Plasmacytoid dendritic cells employ multiple cell adhesion molecules sequentially to interact with high endothelial venule cells – molecular basis of their trafficking to lymph nodes

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Abstract

Plasmacytoid dendritic cells (pDCs) are natural type I IFN-producing cells found in lymphoid tissues, where they support both innate and adaptive immune responses. They emigrate from the blood to lymph nodes, apparently through high endothelial venules (HEVs), but little is known about the mechanism. We have investigated the molecular mechanisms of pDC migration using freshly isolated DCs and HEV cells. We found that pDCs bound avidly to HEV cells and then transmigrated underneath them. Two observations suggested that these binding and migration steps are differentially regulated. First, treatment of pDCs with pertussis toxin blocked transmigration but not binding. Second, pDCs were able to bind but not to transmigrate under non-HEV endothelial cells, although the binding was observed to both HEV and non-HEV endothelial cells. Antibody inhibition studies indicated that the binding process was mediated by $\alpha$L and $\alpha$4 integrins on pDCs and by intercellular adhesion molecule (ICAM)-1, ICAM-2 and vascular cell adhesion molecule-1 on HEVs. The transmigration process was also mediated by $\alpha$L and $\alpha$4 integrins on pDCs, with junctional adhesion molecule-A on HEV cells apparently serving as an additional ligand for $\alpha$L integrin. These data show for the first time that pDCs employ multiple adhesion molecules sequentially in the processes of adhesion to and transmigration through HEVs.

Introduction

In anti-viral immune responses, plasmacytoid dendritic cells (pDCs) directly inhibit viral replication and contribute to the activation of NK cells, T cells, B cells and conventional DCs by producing type 1 IFNs (1, 2). pDCs are constitutively present in lymph nodes (LNs) under steady-state conditions (3) but are substantially reduced in the LNs of L-selectin-deficient mice (4), indicating that their trafficking to LNs is at least partially dependent on L-selectin under physiological conditions. However, detailed studies on adhesion molecules involved in pDC trafficking to lymphoid tissues are scarce and have often depended on in vitro- (5) or in vivo-expanded counterpart (6), because it is difficult to obtain sufficient quantities of unmanipulated pDCs from intact animals. As a result, little information is currently available about the molecular mechanisms regulating the steady-state trafficking of pDCs.

Here we investigated the interactions between pDCs and high endothelial venules (HEVs) directly using purified pDCs and HEV endothelial cells. We show for the first time that pDCs have the full capacity to adhere to and transmigrate...
beneath HEV endothelial cells under steady-state conditions and that they interact with HEVs through a precise sequence of events using specific pairs of adhesion molecules. pDCs show robust transmigration underneath HEV endothelial cells but not non-HEV endothelial cells, indicating that pDC transmigration is at least in part regulated differently from their adhesion to these cells. We also demonstrate the involvement of leukocyte function-associated antigen-1 (LFA-1)/junctional adhesion molecule (JAM)-A adhesion pathway in pDC transmigration.

**Methods**

**Animals**

BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments used experimental protocols that were approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine.

