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Prostaglandin D<sub>2</sub> Regulates CD4<sup>+</sup> Memory T Cell Trafficking across Blood Vascular Endothelium and Primes These Cells for Clearance across Lymphatic Endothelium

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Memory lymphocytes support inflammatory and immune responses. To do this, they enter tissue via blood vascular endothelial cells (BVEC) and leave tissue via lymphatic vascular endothelial cells (LVEC). In this study, we describe a hierarchy of signals, including novel regulatory steps, which direct the sequential migration of human T cells across the blood and the lymphatic EC. Cytokine-stimulated (TNF and IFN) human BVEC preferentially recruited memory T cells from purified PBL. Lymphocyte recruitment from flow could be blocked using a function-neutralizing Ab against CXC<sub>1</sub>R. However, a receptor antagonist directed against the PGD<sub>2</sub> receptor DP2 (formerly chemotactrant receptor-homologous molecule expressed on Th2 cells) inhibited transendothelial migration, demonstrating that the sequential delivery of the chemokine and prostanoid signals was required for efficient lymphocyte recruitment. CD4<sup>+</sup> T cells recruited by BVEC migrated with significantly greater efficiency across a second barrier of human LVEC, an effect reproduced by the addition of exogenous PGD<sub>2</sub> to nonmigrated cells. Migration across BVEC or exogenous PGD<sub>2</sub> modified the function, but not the expression, of CCR7, so that chemotaxis toward CCL21 was significantly enhanced. Thus, chemokines may not regulate all stages of lymphocyte migration during inflammation, and paradigms describing their trafficking may need to account for the role of PGD<sub>2</sub>. The Journal of Immunology, 2011, 187: 000–000.

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ollowing infection, foreign Ag is presented to naive T cells constitutively recirculating through secondary lymphoid organs. They become activated and acquire the effector functions and phenotypic markers associated with Ag-specific memory T cells (reviewed in Refs. 1, 2). A defining aspect of immune memory is the persistence of the response. This is achieved when a fraction of tissue-resident cells leave the inflamed tissue via the lymphatic vasculature and re-enter the peripheral blood during the resolution phase of the inflammatory response. There, in the absence of antigenic stimulation, they patrol the body as long-lived memory T cells, conducting immune surveillance for their cognate Ag (reviewed in Refs. 1, 2).

During inflammation, memory T cells are recruited to tissue after capture by specialized, fast-acting adhesion receptors (i.e., VCAM-1 and E- or P-selectin) on blood vascular endothelial cells (BVEC) of the postcapillary venules. These adhesion molecules are synthesized and presented to the flowing lymphocytes in response to endothelial cell (EC) activation by cytokines such as TNF-α (TNF), IL-1β (IL-1), or IFN-γ (IFN) (3). Initial capture is followed by activation of the lymphocytes by surface-presented chemokine(s). Chemokines induce the rapid activation of the β<sub>1</sub> and β<sub>2</sub> integrin receptors constitutively expressed on the lymphocyte surface. High-affinity interactions between α<sub>1</sub>β<sub>2</sub> integrin (VLA-4) and its EC counterligand, VCAM-1, and between α<sub>4</sub>β<sub>1</sub> integrin and ICAM-1 (4–6) stabilize lymphocyte adhesion to the EC monolayer. There is also a strong presumption that chemokine signals are sufficient to promote lymphocyte migration over and through the endothelial monolayer and into inflamed tissue. In the case of EC treatment with TNF plus IFN, chemokines acting through the lymphocyte-borne receptor CXCR3 have been shown to stabilize attachment, but the signals inducing transendothelial migration have not been defined (7, 8). Transendothelial migration of T cells has been observed within minutes of adhesion to EC activated with TNF (where blockade of β<sub>2</sub> integrins was inhibitory) (4), with TNF plus IFN (7), and for EC stimulated with TNF and to which stromal-derived factor-1α (CXCL12) or CCL19 (ELC) had been added to the EC surface (9). In the last example, the homeostatic chemokines (CXCL12 and CCL19) were not endogenous products of blood vascular endothelium, and it is doubtful whether they contribute to lymphocyte activation in the inflamed vasculature.

