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Inflamed Lymphatic Endothelium Suppresses Dendritic Cell Maturation and Function via Mac-1/ICAM-1-Dependent Mechanism

Simona Podgrabinska,* Okebugwu Kamalu,† Lloyd Mayer,† Motomu Shimaoka,§ Hans Snoeck,‡ Gwendalyn J. Randolph,‡‡ and Mihaela Skobe‡*  

The lymphatic system is essential for the generation of immune responses by facilitating immune cell trafficking to lymph nodes. Dendritic cells (DCs), the most potent APCs, exit tissues via lymphatic vessels, but the mechanisms of interaction between DCs and the lymphatic endothelium and the potential implications of these interactions for immune responses are poorly understood. In this study, we demonstrate that lymphatic endothelial cells (LECs) modulate the maturation and function of DCs. Direct contact of human monocyte-derived DCs with an inflamed, TNF-α-stimulated lymphatic endothelium reduced expression of the costimulatory molecule CD86 by DCs and suppressed the ability of DCs to induce T cell proliferation. These effects were dependent on adhesive interactions between DCs and LECs that were mediated by the binding of Mac-1 on DCs to ICAM-1 on LECs. Importantly, the suppressive effects of the lymphatic endothelium on DCs were observed only in the absence of pathogen-derived signals. In vivo, DCs that migrated to the draining lymph nodes upon inflammatory stimuli, but in the absence of a pathogen, showed increased levels of CD86 expression in ICAM-1-deficient mice. Together, these data demonstrate a direct role of LECs in the modulation of immune response and suggest a function of the lymphatic endothelium in preventing undesired immune reactions in inflammatory conditions. The Journal of Immunology, 2009, 183: 1767–1779.

The lymphatic system plays a vital role in maintaining tissue homeostasis by regulating tissue fluid and protein balance and by performing immunological functions (1, 2). Traditionally, the lymphatic system has not been considered actively involved in the regulation of immune responses and has been viewed primarily as a transportation system. Lymphatic vessels serve as a route for the transport of dendritic cells (DCs),3 memory T cells, macrophages, and Ags from the periphery to lymph nodes (LNs) and therefore play an important role in initiating immune responses (3–6). In the steady state, the DC is the major APC found in afferent lymph (5–7). During inflammation, DC transit through the afferent lymphatics is increased by approximately an order of magnitude. It is well established that inflammatory mediators such as TNF-α and IL-1β rapidly induce mobilization of DCs to the LNs by stimulating the production of chemokines and chemokine receptors that direct DC migration (2, 6, 7). Inflammatory signals also lead to remodeling of the lymphatic network at the periphery and in the LN. Lymphangiogenesis has been described in several inflammatory conditions and also in the draining LNs following immunization (8–13). Thus, lymphatic system activation is apparently an integral part of inflammation and immunity. It is believed to aid in the resolution of inflammation by removing extravasated fluids, inflammatory mediators, and cells (2, 14, 15); but whether the lymphatic system has a function in the regulation of immunity and inflammation beyond its role as a transport system is poorly understood.

DCs typically acquire Ags in peripheral tissues and migrate to LNs where they present them to T cells. Exposure of DCs to danger signals such as microbial agents and inflammatory mediators induces their maturation, resulting in increased expression of MHC class II, costimulatory molecules like CD80 and CD86, and cytokines, ultimately resulting in induction of immunity (6, 16). In the absence of pathogens, immature DCs capture autoantigens from apoptotic cells, migrate to secondary lymphoid organs, and trigger T cell tolerance (17–21). Immature DCs continuously traffic from peripheral tissues to LNs under steady-state conditions, and it has been proposed that they play a critical role in establishing peripheral tolerance to innocuous environmental proteins and self-Ags (22). The risk of autoimmunity is particularly heightened during inflammation and infection, which are associated with extensive cell death and increased flux of DCs to the LNs. In inflammation it is therefore of particular importance to prevent undesired immune reactions, but the mechanisms that control the ability of DCs to suppress immune responses are poorly understood.

DCs must come into close contact with lymphatic endothelial cells (LECs) as they migrate in and out of the lymphatic vessels, but little is known about direct interactions between DCs and...
LEC. Recent study showed that in inflammation, lymphatic vessels express several key adhesion molecules involved in the transmigration of leukocytes from the blood (23), and some studies suggest that ICAM-1 may be important for DC migration to the LNs (23–25). In this study, we investigated the role of ICAM-1 and its β2 integrin ligands LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) (26) in mediating adhesive interactions of DCs with LECs and demonstrate that ICAM-1-mediated contact of DCs with LECs suppresses DC maturation and its ability to stimulate T cells in the absence of a pathogen-derived signals.