**Reagents**

Pertussis toxin (PTX) and polymyxin B were obtained from Calbiochem (La Jolla, CA, USA). Hybridomas producing mAbs against peripheral node addressin (PNAd) (MECA-79) (7) and mucosal adressin cell adhesion molecule-1 (MADCAM-1) (MECA-89 and MECA-367) (8) were kindly provided by E. C. Butcher (Stanford University, Stanford, CA, USA). Hybridomas for anti-mouse vascular cell adhesion molecule-1 (VCAM-1) (M/K-1) (9) and CD49d (PS/2) (10) were generous gifts of K. Miyake (University of Tokyo, Tokyo, Japan). mAbs to PNAd antibody to VCAM-1 or CD49d were used as hybridoma culture supernatants. Pertussis toxin (PTX) and polymyxin B were obtained from Cappel (Durham, NC, USA). Allophycocyanin-conjugated anti-CD11c (HL-3), Alexa 647-conjugated anti-CD34 (RAM34) and FITC-conjugated anti-CD45R/B220 (RA3-6B2) or CD49d (R1-2) were from eBioscience (San Diego, CA, USA). FITC-conjugated anti-CD11a (M17/4), CD18 (C71/16), CD29 (HA2/5), β7 (M293) or CD62L (MEL-14), PE-conjugated anti-B220 (RA3-6B2) or CD11c (HL3), Fc block CD16/CD32 (2.4G2) and mAb to CD62L (MEL-14) were from BD Pharamingen (San Diego, CA, USA). Reagents to CD11a (KBA) and CD54 (KAT-1) were described previously (13). Biotin-conjugated anti-mPDCA-1 (JF05-1C2.4.1) was from Miltenyi Biotec (Bergisch Gladbach, Germany). Purified rat IgG and FITC-conjugated goat anti-rat IgG were from Cappel (Durham, NC, USA). Allophycocyanin-conjugated anti-CD11c (HL-3), Alexa 647-conjugated anti-CD34 (RAM34) and FITC-conjugated anti-CD45RB220 (RA3-6B2) or CD49d (R1-2) were from eBio-science (San Diego, CA, USA). FITC-conjugated anti-CD11a (M17/4), CD18 (C71/16), CD29 (HA2/5), β7 (M293) or CD62L (MEL-14), PE-conjugated anti-B220 (RA3-6B2) or CD11c (HL3), Fc block CD16/CD32 (2.4G2) and mAb to CD62L (MEL-14) were from BD Pharamingen (San Diego, CA, USA).

**Purification of HEV cells**

MADCAM-1⁺ endothelial cells of HEVs were purified from mouse mesenteric LNs by MACS (Miltenyi Biotec), using biotin-conjugated anti-MADCAM-1 mAb (MECA-89), as previously described (14). Some experiments used CD34⁺ PNA⁺ MADCAM-1⁺ HEV endothelial cells and CD34⁺ PNA⁻ MADCAM-1⁻ non-HEV endothelial cells that had been sorted by FACS(Aria) (BD Biosciences). The purity of the isolated HEV cells was routinely >90%.

**Preparation of pDCs**

Spleens removed from mice were digested with 400 Mandl U/ml collagenase D (Roche Diagnostics, Mannheim, Germany) and 10 µg/ml DNase I (Roche Diagnostics) in RPMI 1640/10% FCS, with continuous stirring at 37°C for 30 min. After the addition of 10 mM EDTA, the cell suspension was incubated at 37°C for an additional 5 min. The cells obtained were then spun through a 17.5% Accudenz solution (Accurate Chemical & Scientific, Westbury, NY, USA) to enrich for pDCs. These cells were then incubated with anti-CD16/CD32 for FcR blocking followed by an incubation with biotin-conjugated anti-mPDCA-1 (10 µg/ml) for 15 min. After being washed with PBS containing 10% FCS, 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 10 mM EDTA and 10 µg/ml polymyxin B, the cells were incubated with a mixture of 10-fold-diluted streptavidin-conjugated microbeads (Miltenyi Biotec) and subjected to two rounds of MACS (Miltenyi Biotec). The purity of the sorted pDCs was routinely >95%.

**Flow cytometry**

For phenotypic analysis, cells were incubated with a combination of allophycocyanin-conjugated anti-CD11c, PE-conjugated anti-B220 and FITC-conjugated anti-CD11a, -CD18, -CD29, -CD49d, -CD62L or -integrin β7 mAbs, and analysed on a flow cytometer (FACSCalibur, BD Biosciences). In some experiments, the cells were first incubated with anti-JAM-A or JAM-C mAb, followed by FITC-conjugated goat anti-rat IgG (Cappel). After blocking with rat IgG, cells were further incubated with a combination of allophycocyanin-conjugated anti-CD11c and PE-conjugated anti-B220 mAbs. To analyse L-selectin expression, pDCs in unseparated cell populations were used. Bone marrow (BM)-derived pDCs prepared as described (15) and CD11c⁺/CD220⁻ DCs from the spleen were also analysed as above.

