells can be grown in vitro from BM precursors with Flt3 ligand (33, 34) and sorted to 90–95% purity. Importantly, these cells appear to function analogously to bloodborne IPCs in regards to type I IFN production and in response to viral challenge (1, 2). One caveat to the use of BM-derived cells in exploring the ability of IPCs to traffic into PLNs is that they must express the identical adhesion molecules and chemokine receptors as their bloodborne counterparts. Thus, we stained IPCs from culture and whole blood with a panel of antibodies specific for adhesion molecules that may participate in trafficking to unperturbed or inflamed PLNs (Fig. 1 A). In regards to adhesion molecules that could potentially promote the attachment and rolling of these cells on HEVs constitutively and/or in response to inflammatory stimuli, both BM-derived and bloodborne IPCs expressed L-selectin and the E- and P-selectin ligand, PSGL-1. Moreover, these two cell populations also expressed the β_1- and β_2-integrin receptors CD49d/CD29 (VLA-4) and CD11a/CD18 (LFA-1), respectively, which are required for firm adhesion. In regards to chemokine receptors, both BM- and blood IPCs are known to express CCR5 and CXCR3, with the latter being implicated in the transmigration of IPCs into PLNs (8, 30). These results, together with previously published reports, demonstrate that BM-IPCs display an array of homing molecules that closely resembles those on their bloodborne counterparts. Moreover, identical adhesion molecules have been identified on human blood IPCs (1, 2), suggesting that the molecular requirements for IPC trafficking to PLNs may be similar in both humans and mice.

L-Selectin mediates attachment and rolling of IPCs on HEVs of PLNs

To establish whether or not IPC accumulation in PLNs can occur via a hematogenous route, we assessed the in vivo behavior of purified murine BM-IPCs stained in vitro with the intracellular fluorophor 2′7′,-bis-(2-carboxyethyl)-5(and 6) carboxyfluorescein (BCECF). Labeled IPCs were injected retrograde through the contralateral femoral artery of mice and observed by epifluorescent IVM during their passage through the microcirculation of noninflamed subiliac LNs. Indeed, IPCs were capable of attaching to and rolling on the luminal surface of HEVs (~60%) in a manner previously reported for T lymphocytes (Fig. 2 A) (18). Moreover, BM-IPCs used L-selectin in supporting this interaction, as a substantial percentage of cells derived from the BM of animals deficient in this adhesion molecule failed to attach and roll under identical conditions (>90%). Purified cells activated

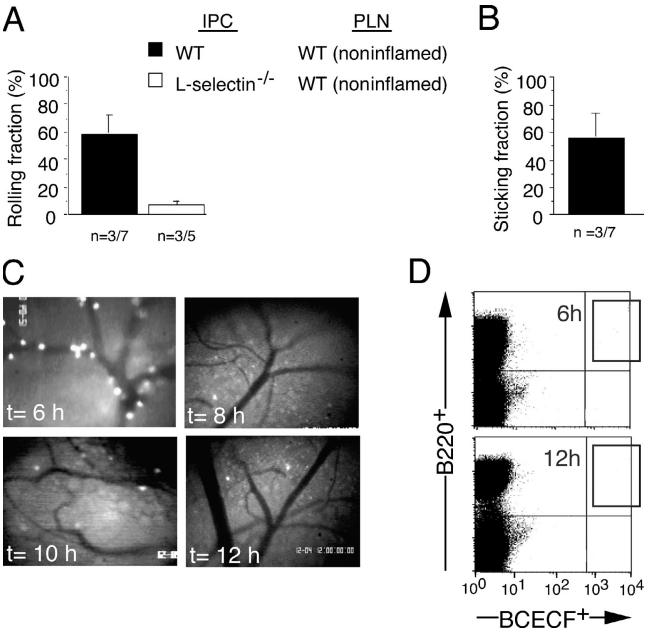

Figure 2. IPC roll and stick but minimally transmigrate across HEVs in noninflamed PLNs. BCECF-labeled IPCs, generated from the BM of WT or L-selectin$^{-/-}$ mice, were injected into the microcirculation of a noninflamed PLN, and the (A) percent rolling fraction and (B) percent sticking fraction were determined by fluorescence microscopy (20×) in identical fields of view. *n* = number of mice per venules analyzed. Data are shown as mean ± SE. (C) Representative micrographs of HEVs in PLNs at 6, 8, 10, or 12 h after injection of labeled IPCs (10×). The skin overlying the subiliac LN was initially left intact until the designated time of viewing. Data are representative of three individual experiments. (D) Representative dot plot to evaluate the accumulation of BCECF-labeled WT IPCs in two noninflamed subiliac PLNs at 6 or 12 h after injection, as determined by flow cytometry (2 vs. 1 BCECF-labeled cells/10^5 total events, respectively).