In addition to signals that permit admission to tissue, T cell egress from the site of inflammation is necessary for immune surveillance and the efficient regulation and resolution of the immune response. For example, memory T cells with Ag reactivity that is not relevant to the ongoing response must achieve efficient release from the inflamed tissue or the immune system risks deletion of their memory repertoire when the immune infiltrate is cleared during inflammatory resolution (10). In addition, T cell helper functions that support the humoral immune response ordinarily occur in inflammatory lymphoid tissue, as it is here that
PGD₂, and Lymphocyte Traffic During Inflammation

PGD₂; all from Cayman Chemicals), or with the G protein-coupled receptor (GPCR) inhibitor pertussis toxin (100 ng/ml; Sigma-Aldrich) prior to the endothelial monolayer required a PGD₂-mediated signal. Importantly, the passage of the BVEC monolayer and, specifically, exposure to PGD₂ also primed CD4⁺ T cells for transmigration across lymphatic endothelium. Efficient translymphatic migration was CCR7 dependent, and migration across BVEC, or the provision of exogenous synthetic PGD₂, greatly increased the number of lymphocytes migrating across lymphatic vascular EC (LVEC) as well as promoting chemotaxis of lymphocytes toward CCL21.

Materials and Methods

Isolation and culture of human PBL and CD4⁺ memory T cells

Venous blood from healthy individuals was collected in EDTA at a final concentration of 1.6 mg/ml (Sarstedt, Leicester, U.K.). PBMC were isolated by centrifugation of blood on Histopaque 1077 (Sigma-Aldrich, Poole, U.K.), and PBL were prepared by panning of PBMC on culture plastic to obtain 95% purity of mononuclear cells. Isolated cells were washed, counted, and adjusted to a final concentration of 1 × 10⁶ cells/ml in Medium 199 (Life Technologies Invitrogen Compounds, Paisley, U.K.) supplemented with 1% BSA (Sigma-Aldrich) or PBS with albumin (0.15%; PBSA).

In some experiments, lymphocytes were pretreated with 10 nM PGD₂ or agonists against the PGD₂ receptors, DP1 (BW 245C; Cayman Chemicals) or DP2 (BAY- u3405 [Ramatroban]; Cayman Chemicals), or with NBD-phallacidin (Molecular Probes) to measure F-actin. The lymphocytes were allowed to settle, adhere, and migrate through EC and the filter at 37°C in a CO₂ incubator for 24 h. From previous experience, little transmigration was detected within a few hours using filters, and 24 h were needed to obtain a proportion of lymphocytes under the filter comparable to that seen in minutes during direct observations of transendothelial migration (17). Transmigrated cells in the lower chamber were removed and counted using a Coulter Multi-sizer II (Coulter Electronics, Essex, U.K.). From the known number of added lymphocytes, the percentage of lymphocytes that transmigrated was calculated. In some experiments, the surface phenotype of transmigrated lymphocytes was assessed by flow cytometry. Lymphocytes were labeled with Becton Dickinson (BD) anti-CD4-PE, anti-CD8-FITC (BD Biosciences, Oxford, U.K.), or anti-CD45RA-CY5 (Seoretch, Oxford, U.K.) for 30 min on ice. Fixed-volume counts for positively labeled cells were made using a Coulter XL flow cytometer (Coulter Electronics) and analyzed using WinMDI. In this way, we calculated the percentage of transmigration for CD4⁺ and CD8⁺ memory T cell subsets. Anticoagulated cells were assessed by DiOC6 and 7-aminoactinomycin D and excluded from counts; we observed little if any apoptosis over the course of the assay.

Migration through LVEC on Transwell filters under static conditions. Lymphocyte migration across LVEC was assessed as described above. The assay of lymphocytes across BVEC with the exception that the LVEC were cultured on the underside of the filter so that lymphocytes migrating across this barrier would contact the monolayer from a physiologically relevant direction. The lymphocyte populations used were: 1) PBL or purified CD4⁺CD45RA⁻ T cells collected from the lower chamber of BVEC migration assays; and 2) control PBL or purified CD4⁺CD45RA⁺ T cells that were maintained in culture flasks in BVEC medium for 24 h. The latter represented lymphocyte populations of equivalent age and prior manipulation to cells migrated across BVEC, but had not migrated through BVEC.