**Cell adhesion and transmigration assay**

Purified HEV cells (1–2 × 10⁴ cells per well) were allowed to adhere to collagen I-coated wells of 8-well chamber slides (BD Biosciences) at 37°C for 2 h. pDCs (1–2 × 10⁵ cells per well) were then added, and the cultures were incubated at 37°C for an additional 3 h. The cells were washed 3 times with PBS, fixed with 4% PFA and stained with May–Grunwald–Giemsa solution. Under the light microscope, we observed pDCs bound to the surface of HEV cells (stained dark blue), and other pDCs that transmigrated underneath the HEV cells (stained pale blue). These cells were scored separately for at least 100 HEV cells. For antibody inhibition studies, purified mAbs were used at a final concentration of 50 µg/ml. Antibodies to VCAM-1 or CD49d were used as hybridoma culture supernatants. In some experiments, pDCs were pre-treated with various concentrations of PTX at 37°C for 1 h.

**Electron microscopy**

For scanning electron microscopy, purified HEV cells (2 × 10⁴ cells per well) and pDCs (4 × 10⁵ cells per well) in the wells of collagen I-coated 8-well culture slides (BD Biosciences) were allowed to interact for 3 h as above. The cell layers were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer, then post-fixed in 1% OsO₄ for 30 min at room temperature. The samples were then dehydrated in graded concentrations of ethanol, and critical-point dried in CO₂. After
sputter coating with gold, the specimens were examined under a Hitachi S-3000H electron microscope operated at 15 kV. For transmission electron microscopy, purified HEV cells (7 x 10^4 cells per well) and pDCs (5 x 10^5 cells per well) were cultured in collagen I-coated 48-well culture slides (BD Biosciences) as above. The cell layers were fixed as above, then dehydrated in graded ethanol and embedded in Quetol 812. Vertically cut ultrathin sections of the layer were stained with uranyl acetate/lead citrate and examined using a JEOL JEM-1230 electron microscope operated at 60 kV.

Statistical analysis
A Student's t-test was applied to compare the statistical difference within two groups.

Results and discussions
Splenic pDCs express an array of adhesion molecules that can interact with HEVs
To study the mechanisms by which pDCs are recruited to LNs under steady-state conditions, we wished to use naturally circulating pDCs rather than in vitro- or in vivo-expanded pDCs, because any cell amplification process may result in changes in the cell phenotype and trafficking patterns. A phenotypic comparison of the pDCs from different lymphoid organs of unperturbed mice showed that pDCs from the spleen, LNs, BM and peripheral blood all expressed readily detectable levels of L-selectin and αL, α4, β1 and β2 integrins, low levels of β7 integrin and were negative for JAM-A and JAM-C (Fig. 1). This pattern corresponds to the phenotype of circulating pDCs in the peripheral blood of mice (5). Myeloid (CD11b^highB220^-) DCs from the spleen showed a similar pattern of adhesion molecule expression except that they showed higher expression of β7-integrin and JAM-A than pDCs. L-selectin expression is apparently susceptible to isolation procedures, because the prolonged exposure of pDCs to digestion enzymes and/or density gradient solutions during their isolation rapidly diminished the L-selectin expression (data not shown). This might at least partly account for the previous observation that pDCs isolated from the secondary lymphoid organs showed negative or very low L-selectin expression in the mouse (4) and human (16). Because the expression pattern of these molecules was almost identical to that seen with pDCs obtained from other lymphoid tissues and peripheral blood, we judged our pDCs to be representative of blood-borne circulating pDCs and used them as such throughout the present investigation. Their localization in the trafficking area of the spleen, i.e. in the periarteriolar lymphoid sheath or at the marginal zone (3) also supports the contention that splenic pDCs represent circulating pDCs.