with CpG oligonucleotides 2 h before administration also did not notably interact with HEVs, which was consistent with their down-regulation of L-selectin expression (unpublished data). We conclude from these observations that circulating BM-derived IPCs can be recruited to HEVs of noninflamed PLNs through an adhesive event requiring L-selectin, as described for naive T cells. In regards to their bloodborne counterparts that express lower levels of this adhesion molecule, it is conceivable that such interactions may be curtailed. However, this may not prevent the constitutive trafficking of endogenous IPCs into PLNs, as these cells can also be found in PLNs of germ-free mice (Fig. 1 B). Thus, in the absence of any external inflammatory stimuli that could alter adhesion molecule expression on IPCs or HEVs, L-selectin would be the primary adhesion receptor responsible for the attachment and rolling of these cells on this specialized vascular endothelium. This is consistent with a previous report that, under noninflamed conditions, PLNs in mice lacking L-selectin have reduced numbers of IPCs (27). Moreover, we have found that the ratio of IPCs to myeloid DCs in the LNs of these animals to be reduced by ~50% (unpublished data).

IPCs firmly adhere to noninflamed HEVs but undergo limited transendothelial cell migration

In addition to selectin-mediated attachment and rolling, circulating hematogenous cells such as lymphocytes must be able to stably interact with HEVs, a prerequisite to chemoattractant-induced migration. To determine if IPCs are capable of firmly adhering to this specialized endothelium, we measured the fraction of rolling cells that subsequently became stationary on HEVs for 30 s. ~50% of all rolling IPCs eventually stuck to HEVs, with the remainder exhibiting transient arrest and release (Fig. 2 B). To test whether IPCs that became stably adherent to the endothelium eventually migrate into the lymphoid tissue underlying HEVs, we followed IPC extravasation by IVM over a period of 12 h. WT animals were injected with BCECF-labeled cells via the femoral artery, and the nonmanipulated subiliac PLNs were exposed and viewed by IVM only at 6-, 8-, 10-, or 12-h time points. This was done to prevent possible alterations in IPC trafficking caused by the effect of prior tissue manipulation, which may impair the flow of blood and lymphatic fluid. Although cells were still firmly attached to HEVs at 6 h, no substantial amount of transmigration was observed at later time points (Fig. 2 C). In fact, continuous viewing of IPC behavior in surgically exposed HEVs of PLNs from 10 min to 6 h after infusion revealed that the majority of adherent cells eventually detached from HEVs and returned back into the circulation (unpublished data). Moreover, retrograde injection of BCECF-labeled cells into the femoral arteries of mice and subsequent harvesting of two unperturbed subinguinal PLNs at 6 and 12 h yielded little evidence of IPC accumulation, as demonstrated by flow cytometry (Fig. 2 D). Thus, unlike the constitutive trafficking of lymphocytes, IPC migration into noninflamed LNs appears to be limited under steady-state conditions in our intravital system, which is reflective of their overall small numbers in noninflamed PLNs (<0.1–0.5% of all cells). This is also consistent with the relatively slow turnover of these cells in noninflamed LNs draining cutaneous tissues as compared with T lymphocytes (35).