Microscopic observation of migration through EC under static conditions. Adhesion and transmigration were assessed by direct microscopic observation as previously described (19). BVEC in six-well plates were washed with PBSA to remove residual cytokines, and purified PBL were added for 5 min. Nonadherent cells were removed from the EC by gentle washing with PBSA, and video recordings of five fields of view of the endothelial surface were made using phase-contrast videomicroscopy so that the number of adherent cells and their position above or below the EC monolayer could be assessed. Manipulations and microscopy were carried out at 37°C. The video recordings were digitized and analyzed offline using Image-Pro Plus software (DataCell, Finchampstead, U.K.). The numbers of adherent cells were counted in the video fields, averaged, converted to cells per mm² using the calibrated microscope field dimensions, and multiplied by the known surface area of the EC monolayer to calculate the total number adherent. This number was divided by the known total number of lymphocytes added to obtain the percentage of the lymphocytes that had adhered. Each lymphocyte was classified as either: 1) phase bright and adherent to the surface of the EC; or 2) phase dark and spread and migrating below the EC. The percentage of adherent lymphocytes that had transmigrated was calculated.

Microscopic observation of migration through EC under conditions of flow. B cell or T cell populations (1 × 10⁶ cells) were placed in culture flasks in BVEC medium for 24 h. At one end, they were connected to a Harvard withdrawal syringe pump and delivered flow equivalent to 1.0 to 1.5 Pa. At the other end, they were connected to an electronic switching valve (Lee Products, Gerards Cross, U.K.), which selected flow from two reservoirs containing PBL in PBSA or cell-free PBSA. A 4-min bolus of PBL

Analysis of lymphocyte migration

Migration through BVEC on Transwell filters under static conditions. Lymphocyte migration was assessed using 24-well format Transwell filters as described (17). BVEC were washed to remove residual cytokines, fresh M199+BSA was placed in the lower chamber, and PBL were added to the upper chamber. In some experiments, lymphocytes were pretreated with Abs at 10 μg/ml (unless otherwise stated) against the integrin subunits CD29 (anti-β₁; clone Mab13 a gift from M. Humphries, University of Manchester, Manchester, U.K.), CD18 (anti-β₂; clone R6.5E a gift from Dr. Tony Shock; Cell Tech, Slough, U.K.), CD61 (anti-β₃ integrin; clone S2Z1 from Immunotech, Marseille, France), or CD18 activation epitope (anti-β₂; clone KIM127 a gift from Martin Robinson, Cell Tech) (18) against the chemokine receptors CXCR3 (clone 1C6; R&D Systems) or CCR7 (clone 150503; R&D Systems), with antagonists of the PGD₂ receptors DP1 (BW868c; Cayman Chemicals) or DP2 (BAY-u3405 [Ramatroban]; Cayman Chemicals), or with NBD-phallacidin (Molecular Probes) to measure F-actin. The lymphocytes were allowed to settle, adhere, and migrate through EC and the filter at 37°C in a CO₂ incubator for 24 h. From previous experience, little transmigration was detected within a few hours using filters, and 24 h were needed to obtain a proportion of lymphocytes under the filter comparable to that seen in minutes during direct observations of transendothelial migration (17). Transmigrated cells in the lower chamber were removed and counted using a Coulter Multi-sizer II (Coulter Electronics, Essex, U.K.). From the known number of added lymphocytes, the percentage of lymphocytes that transmigrated was calculated. In some experiments, the surface phenotype of transmigrated lymphocytes was assessed by flow cytometry. Lymphocytes were labeled with Becton Dickinson (BD) anti-CD4-PE, anti-CD8-FITC (BD Biosciences, Oxford, U.K.), or anti-CD45RA-CY5 (Seoretch, Oxford, U.K.) for 30 min on ice. Fixed-volume counts for positively labeled cells were made using a Coulter XL flow cytometer (Coulter Electronics) and analyzed using WinMDI. In this way, we calculated the percentage of transmigration for CD4⁺ and CD8⁺ memory T cell subsets. Anticoagulated cells were assessed by DiOC6 and 7-aminoactinomycin D and excluded from counts; we observed little if any apoptosis over the course of the assay.