pDCs bind readily to purified HEV cells and subsequently transmigrate beneath them
To establish whether pDCs can interact with HEV endothelial cells, we purified pDCs and HEV endothelial cells and examined their interactions in vitro. After being seeded on collagen I-coated slides, most HEV endothelial cells spread well, but some retained a plump morphology and adhered to the substratum by extending pseudopods from the cell surface. Scanning electron microscopy showed that purified pDCs bound avidly to both the flat and plump types of HEV endothelial cells, which occurs within 30 min of co-incubation (Fig. 2A and B), and the pDCs occasionally induced distinct morphological changes, such as pseudopod protrusion, in the endothelial cells in the vicinity of their adhesion (Fig. 2A). As shown in Fig. 2(C and D), pDCs often transmigrated beneath HEV endothelial cells from the site where the morphological

![Fig. 1](https://academic.oup.com/intimm/article-abstract/19/9/1031/666122) **pDCs obtained from various lymphoid tissues express a variety of adhesion molecules that can interact with HEVs.** pDCs were obtained from the spleen, mesenteric LNs (MLN), peripheral LNs (PLN), BM and blood, and were examined for the expression of L-selectin and integrin αL, α4, β1, β2, β7 chains, JAM-A and JAM-C. Myeloid (CD11b^highB220^-) DCs from the spleen were also analysed in the same way. Note that the pDCs from various tissues showed comparable expression profiles of the trafficking-associated adhesion molecules.
of transmission electron micrographs in Fig. 3(A–E), pDCs bodies beneath the HEV endothelial cells. As seen in the set
After adhesion, pDCs appeared to swiftly insinuate their cell
manner cell or between adjacent endothelial cells in a PTX-sensitive
pDCs transmigrate along the periphery of an endothelial
cell or between adjacent endothelial cells in a PTX-sensitive
changes were observed. These findings are consistent with
the process by which leukocyte adhesion is thought to trigger
signals in the endothelial cells that promote subsequent cellular interactions such as transmigration (17). Fig. 2(E) illustrates
numerous pDCs, identified by their unique cytoplasmic tail, binding to and transmigrating underneath HEV endothelial cells. The transmigration was readily observed soon after pDC adhesion, reaching a plateau at 2 h after adhesion.

pDCs transmigrate along the periphery of an endothelial cell or between adjacent endothelial cells in a PTX-sensitive manner

After adhesion, pDCs appeared to swiftly insinuate their cell bodies beneath the HEV endothelial cells. As seen in the set of transmission electron micrographs in Fig. 3(A–E), pDCs appeared to crawl underneath the HEV endothelial cells along the cell periphery. Once more than half of the pDC cell body was underneath the endothelial cell, a thin protrusion from the endothelial cell appeared that was tightly apposed to the transmigrating pDCs (Fig. 3D and E). Eventually, this protrusion surrounded the entire apical surface of the pDCs, completing the process of pseudopermeoposis (18) by the HEV endothelial cells. As shown in Fig. 3(F), pDCs sometimes appeared to make their way to endothelial junctions and then transmigrate between tightly apposed HEV endothelial cells. During this process, an endothelial flap appeared to lift in a way that would facilitate the pDC’s passage through the junction, indicating that pDC adhesion may initiate the opening of endothelial cell junctions to expedite the pDC transmigration process. In the scanning of >1000 HEV endothelial cells interacting with pDCs, there was no indication that pDCs penetrated the HEV endothelial cell body or passed through the cytoplasm of the endothelial cell during the course of diapedesis. This is consistent with the idea that pDCs use mainly the paracellular and not the transcellular route to extravasate through HEVs, as do naive lymphocytes (19, 20).

The transmigration by pDCs was apparently regulated by Gq/11-protein-mediated signaling, because PTX treatment of the pDCs almost completely abrogated the transmigration underneath HEV endothelial cells (Fig. 3G). It is also of note that no transmigration was observed when non-HEV endothelial cells from LNs were used instead of HEV endothelial cells, although appreciable pDC binding to the non-HEV endothelial cells was observed (Fig. 3H). These findings indicate that pDC transmigration occurs with a specific type of endothelial cell and that pDC transmigration is, at least in part, regulated differently from pDC adhesion to endothelial cells.