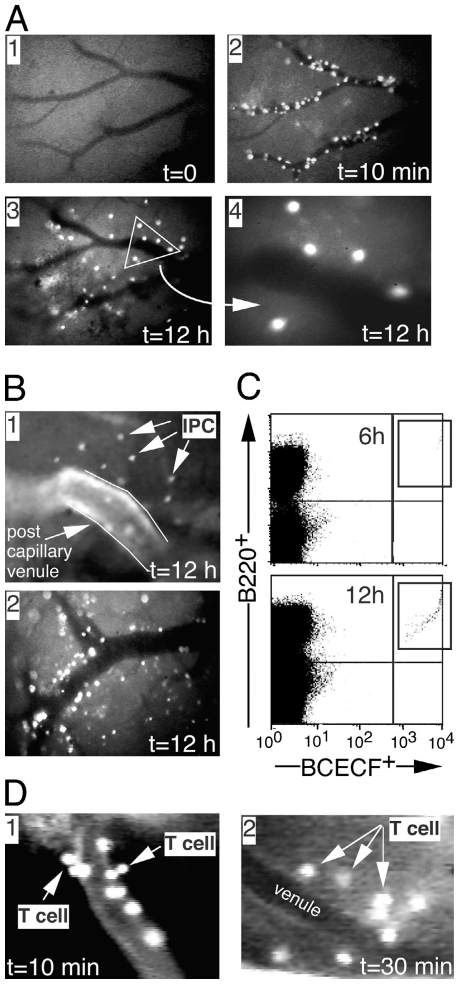

Figure 3. IPC transmigration into PLNs is enhanced by inflammation. Representative micrographs depicting (A) HEVs alone (panel 1, 10×), HEVs 10 min after injection of BCECF-labeled WT IPCs (panel 2, 10×), HEVs 12 h after injection of labeled cells (panel 3, 10×), and an enlarged section of PLNs (panel 4, 20×). (B) Representative micrographs of an inflamed PLN after i.v. injection of 150 kD FITC–dextran and 12h after injection of BCECF-labeled WT IPCs (panel 1, 40×). Lines demarcate lateral borders of the lumenal compartment. A micrograph demonstrating IPC transmigration at 12 h without prior surgical intervention is shown for comparison (panel 2). PLN inflammation was induced by injection of *Mtb* as described in Materials and methods. (C) Representative dot blot to evaluate the accumulation of BCECF-labeled WT IPCs in inflamed subiliac PLNs at 6 or 12 h after injection, as determined by flow cytometry (2 vs. 13 BCECF-labeled cells/10^5 total events, respectively). (D) Representative micrographs of an inflamed PLN after i.v. administration of 150 kD FITC–dextran at 10 and 30 min after injection of BCECF-labeled T cells (panels 1 and 2, respectively; 40×).

IPCs transmigrate into inflamed PLNs

Although IPCs did not show remarkable transendothelial migration under noninflamed conditions in our system, they clearly are capable of emigrating from the blood into inflamed PLNs. In fact, it has been well established that these cells are abundant in inflamed LNs of patients with particular infections or autoimmune diseases, as well as in draining LNs found at sites of inflammation in mice (30, 36, 37). Moreover, we have previously shown that repeated s.c. injections of heat-killed *Mycobacterium tuberculosis* (*Mtb*) into the mouse hind legs over a 72-h period of time leads to considerable recruitment of IPCs in the draining LNs as compared with the control, although the route of entry of these cells remained elusive (37). Therefore, we used this model to determine whether IPC recruitment into PLNs via a hematogenous route is enhanced by inflammation. Labeled IPCs were infused 24 h after the last of two injections of *Mtb,* and their adhesive behavior was followed by IVM during the initial injection and for the subsequent 24 h. Direct observation of fluorescently labeled cells in HEVs of *Mtb*-treated mice revealed that firmly adherent cells (Fig. 3 A, panel 2) did transmigrate into PLNs, an event that occurred maximally at 12 h (panels 3 and 4). This was evident upon the i.v. administration of FITC–dextran that demarcated the boundaries of the HEV lumen (Fig. 3 B, 1). Moreover, transmigration under these conditions did not appear to be influenced by prior surgical manipulation of PLNs, as this process was still evident upon viewing of unperturbed LNs 12 h after the infusion of IPCs (Fig. 3 B, panel 2). Similarly, retrograde injection of BCECF-labeled cells into the femoral arteries of mice and the subsequent harvesting of inflamed subinguinal PLNs at 6 and 12 h demonstrated considerable IPC accumulation only at the latter time point (sixfold increase), as demonstrated by flow cytometry (Fig. 3 C). This delay in IPC migration did not appear to be caused by surgical manipulation of the PLNs, as migration of purified T cells was observed to occur within 10 min after injection (Fig. 3 D).