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was perfused over the HUVEC followed by cell-free wash buffer. Video recordings were made of a series of microscope fields along the centerline of the flow channel after 11 min of washout. Video recordings were analyzed as above, except that lymphocytes adherent to the HUVEC could be classified as rolling adherent (spherical cells moving over the surface much slower than free-flowing cells), stationary adherent (typically with distorted shape and actually migrating slowly on the surface), or phase-dark transmigrated cells. The sum of the numbers adherent in all categories was divided by the number perfused during the bolus to obtain total PBL adhesion as percent of cells perfused.

**Lymphocyte chemotaxis toward CCL21**

Lymphocyte chemotaxis was assayed in 96-well MultiScreen plates with 5-μm pores (Millipore). Freshly isolated PBL, PBL that had migrated through BVEC or PBL pretreated with exogenous PGD₂, were allowed to migrate across uncoated filters toward CCL21 (80 ng/ml added to lower well; R&D Systems) for 4 h to allow sufficient time for lymphocytes to cross the filter. Transmigrated cells in the lower chamber were removed and counted using a Coulter MultiSizer II (Coulter Electronics). From the known number of added lymphocytes, the percentage of lymphocytes that migrated across the filter was calculated.

**mRNA quantification by PCR**

Trypsin/EDTA was used to detach HUVEC from the inside of filters or LVEC from the underside of the filters, and mRNA was isolated from the cells using the RNeasy Mini Kit (Qiagen, Crawley, U.K.). CCL19 FAM-labeled and 18S VIC-labeled primers were bought as Assay on Demand kits from Applied Biosystems (Warrington, U.K.) to analyze mRNA. mRNA were analyzed by quantitative real-time PCR using the QuantiTect probe RT-PCR kit according to the manufacturer’s instructions (Qiagen). Samples were amplified using the 7900HT Real-Time PCR machine (Applied Biosystems) and analyzed using the software package SDS 2.2 (Applied Biosystems). Data were expressed as relative expression units relative to 18S.

**Statistical analysis**

Effects of multiple treatments were tested using ANOVA, followed by post hoc comparison with control by Dunnett test. Single treatments were compared with controls by paired t test.

**Results**

**Vascular EC stimulated with TNF and IFN preferentially recruit memory T cells**

When unfractionated PBL were added to unstimulated BVEC cultured in Transwell with 3-μm pores, ~40% became adherent, and 10% of all lymphocytes added migrated across the endothelial monolayer into the bottom chamber of the assay (Fig. 1A, 1B). Activation of the EC with TNF, IFN, or a combination of these agents did not lead to a significant increase in lymphocyte adhesion, although the combination of TNF and IFN did increase the efficiency of migration significantly (Fig. 1A, 1B). CD4 and CD8 memory (CD45RA⁻) T cells preferentially migrated through EC stimulated with TNF and IFN compared with naive cells of the same types (Fig. 1C), in agreement with previous data from static (17) and flow assays (9). Memory cells constituted ~50% and 20% of the CD4 and CD8 populations in peripheral blood, respectively. Both CD4 and CD8 memory populations were enriched after migration so that they represented ~90% and 50% of the migrated CD4 and CD8 populations, respectively (Fig. 1C).

**A CXCR3-mediated signal immobilizes lymphocytes on cytokine-stimulated vascular EC, but signaling through the PGD₂ receptor DP-2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) is essential for efficient transmigration of recruited lymphocytes**