\( \alpha_\text{L} \) and \( \alpha_\text{4} \) integrins on pDCs and ICAM-1, ICAM-2, VCAM-1 and JAM-A on HEVs were involved in pDC–HEV interactions

We next performed antibody inhibition studies to identify the adhesion molecules involved in the interaction between pDCs and HEV endothelial cells. As shown in Fig. 4(A), mAbs to \( \alpha_\text{L} \) or \( \alpha_\text{4} \) integrin inhibited the pDC adhesion to HEV endothelial cells by about 30–40% compared with control rat IgG, and a mixture of the two mAbs inhibited the adhesion by \(~70\%\). Correspondingly, mAbs to the ligands for these integrins, intercellular adhesion molecule (ICAM)-1, ICAM-2 or VCAM-1 inhibited pDC adhesion to HEV endothelial cells by 30–50% individually and by \(~60\%\) when applied in combination. In contrast, mAbs to L-selectin and MadCAM-1 did not inhibit the adhesion. These results indicate that pDC adhesion to HEV endothelial cells is regulated mainly by the \( \alpha_\text{L} \beta_2 \) integrin/ICAM-1, -2 and \( \alpha_\text{4}\beta_1 \) integrin/VCAM-1 pathways, although another adhesion pathway is also involved. As shown in Fig. 4(B), the transmigration process also appears to be regulated by the \( \alpha_\text{L} \beta_2 \) integrin/ICAM-1, -2 and \( \alpha_\text{4}\beta_1 \) integrin/VCAM-1 pathways, because individual mAbs to these molecules inhibited transmigration by \(~50\%\): a mixture of mAbs to \( \alpha_\text{L} \) and \( \alpha_\text{4} \) integrins almost completely abolished the transmigration of pDCs, while mAbs to ICAM-1, -2 plus VCAM-1 inhibited pDC transmigration by \(~80\%\). The effect of these mAbs on transmigration might in
part reflect their inhibitory effects on adhesion and/or cell movement: firm adhesion is a prerequisite for transmigration, so blocking adhesion necessarily blocks transmigration (21). In addition, blocking α4β1 integrin inhibits leukocyte chemotaxis (Y. Srinoulprasert and M. Miyasaka, unpublished observation). Nevertheless, the observation that these mAbs invariably inhibited transmigration far more efficiently than adhesion argues for a role of these molecules in the transmigration process, and the involvement of other molecules is not ruled out.

Previous studies by Ostermann et al. (22) showed that JAM-A could serve as a functional ligand for LFA-1 and mediate transendothelial migration of certain leukocytes. We therefore sought to test the functional contribution of JAM-A expressed in HEV endothelial cells (23) in pDC–HEV interactions. As shown in Fig. 4(C), mAb to JAM-A inhibited transmigration of pDCs underneath HEV endothelial cells without significantly affecting the pDC binding to HEV cells, whereas mAb to JAM-C affected neither adhesion nor transmigration. In addition, mAbs to JAM-A and ICAM-1 additively inhibited transmigration of pDCs to a similar extent that mAb to αL integrin did (Fig. 4D). These observations are consistent with previous findings that JAM-A can function as an additional ligand to LFA-1 mediating transendothelial migration of leukocytes and suggest an important role of JAM-A in the transmigration of pDCs. The possible involvement of CD31 (24), CD99 (25) and other JAM family members (26), such as JAM-B and ESAM, in pDC transmigration needs to be investigated in future studies.