L- and E-selectin are required for IPC rolling on inflamed HEVs

After establishing that inflammation enhances the accumulation of IPCs in PLNs, we next evaluated whether the attachment and rolling of these cells on HEV in *Mtb*-treated mice relied solely on L-selectin. ~50% of circulating BM-IPCs attached and rolled on HEVs contained within the microcirculation of inflamed LNs (Fig. 4 A), paralleling their behavior under noninflammatory conditions. In contrast, a deficiency of L-selectin resulted in only a partial reduction in the rolling fraction of IPCs (~50%) without affecting firm adhesion, suggesting that other adhesive interactions may contribute to this process during inflammation. A potential candidate includes E-selectin, which is known to be up-regulated on the surface of HEVs in response to inflammatory stimuli (38). However, it is not known whether selectin ligands expressed on IPCs, such as PSGL-1, are functional and can thus contribute to their recruitment to inflamed PLNs. As we established that our BM-derived IPCs do express high levels of PSGL-1 (Fig. 1), it was also necessary to determine if they can interact in flow with purified selectin molecules. Indeed, these cells can bind to surface-immobilized E- or P-selectin under hydrodynamic conditions in vitro (Fig. 4 B). Consistent with these findings was a >90% reduction in the attachment and rolling of L-selectin$^{-/-}$ IPCs in HEVs of mice deficient in E-selectin as compared with WT control cells and PLNs (rolling fraction of 4.2 ± 3.4% vs. 48.7 ± 14.4%, respectively; Fig. 4 A). Moreover, these cells were not capable of becoming firmly adherent to HEVs and, thus, could not undergo transmigration (unpublished data). In contrast, the absence of E-selectin alone did not significantly ($P < 0.05$) impair this process (rolling fraction of 44.7 ± 9.5%), demonstrating the importance of L-selectin–dependent interactions. Of note, CpG-activated IPCs, which down-regulate L-selectin, are also capable of interacting with and transmigrating across HEVs of inflamed PLNs (unpublished data). Thus, the contribution of L-selectin is not absolute in regards to IPC recruitment under these conditions. We conclude that IPC attachment and rolling under these inflammatory conditions rely on interactions

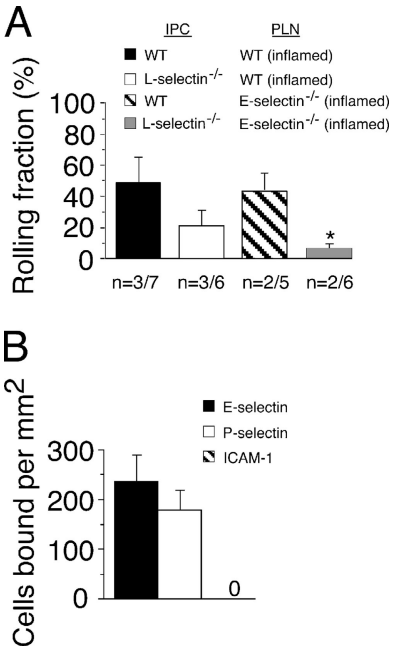

Figure 4. L- and E-selectin are required for IPC attachment and rolling on HEVs of inflamed PLNs. BCECF-labeled IPCs generated from the BM of WT or L-selectin$^{-/-}$ mice were injected through the femoral artery of WT or E-selectin$^{-/-}$ animals, and the rolling fraction was quantified as they passed through HEVs in inflamed PLNs. Data are shown as mean ± SE. *, $P < 0.05$; n = number of mice per venules analyzed. (B) Attachment of WT IPCs to surface-immobilized E- or P-selectin Ig chimeras at a wall shear rate of 200 s^{-1}. Adhesion to the β_2-integrin ligand ICAM-1–Ig chimera is shown as a negative control. Results represent the mean ± SE for three experiments performed in duplicate.

mediated by L-selectin and glycoproteins on IPCs, with their cognate ligands PNAd and E-selectin on HEVs.

Firm adherence of IPCs to inflamed PLNs/HEVs involves β_1- and β_2-integrins

It has been established that firm adhesion of lymphocytes to HEVs is mediated by the β_2-integrin LFA-1 (CD11a/CD18) (18). As IPCs also express this adhesion molecule (Fig. 1), it is reasonable to assume that LFA-1 would serve an identical function in supporting the firm adhesion of these cells to inflamed HEVs. In contrast to T lymphocytes, firm adhesion of IPCs did not appear to require this adhesion molecule, as BM cells derived from animals deficient in this β_2-integrin stuck to inflamed HEVs at levels comparable with those of WT control cells (48.8 ± 12.4% vs. 53.1 ± 16.8%, respectively; Fig. 5 A). Among potential secondary adhesion receptors that may also participate in firm adherence of IPCs to inflamed HEVs, the β_1-integrin VLA-4 is a likely candidate, as it is expressed on this subset of DCs (Fig. 1). Moreover, the counterligand for the former, vascular endothelial adhesion molecule (VCAM)–1, is up-regulated on HEVs during inflammation (39). Despite the i.v. administration of a function-blocking antibody to mouse VCAM-1, however, rolling IPCs continued to undergo firm adhesion at levels comparable with both WT and LFA-$1^{-/-}$ cells (43 ± 4.5%; Fig. 5 A). In contrast, LFA-1–deficient IPCs injected into WT animals pretreated with the VCAM-1 antibody were unable to firmly adhere to inflamed HEVs. However, IPC attachment and rolling was unaffected under these conditions (Fig. 5 B). Thus, both β_1- and β_2-integrins contribute to the stable attachment of IPCs to HEVs in inflamed PLNs.