In agreement with previous observations (7, 8), we found that a function-blocking Ab against CXCR3 nearly abolished the adhesion of flowing PBL to TNF- and IFN-stimulated EC (Fig. 2A). These data implied that a CXCR3-mediated signal was required for stable adhesion to EC, but could not reveal whether it was necessary to promote passage across the EC barrier. Previously, we found that chemokine-activated neutrophils required a signal delivered through the PGD₂ receptor DP-1 to efficiently migrate across EC (14). In this study, inclusion of antagonists of the PGD₂ receptors DP-1 or DP-2 in a Transwell migration assay demonstrated that the latter but not the former could effectively ablate the migration of lymphocytes (Fig. 2B). We also tested the efficacy of the DP-2 receptor antagonist in a flow-based assay. On cytokine-stimulated EC, lymphocytes were recruited, activated, and migrated on the surface and underneath the monolayer after diapedesis (Supplemental Video 1). DP-2 receptor antagonist did not reduce the number adhering (data not shown) but was able to inhibit >50% of lymphocyte migration across the monolayer (Fig. 2C–E, Supplemental Video 2). Of those cells that migrated, movement across the endothelium occurred in a paracellular and not transcellular manner. The adherent lymphocytes recruited from flow did not roll, implying that they received the CXCR3-mediated chemokine signal necessary for immobilization, but lacked the prostanoi signal required for transit of the monolayer.

**Transit of blood vascular EC primes lymphocytes for migration across lymphatic vascular EC**

PBL were allowed to migrate across TNF- and IFN-stimulated BVEC for 24 h and collected from the lower chamber of the assay. These cells (predominantly CD4 and CD8 memory T cells)
were then placed on LVEC that were unstimulated or activated with TNF and IFN. As a control, freshly isolated PBL were also cultured in dishes for the same period prior to adhesion assay on LVEC. The cultured lymphocytes could adhere to LVEC, and a small proportion migrated across the monolayer (Fig. 3A). Stimulation of the LVEC with cytokines did not increase the efficiency of migration of these cells (Fig. 3A). However, when lymphocytes had previously migrated across BVEC, the efficiency of migration across LVEC was significantly increased (Fig. 3A). Importantly, passage of lymphocytes across cytokine-stimulated BVEC did not increase the efficiency of migration across a second monolayer of BVEC (Fig. 3B), implying that the priming process modified lymphocyte responses that were specific to the transit of LVEC.

As PBL that migrate across cytokine-stimulated BVEC are highly enriched for CD4 memory T cells, it was possible that the enhanced capacity to migrate across LVEC was an intrinsic property of this subset of cells that was not reliant on a priming signal. To investigate this, we repeated the above experiment using CD4 memory T cells isolated from the mixed lymphocyte populations found in peripheral blood. Fig. 3C shows that cultured CD4 memory T cells were poor at migrating across LVEC. Importantly, CD4 memory T cells that had experienced migration across BVEC again displayed significantly increased migration across LVEC, demonstrating that a priming signal was indeed necessary for efficient T cell migration across LVEC.

**Efficient migration of CD4 memory T cells across lymphatic endothelium is dependent on CCR7 and supported by β1 and β2 integrins**

The migration of CD4+ T cells from skin into draining lymphatics has been shown to depend on the chemokine receptor CCR7 (12). In this study, when we took CD4 memory T cells that had migrated through BVEC and treated them with a function-neutralizing Ab against CCR7, migration through a LVEC monolayer was significantly inhibited (Fig. 4A). As controls, we used a function-neutralizing Ab against CXCR3 and an isotype-matched nonspecific Ab, neither of which had any consistent effect on migration across LVEC (Fig. 4A). We confirmed the expression of CCL21 mRNA in LVEC but not in HUVEC by quantitative PCR (data not shown). In addition, pretreatment with pertussis toxin inhibited PGD2-induced lymphocyte migration across LVEC by 41 ± 11% (mean ± SEM, n = 4; p < 0.05 by paired t test), demonstrating a role for GPCR in the movement of lymphocytes across these EC.

The lymphocyte adhesion receptors that support transit of LVEC have not been described. Because integrins are crucial for transit through BVEC, we tested the effect of function-neutralizing Abs against β1, β2, and β3 integrins. Blockade of β1 and β2 integrins had a significant effect on the efficiency of transmigration (≈40% and 80% inhibition, respectively; Fig. 4B). A β3 integrin-blocking reagent had no more effect than an isotype-matched control Ab (Fig. 4B). In addition, the priming effect of BVEC migration did not alter the activation status of β2 integrins or F-actin on migrated lymphocytes compared with nonmigrated controls (data not shown).