Although mAbs to MAdCAM-1 or L-selectin had little effect on adhesion and the anti-MAdCAM-1 did not affect transmigration in our analysis (Fig. 4A and B), it does not rule out the possible involvement of these molecules in pDC–HEV interactions in vivo. Our in vitro static assay system may bypass the initial shear-dependent tethering processes mediated by L-selectin and/or MAdCAM-1 that may be necessary for firm adhesion. In support of this idea, our preliminary analysis indicated that pDCs could adhere to and transmigrate underneath HEV endothelial cells independently of shear stress (data not shown). Although we could not formally address the role of L-selectin under shear stress conditions in the present investigation, a previous study demonstrated the critical role of L-selectin as a tethering receptor in pDC–HEV interactions in vivo (5).

Finally, it has been generally thought that trafficking of circulating pDCs from the blood to LNs is primarily driven by inflammatory stimuli (1, 5, 27). However, our study clearly demonstrates that circulating pDCs are fully capable of interacting with HEV endothelial cells under physiological conditions and are likely to use a mechanism very similar to that described for T and B naive lymphocytes (28). Our study also indicates that pDCs interact with HEVs via a specific

**Fig. 3.** pDCs transmigrate under HEV endothelial cells in a PTX-sensitive manner. After their adhesion to HEV endothelial cells, pDCs appeared to crawl along the cell periphery and transmigrate beneath the HEV cells. Panels (A–E) show the presumed sequence of events in the pDC–HEV endothelial cell interactions. Toward the end of this process, HEV endothelial cells provide a cellular protrusion in the form of a thin flap in close apposition to the pDCs, as seen in (D and E). A dotted line in (C–E) demarcates a junction between the endothelial thin flap and the pDC cell body. (F) pDCs sometimes showed transmigration through the junctions of HEV endothelial cells. This panel shows a pDC that was just about to migrate into the junction formed by two HEV endothelial cells. A cell on the left adhering to the surface of one HEV cell appears to be a lymphocyte. Note the prominent flap sticking out from the endothelial cell on the right, which made close contact with the transmigrating pDC. Scale bars, 1 μm. (G) Pre-treatment of pDCs with PTX strongly inhibited their transmigration underneath HEV endothelial cells. (H) pDCs transmigrated under HEV endothelial cells but not under non-HEV endothelial cells.
Fig. 4. Involvement of multiple cell adhesion pathways in pDC–HEV interactions. (A) pDC adhesion to HEV endothelial cells was inhibited by mAbs against integrin αL, α4, ICAM-1, ICAM-2 or VCAM-1. (B) pDC transmigration was also inhibited by mAbs against integrin αL or α4, or ICAM-1, ICAM-2 or VCAM-1. (C) pDC transmigration underneath HEV endothelial cells without affecting pDC binding to HEV endothelial cells was inhibited by mAb against JAM-A. (D) Similar inhibitory effects on pDC transmigration were observed with anti-αL integrin mAb and anti-ICAM-1 plus anti-JAM-A mAbs. Data are presented as mean number of pDCs per 100 HEVs ± SD (n = 3). *P < 0.001, **P < 0.01, ***P < 0.05 compared with controls.
order of events: they first bind to HEV endothelial cells, then invoke morphological changes in the endothelial cells and subsequently transmigrate underneath them. Because it is technically difficult to monitor continuously the process of pDC diapedesis/transmigration with the currently available intravital microscopy technique (5), our assay system may provide a useful new tool for studying the entire process of pDC transmigration in detail in vitro. Although the present study did not address the mechanisms of integrin activation in pDC–HEV interactions, future studies using our in vitro system may help define the chemokines and lysophospholipid mediators, such as sphingosine-1-phosphate and lysophosphatic acid, that might be involved in this process under physiological and pathological conditions. Such findings would undoubtedly contribute to the development of techniques for manipulating pDC trafficking in vivo.

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Abbreviations
BM bone marrow
HEV high endothelial venule
ICAM intercellular adhesion molecule
JAM junctional adhesion molecule
LFA-1 leukocyte function-associated antigen-1
LN lymph node
MAaCAM-1 mucosal addressin cell adhesion molecule-1
pDC plasmacytoid dendritic cell
PNAd peripheral node addressin
PTX pertussis toxin
VCAM-1 vascular cell adhesion molecule-1

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