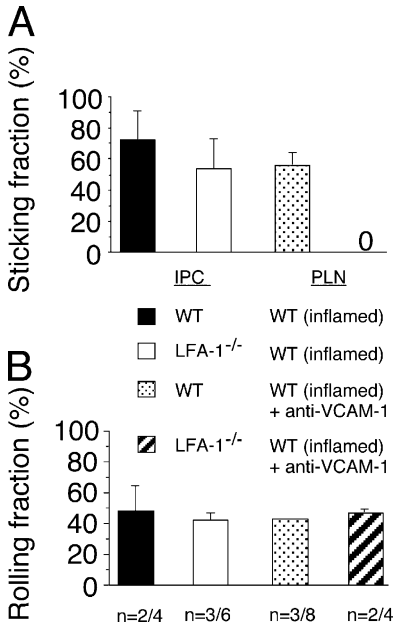

Figure 5. β_1- and β_2-integrins on IPCs participate in firm adhesion. The percent sticking fraction of WT or LFA-$1^{-/-}$ IPCs in HEVs of inflamed PLNs in the absence or presence of the VCAM-1 function blocking F(ab′)$_2$ M/K-2.7 was evaluated during their initial passage through the microcirculation. Data are shown as mean ± SE. *n* = number of mice per venules analyzed. (B) The percent rolling fraction of IPCs under the identical conditions.

The role of CCR5 versus CXCR3 in IPC transmigration

IPCs are known to express multiple chemokine receptors, including CCR5 and CXCR3 (30). Moreover, it has been shown that ligands for these receptors (i.e., CCL3, CCL4, CCL5, and CXCL9) are produced in the inflamed LNs of humans and/or mice (25, 26, 30). In fact, it has been suggested that CXCR3 is the major chemokine receptor that supports IPC transmigration into the inflamed PLNs of mice (30). To confirm this finding and elucidate the role of other chemokine receptors in this process, we studied the interaction of cells deficient either in CXCR3 (40) or CCR5 (41) with HEVs of inflamed PLNs. In contrast to this previous study (30), CXCR3$^{-/-}$ IPCs were able to transmigrate across HEVs, with the results consistent in four individual experiments (Fig. 6 A, panels 1–4). The absence of CCR5 on purified cells, however, did impede their ability to transmigrate under identical conditions. IPCs either remained within the lumen of HEVs (Fig. 6 B, panels 1 and 2) or were not present at all (panels 3 and 4). Confirmation that IPCs do respond to ligands for CCR5 is demonstrated by their ability to transmigrate across a bare filter insert in response MIP-1α and regulated on activation, normal T cell expressed and secreted (RANTES; Fig. 6 C).

To further demonstrate that IPCs do indeed use a G protein–coupled receptor to undergo transmigration, we evaluated the ability of pertussis toxin (PTX), an inhibitor of Gαi-linked signal transduction pathways (42), to prevent extravasation of these cells into PLNs. Indeed, PTX treatment of IPCs did prevent their transmigration into inflamed LNs (Fig. 6 D, panel 2), supporting a role for this signal transduction pathway in this process. Further evidence is provided by the ability of this toxin to abrogate IPC chemotaxis in vitro (Fig. 6 D).

DISCUSSION

Our findings provide the first direct demonstration that IPCs are recruited from the blood into PLNs through a multistep process that involves rolling, firm adhesion, and transmigration across HEVs, the latter event being enhanced by inflammation. This observation is paramount to deciphering our previous results using the IPC-specific mAb 440c, which enabled us to detect by immunohistology an increase in IPC numbers in PLN draining sites of inflammation (37). As these cells possess the adhesion molecules and chemokine receptors necessary to support trafficking into PLNs, it is highly probable that the enhanced localization of IPCs in these secondary lymphoid organs is caused by their recruitment from the circulation rather than from proliferation of resident cells in response to inflammatory stimuli. Thus, it appears that a multi-