**Migration across BVEC or delivery of exogenous PGD2 increases the efficiency of CD4 memory T cell migration across LVEC and chemotaxis toward the CCR7 ligand CCL21**

As lymphocyte transit of BVEC was a PGD2-dependent process, we speculated that this prostanoid signal might also be an important signal for increasing the efficiency of migration across LVEC. In a separate series of experiments, passage of CD4 memory T cells across BVEC again delivered a considerable increase in migration efficiency across LVEC compared with CD4 memory T cells cultured on plastic (Fig. 5A). However, if isolated CD4 memory T cells were cultured on plastic in the presence of exogenous PGD2 for 24 h, then an increase in efficiency of migration across LVEC was also obtained (Fig. 5A). Some increase

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**FIGURE 2.** IFN-γ-inducible chemokines and PGD2 are both essential for the efficient migration of lymphocytes across BVEC. **A**, The effect of blocking the chemokine receptor CXCR3 on the recruitment of flowing lymphocytes to BVEC stimulated with TNF and IFN. **B**, The effect of blocking the PGD2 receptors DP-1 or DP-2 on lymphocyte migration across BVEC stimulated with TNF and IFN under static assay conditions. ANOVA showed a significant effect of inhibitor treatment on lymphocyte migration (p < 0.01). **C** and **D**, Micrographs from flow-based adhesion assays of lymphocytes adherent to TNF- and IFN-stimulated BVEC in the absence or presence of 1 μM DP-2 antagonist (original magnification ×200). Arrows refer to surface-adherent phase-bright lymphocytes (SA) and transmigrated phase-dark lymphocytes (TM). **E**, The effect of DP-2 antagonist on lymphocyte behavior following recruitment from flow. Cells were classified as rolling (white bars), surface adherent (black bars), or transmigrated (gray bars). All data are mean ± SEM of at least three independent experiments. *p < 0.05 compared with untreated lymphocytes by paired t test, **p < 0.01 by Dunnett post hoc test.
The process of CCR7-mediated diapedesis across LVEC is more efficient after PGD2 stimulation, but this is not due to increased chemokinesis.

As CD4 memory T cell migration across LVEC was driven by CCR7, we examined the effect of transit of these cells across BVEC on the levels of expression of this receptor. We also analyzed other chemokine receptors reported to support migration of different lymphocyte subsets into lymph nodes via the high endothelial venule or into the afferent lymphatic vasculature via the tissue. Although lymphocytes in peripheral blood expressed CCR7, CXCR5, and CXCR3, expression levels were not altered by the process of migration across BVEC (Table I). Importantly, however, we could show that in a Transwell chemotaxis assay, CD4 memory T cells that had previously been recruited across BVEC or had been stimulated with exogenous PGD2 migrated with significantly increased efficiency to the CCR7 ligand CCL21 (Fig. 5C). Thus, the migration of CD4 T cells across BVEC appears to enhance the function of CCR7 without increasing its surface expression on primed cells (Fig. 6). Interestingly, using LVEC cultured on plastic to track the migration velocity of lymphocytes during migration, we could show that stimulation of DP2 with a specific agonist significantly reduced the velocity of migration underneath LVEC (6.4 ± 0.2 μm/min for untreated compared with 4.2 ± 0.2 μm/min for DP2 agonist pretreated PBL; mean ± SEM, n = 3; p < 0.001 by paired t test). These data show that the process of CCR7-mediated diapedesis across LVEC is more efficient after PGD2 stimulation, but this is not due to increased chemokinesis.

Discussion

In the current model of lymphocyte trafficking, we have identified new steps that regulate the migration of memory T cells across the EC of both the blood and lymphatic vasculature. Thus, PGD2 is required for the efficient transendothelial migration of memory T cells across BVEC, and this PGD2-driven step of recruitment also primes CD4 memory T cells for efficient migration across LVEC (Fig. 6). During migration across BVEC, the PGD2 signal is
Subordinate to a primary activating stimulus delivered by chemokines of the IFN inducible family (CXCL9–11), which promote stable integrin-mediated adhesion via the CXCR3 chemokine receptor. However, without receipt of the PGD2 signal, memory T cells do not traffic efficiently across BVEC or LVEC. PGD2-mediated priming for efficient migration across LVEC did not cause upregulation of integrin adhesion receptors or chemokine receptors on CD4 memory T cells, but did modify the function of CCR7 so that the efficiency of chemotaxis toward CCL21 was significantly increased.