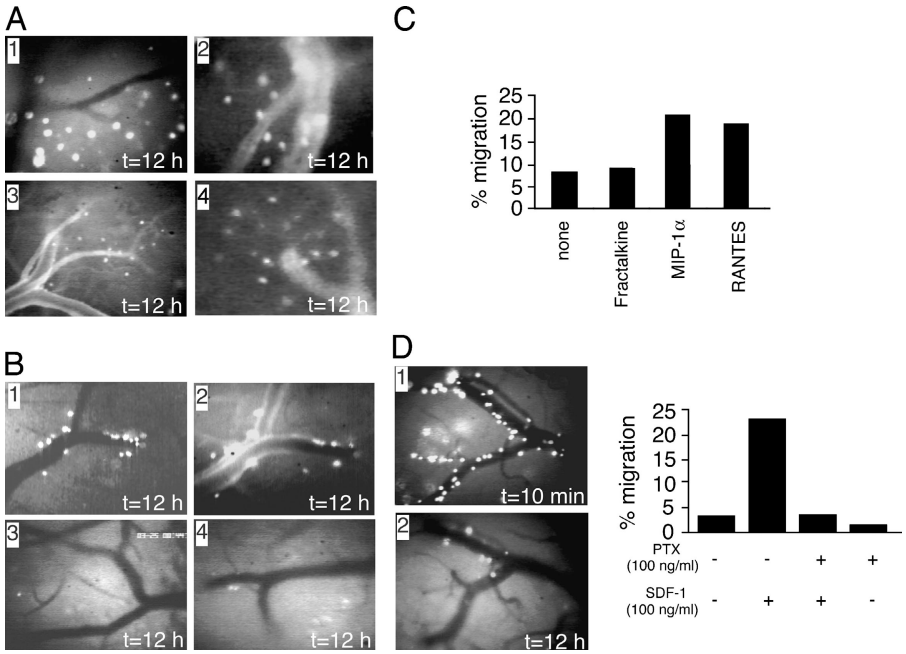

Figure 6. IPC transmigration across HEVs in inflamed PLNs is CCR5-dependent. (A) Representative micrographs of HEVs in inflamed PLNs 12 h after injection of CXCR3$^{-/-}$ IPCs in the absence of (panel 1, 10×) or after i.v. administration of FITC-dextran to delineate the vascular compartment (panel 2, 10×; panels 3 and 4, 20×). All panels are from individual animals ($n = 4$). (B) Representative micrographs of HEVs in inflamed PLNs 12 h after injection of CCR5$^{-/-}$ IPCs in the absence of (panels 1, 3, and 4; 10×) or after i.v. administration of (panel 2, 10×) FITC-dextran to delineate the vascular compartment. All panels are from individual animals ($n = 3$). (C) Migration of BM-derived IPCs across bare transwell filters in response to fractalkine, MIP-1α, or RANTES. (D) Representative micrographs depicting the same HEV either 10 min (panel 1, 10×) or 12 h (panel 2, 10×) after the injection of BCECF-labeled WT IPCs pretreated with 100 ng/ml PTX. The effect of 100 ng/ml PTX on SDF-1–induced chemotaxis in an in vitro transwell assay system is shown for comparison.

step adhesive process akin to that of T lymphocytes is key to regulating the localization of IPCs in PLNs.

Importantly, the use of IVM, rather than immunohistology, has enabled us to identify the adhesion molecules that contribute to IPC trafficking into PLNs in flowing blood, of which E-selectin and VCAM-1 are only expressed on HEVs in response to inflammatory stimuli. This is in contrast to the constitutive expression of the L-selectin ligand known as PNAd. Thus, these cells are well suited for both constitutive and inflammation-directed homing to PLNs, as IPCs express both L-selectin and functional ligands for E-selectin on their surface (i.e., PSGL-1). Similarly, they bear the appropriate set of integrin receptors (i.e., LFA-1 and VLA-4) required for entry into these secondary lymphoid organs under the specified conditions. Although evidence exists suggesting that IPCs may constitutively home to LNs, as is the case for naive T lymphocytes, the more robust accumulation of these cells in response to an inflammatory stimulus is also consistent with their ability to regulate an adaptive immune response under such conditions. This would explain why IPCs are most abundant in the T cell area of inflamed secondary lymphoid organs in the human and mouse, particularly around HEVs (30, 36, 37). Moreover, the requirement for CCR5 to promote IPC migration into inflamed PLNs is consistent with the fact that ligands for this chemokine receptor (i.e., CCL3, CCL4, and CCL5) are only expressed under such conditions. In fact, these chemokines are known to contribute to the trafficking of T cell subsets into LNs during an immune response (25). That said, the presence of IPCs in noninflamed PLNs of normal and germ-free mice, albeit <0.1–0.5% of the total population of cells, suggests that constitutive trafficking from the blood into LNs can occur. Although in our study CXCR3 was not required for IPC migration in response to *Mtb*, it is possible that in different inflammatory conditions, such as those reported by Yoneyama et al. (30), CXCR3 and CCR7 may play an important role because of the induction of CXCR3 ligands and up-regulation of CCR7 (31). This would be consistent with a previous study demonstrating that the particular chemokine produced in inflamed LNs affected the prevailing disease state (26). For instance, CXCR3 ligands were shown to drive IPC migration into all LNs after i.v. injection of *Propioni bacterium acnes*, which can induce systemic inflammation as well as up-regulation of CCR7 on this cell type (30). Moreover, CXCR3 ligands have been shown to promote IPC migration into LNs draining a cutaneous HSV-1 infection (30). Similarly, murine CMV infection and systemic injection of TLR7 and TLR9 ligands induced migration and clustering of splenic plasmacytoid DCs in the spleen marginal zone. This migration was dependent on CXCR3

ligands induced by type I IFNs during inflammation. In this system, type I IFN was also required for up-regulation of CCR7 on IPCs, which allows them to migrate in response to CCR7 ligands (31).

It is reasonable to speculate that circulating IPCs can use the same adhesion and chemokine receptors as described here to accumulate in inflamed tissues other than PLNs. For instance, IPCs are not normally found in skin, but readily accumulate in this organ system during inflammation as a result of autoimmune diseases such as systemic lupus erythematosus (43). Moreover, E-selectin and VCAM-1 are coexpressed on vessels contained within dermal lesions associated with this disease state, as well as the chemokine chemerin (44, 45). Most recently, IPCs have been shown to undergo chemotaxis in response to chemerin (46), a novel protein identified as the natural ligand of ChemR23, which is a G protein–coupled receptor (45, 47). Thus, identification of the adhesive pathways involved in IPC recruitment by inflamed tissues such as LNs or skin may provide an opportunity to regulate the trafficking pattern of these cells such that one may modulate the immune response.

MATERIALS AND METHODS

Reagents and mice. Flt3 ligand and *Mtb* were purchased from R&D Systems and Difco Laboratories, respectively. Murine SDF-1, MIP-1α, RANTES, and fractalkine were obtained from PeproTech, and CpG oligonucleotide 2216 was obtained from QIAGEN. Murine E-selectin, human P-selectin, or human ICAM-1 expressed as Fc chimeric proteins were obtained from R&D Systems, Genetics Institute, or ICOS Corp., respectively. PTX was purchased from Sigma-Aldrich. L-selectin$^{-/-}$ mice were obtained from M. Siegelman (University of Texas Southwestern Medical Center, Dallas, TX), CXCR3$^{-/-}$ mice were provided by C. Gerard (Harvard Medical School, Boston, MA), and germ-free mice were obtained from J. Gordon (Washington University School of Medicine, St. Louis, MO).

Antibodies. The following mAbs to mouse proteins were purchased from BD Biosciences: CD62L, CD162 (PSGL-1), CD29, CD49d (VLA-4), CD11b (Mac-1), CD11c (p150,95), and B220. Function-blocking anti–mouse VCAM-1 (M/K-2.7) antibody was purchased from the American Type Culture Collection. mAb 440c and anti-CXCR3 antibody (provided by R.D. Schreiber, Washington University School of Medicine, St. Louis, MO) have been previously described (8, 37, 45, 47).

Preparation of cells. Murine BM cells from WT C57BL/6, L-selectin$^{-/-}$, CXCR3$^{-/-}$, CCR5$^{-/-}$, or LFA-1$^{-/-}$ mice were cultured in 10 ng/ml Flt3 ligand at 2–4 × 10^6 cells/ml for 9–10 d (8, 34). IPCs were isolated by magnetic cell sorting (MACS; Miltenyi Biotec) by depletion of CD19$^+$ cells followed by enrichment of B220$^+$ cells. Purity, as measured by flow cytometry, was >95% B220$^+$, CD19$^-$, CD11c$^+$, CD11b$^{low/-}$, and 440c$^+$. Cells were stimulated with 6 μg/ml CpG oligonucleotide 2216 for 2 h where indicated in the figures. T cells were purified from PLNs of mice by negative selection using magnetic cell sorting for B220$^+$ cells. Flow-through material was >90% for CD3$^+$.