Current paradigms of T cell trafficking during an inflammatory response assume that the delivery of a chemokine signal is sufficient to promote integrin-mediated stable adhesion, followed by reorganization of the actin cytoskeleton during spreading on the EC surface, eventually leading to transendothelial migration. However, blockade of the PGD2 receptor DP-2 [formally known as chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2)] greatly reduces the transmigration of memory T cells, leaving the great majority attached to the apical surface of the EC monolayer. These observations strongly imply the presence of a hierarchy of activation signals that are required for efficient recruitment. Thus, we propose an initial chemokine signal activates lymphocyte integrins allowing immobilization on the endothelium. In turn, PGD2 permits downstream functions such as migration and diapedesis. Such a model for the recruitment of lymphocytes during inflammation resembles our recently published observations of the recruitment of neutrophils (14). In that study, we showed that migration across EC of the blood vascular required PGD2-mediated signaling through the DP-1 receptor. This prostanooid signal was again subordinate to an initial chemokine stimulus delivered to the neutrophil by CXCR2. Taken together, these observations indicate that leukocyte access to inflamed tissue may require both chemokine and prostanooid signals, although the source of PGD2 in these assays is still to be definitively identified. Previously, we showed that PGD2 production by cultured EC was at the limits of detection by mass spectrometry (14). However, the use of cyclooxygenase inhibitors in EC cultures was very effective at reducing PGD2-mediated leukocyte migration. The implication of these observations is that formation of small amounts of this agent by EC may be highly localized and occur in response to leukocyte adhesion to the endothelial cell surface rather than being an endogenously synthesized agent released constitutively. This aspect of prostanooid production requires further elucidation in future studies.

The process of migrating across BVEC primed CD4 memory T cells for efficient migration across LVEC. This phenomenon could be recapitulated by treating PBL with PGD2, strongly implying that this eicosanoid promoted passage across both EC monolayers. The mechanism by which T cell migration across LVEC was expedited was also identified. Thus, either migration across BVEC or exogenous PGD2 could promote the function of the CCR7 without increasing its surface expression. Evidence for this came through the use of a function-neutralizing Ab against CCR7 and also blockade of GPCR signaling, both of which significantly retarded CD4 memory T cell migration across LVEC. In addition, CD4 memory T cells, which had migrated across BVEC or received an exogenous PGD2 stimulus, were capable of migrating more efficiently toward the CCR7 ligand CCL21 in a Transwell chemotaxis assay. The use of CCR7 by CD4 T cells for migration across LVEC is in accordance with previous observations made in murine models, in which T cells lacking the receptor were unable to efficiently traffic into the lymphatic vasculature from the skin (12) or the lung (13). A role for prostanooids in regulating the function of CCR7 in T cells has not previously been demonstrated, although the phenomenon has close parallels with the regulated migration of DCS out of peripheral tissue and into the afferent lymphatic vasculature. In this case, the differentiation and maturation of DCS within peripheral tissue is driven by PGE2 (21–24). This prostanooid signal promotes the function (but not the

Table I. Mean fluorescent intensities for flow cytometry of chemokine receptor expression on peripheral blood lymphocytes before and after migration across BVEC

<table>
<thead>
<tr>
<th>Chemokine Receptor</th>
<th>Nonmigrated PBL</th>
<th>PBL Migrated Across BVEC</th>
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<tbody>
<tr>
<td>CCR7</td>
<td>46.12 ± 10.14</td>
<td>36.23 ± 6.271</td>
</tr>
<tr>
<td>CXCR3</td>
<td>22.79 ± 2.481</td>
<td>16.77 ± 1.227</td>
</tr>
<tr>
<td>CXCR5</td>
<td>33.34 ± 6.286</td>
<td>22.56 ± 9.035</td>
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Data are mean ± SEM of five experiments. ANOVA showed no significant effect of migration on the expression of chemokine receptors.
expression) of the chemokine receptor CCR7, so that DCs also respond more efficiently to the chemokines CCL19 and CCL21. The striking similarity of the pathways used by DCs and CD4 memory T cells for exit from peripheral tissue may indicate that as the adaptive immune system has evolved, the different populations of effector cells have adopted a similar mechanism for localization in secondary lymphoid tissue, except that their emigration from tissue is regulated by different prostanoids.