Flow cytometry. For evaluating adhesion molecule expression on BM- and blood IPCs, we performed three-color analysis using (a) allophycocyanin- or PE-conjugated 440c; (b) FITC–B220 or PE–B220; or (c) PE–CD62L, PE–CD162, biotinylated LFA-1, biotinylated CD29, biotinylated CD49d, or FITC–CXCR3. For determination of IPC trafficking into PLNs, inflamed and noninflamed subinguinal LNs were harvested bilaterally, digested with collagenase D for 1 h at 37°C, and stained with 440c–biotin, B220–PE, and propidium iodide (PI). The entire cell suspension was analyzed by flow cytometry. A gate was set on live cells (PI negative). All samples were analyzed on a flow cytometer (FACSCalibur; BD Biosciences).

Chemotaxis assay. Chemotaxis was measured in a 2-h transwell migration assay using 24-well chambers with 5-μm pores (Costar Transwell; Corning Costar). 100 ng/ml recombinant SDF-1, 10 ng/ml MIP-1α, 100 ng/ml RANTES, or 100 ng/ml fractalkine was added to the lower wells in 600 μl of chemotaxis medium (RPMI 1640 with 1% human serum albumin), and 2 × 10^5 cells in 100 μl were added to the Transwell insert. Migrated cells were counted by flow cytometry. Where indicated in the figures, cells were pretreated with 100 ng/ml PTX for 1 h at 37°C.

Induction of LN inflammation. Mice were injected with nonviable desiccated *Mtb* s.c. in both hind legs (500 μg/injection) at 72 and 24 h before the experiments. All animals were handled in accordance with policies administered by the National Institutes of Health and the Washington University Institutional Animal Care and Use Committee.

Subiliac LN preparation and IVM. The left subiliac LN of anesthetized mice was prepared for IVM as previously described (48). A polyethylene catheter inserted in the right femoral artery was used for injection of cell suspensions. IPCs were fluorescently labeled with BCECF (Molecular Probes, Inc.) and visualized in the microcirculation of the node through a 10, 20, or 40× water immersion objective (Carl Zeiss MicroImaging, Inc.) using an intensified camera (VE1000SIT; Dage-MTI) and epifluorescence illumination (49). Rolling fraction (RF) was defined as the percentage of cells that interact with a given region of venule as compared with the total number of cells that enter that vessel (interacting and noninteracting) during the same 1-min time period. The sticking fraction (SF), the percentage of rolling IPCs that became stationary for a minimum of 30 s, was determined during the same time period and segment of vessel. Some experiments included injection of 10 mg/ml FITC–dextran (150 kD; Sigma-Aldrich) after recordings of cell behavior to delineate the lumen of vessels. For experiments evaluating IPC transmigration, the skin flap was sutured in place, closing the wound, after collecting data on RF and SF, and subsequently reopened after a time period of 6, 8, 10, or 12 h for viewing by epifluorescent IVM. For comparison purposes, fluorescently-labeled IPCs were inject through the femoral catheter, and the skin overlying the subiliac LN was left intact until the designated viewing times. For experiments evaluating the role of β_1-integrins in mediating IPC firm adhesion, a function-blocking F(ab′)$_2$ to 200 μg mouse VCAM-1 was administer i.v. 1 h before the injection of BCECF-labeled IPCs. Video images were recorded using a Hi8 VCR (Sony), and analysis was performed using a PC-based image analysis system as previously described (50).

Laminar flow assays. For flow studies involving recombinant proteins, polystyrene plates were coated overnight with PBS, pH 7.4, containing 100 μg/ml protein A (Sigma-Aldrich) at 4°C, washed, and incubated with E- or P-selectin or ICAM-1–Fc chimeric proteins diluted to a concentration of 20 μg/ml (PBS, 0.1% BSA, pH 7.4) for 2 h at 37°C (50). Nonspecific interactions were blocked with PBS containing 50 μg/ml rabbit Ig for 30 min at 37°C. 10^6 IPCs/ml (HBSS, 10 mM Hepes, 1 mM $CaCl_2$, 0.5% BSA, pH 7.4) were infused over the selectin substrates that had been incorporated into a parallel plate flow chamber (GlycoTech) at a shear rate of 200 s^{-1}. IPC accumulation was recorded on Hi8 videotape using an inverted microscope (TE300; Nikon) with a plan 10× objective. The number of cells that attached over 5 min was determined and expressed per unit area.

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