Although CCR7 regulates CD4 memory T cell trafficking across LVEC, other aspects of adhesion and migration of these cells across LVEC are less well characterized. However, it does appear that sphingosine 1-phosphate (S1P) is a potent retention signal for tissue-resident T cells (25). In vivo activation via the S1P receptor, S1P1, retarded migration into the lymphatic vasculature, whereas in vitro, activation of polarized T cells through the S1P1 receptor caused firm adhesion of these cells to the basal surface of lymphatic but not vascular EC (25). Such retention signals do not preclude a role for PGD2 in encouraging migration away from tissue. Indeed, they might represent an additional step in the relay of directional cues that control entry, residence, and removal of leukocytes from tissue during the evolution of an inflammatory response. Other studies have identified adhesion receptors such as E-selectin, VCAM-1, and ICAM-1 on cytokine-stimulated LVEC and demonstrated that they function to support migration of DCs across LVEC (26–28). In this study, we show for the first time, to our knowledge, that passage of CD4 memory T cells across LVEC requires both $\beta_1$ and $\beta_2$ integrins. Interestingly, the PGD2-dependent priming of these cells for efficient transit of the LVEC did not require upregulation of integrin receptors that were highly expressed on this subset of T cells. However, taken together, these studies indicate that migration of leukocytes across LVEC uses adhesion receptors that are remarkably similar to those used for transit of the BVEC barrier.

Although the importance of PGD2 for successful navigation of both blood vascular and lymphatic vascular EC by memory T cells is a novel observation, an important role for PGD2 and its receptor DP-2 in memory T cell biology has been previously reported. Indeed, Nagata and colleagues (29) demonstrated the presence of a G protein-coupled orphan receptor CRTh2 on CD4+ and CD8+ memory T cells before they identified its cognate ligand, PGD2 (30). Interestingly, others claimed that this receptor was the most reliable marker for some memory T cell subsets (31). In these studies, signaling by PGD2 via the DP-2 receptor could upregulate the $\beta_2$ integrin CD11b and the TNF superfamily member CD154.

**FIGURE 6.** New steps in the regulation of CD4+CD45RO+ T cell trafficking that are dependent upon PGD2. 1, BVEC stimulated with cytokines (TNF and IFN) selectively recruit CD4+CD45RO+ T cells from flowing blood. 2, The T cells receive an activating stimulus from IFN-γ-inducible chemokines (CXCL9–11), which operate exclusively through the CXCR3 receptor. 3, T cell integrins are transiently activated and immobilize the cell on the surface of BVEC. 4, PGD2 stimulates the DP-2 receptor (formally CRTh2), a signal that is essential for efficient passage across the BVEC monolayer. 5, Receipt of a PGD2 signal during passage of the BVEC or stimulation with exogenous PGD2 promotes the function, but not the expression, of the lymphocyte chemokine receptor CCR7. 6, CD4+CD45RO+ T cells migrate across LVEC in response to the chemokines CCL19 and CCL21 with significantly increased efficiency.
(CD40L) and could induce chemotactic responses in some memory T cells (30, 32). Moreover, PGD₂ signaling through DP-2 was shown to exacerbate cutaneous (33) and pulmonary (34) allergic inflammation in rodent models. Thus, it is now becoming apparent that prostanoid signals operating through the DP-2 receptor are important regulators of both memory T cell trafficking and immune function.

In conclusion, the current paradigms that postulate an exclusive role for chemokines in the recruitment of memory T cells from the blood require updating (see Fig. 6 for details). Although an initial chemokine signal is essential for the activation of T cells on BVEC, a downstream prostanoid signal, delivered by PGD₂ via the DP-2 receptor, is required to drive the process of diapedesis. In addition, passage of the blood vasculature and receipt of a PGD₂ signal significantly increases the efficiency of passage across EC of the lymphatic vasculature by modulating the function of CCR7.

Disclosures

The authors have no financial conflicts of interest, with the exception of M.R., who is employed by AstraZeneca but will not benefit financially from this work.

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