Materials and Methods

Antibodies

Allophycocyanin-conjugated mouse anti-human CD14, CD83, CD86, and HLA-DR Abs and IgG1 and IgG2a isotype controls were purchased from BD Biosciences. Allophycocyanin-conjugated rat anti-mouse CD86 Ab and IgG2a isotype control were purchased from eBioscience. Mouse anti-human podoplanin used for FACS was from angioBio. Abs used for immunostaining were as follows: rat anti-mouse ICAM-1 (SouthernBiotech); anti-human ICAM-1, VCAM, and E-selectin (R&D Systems); ICAM-2 (BioSource); FcTc-conjugated mouse anti-α-smooth muscle actin (Sigma-Aldrich); and rabbit anti-human LYVE-1 and podoplanin ( Fitzgerald Indusries International). Mouse anti-human Abs used in blocking studies were the following: anti-ICAM-1, clone P2A4 (Chemicon) and clone 15.2 from Santa Cruz Biotechnology (Santa Cruz Biotechnology); anti-LFA-1, clone TS1/22, (Pierce Biotechnology); and anti-Mac-1, clone CBRM1/5 (eBiosciences). Isotype-matched control used for blocking studies was mouse IgG1 from R&D Systems.

Cell isolation and culture

Primary cultures of human LECs were established from neonatal foreskins and cultured on collagen-coated dishes in endothelial basal medium (EBM; Clonetics) with gluta mine and 20% FBS (Sigma-Aldrich) as described (27). In all experiments involving coculture of LECs with leukocytes, hy drocortisone acetate, cAMP, penicillin, streptomycin, and amphotericin B, which are supplemented for routine culture, were omitted from the medium. The human monocyteic cell line THP-1 and human T cell leukemia Jurkat cells were obtained from American Type Culture Collection and cultured in RPMI 1640 (Invitrogen) with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen).

Conditioned medium preparation

Confluent LECs were prestimulated with TNF-α (1 ng/ml for 24 h) in EBM with 1% FBS; the cells were washed to remove TNF-α and fresh EBM with 1% FBS was added. Conditioned medium was collected after 24 h and filtered with a 0.22-μm polyvinylidene difluoride filter (Millipore).

Preparation of human DCs

Blood was obtained from healthy donors according to the guidelines approved by the Institutional Review Board of the Mount Sinai School of Medicine (New York, NY). PBMCs were isolated by density gradient centrifugation on Ficoll-Hypaque (GE Healthcare) and CD14+ monocytes were purified using anti-CD14 magnetic beads (Miltenyi Biotec). DCs were generated by culturing CD14+ monocytes for 5 days in RPMI 1640 supplemented with heat-inactivated 10% FBS, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 ng/mL GM-CSF, and 50 ng/mL IL-4 (Peprotech). For DC maturation, cells were treated with 100 ng/mL LPS (Escherichia coli, serotype 0111:B4, Sigma-Aldrich) or 50 ng/mL TNF-α (Peprotech), for an additional 48 h.

Adhesion to immobilized ICAM-1/Fc

Flat-bottom, 96-well microtiter plates (MaxiSorp; Nalg Nunc) were coated with 15 μg/ml recombinant human ICAM-1Fc chimERIC protein (R&D Systems) for 2 h at room temperature, washed with PBS using a microplate washer (EL404; Bio-Tek Instruments), and incubated with 0.2% gelatin in PBS without Ca2+ and Mg2+ for 1 h at room temperature to minimize nonspecific binding. Cells were labeled with 5 μM CFSE (Molecular Probes), resuspended in HBSS without Ca2+ and Mg2+, and added to the coated 104 cells/well in triplicate. To activate integrins, cells were seeded in HBSS containing 2 mM EGTa and 5 mM MgCl2 (28). For blocking studies, cells were preincubated with blocking Abs to LFA-1 (10 μg/ml; clone TS1/22), Mac-1 (20 μg/ml; clone CBRM1/5), or mouse IgG1 (20 μg/ml) for 30 min on ice. After seeding, plates were centrifuged at 200 × g for 2 min at 4°C and the cells were allowed to adhere for 30 min at 37°C. Cell fluorescence was measured using a Synergy HT microplate reader (BioTek) before and after two washes with PBS using a microplate washer. Results were expressed as a percentage of input cells.

Adhesion to LECs

LEC were grown to confluence on collagen-coated wells of 6-well plates (BD Biosciences) in EBM with 20% FBS. Medium was changed to EBM with 1% FBS, with or without 2 ng/ml TNF-α for 24 h. This concentration of TNF-α was chosen because it induced high levels of ICAM-1 in LECs but did not alter cell shape as observed at the concentrations >50 ng/ml. LECs were then washed twice with HBSS and DCs were labeled with CFSE seeded on top of the LEC monolayers at 3 × 104 cells/well in triplicate. For blocking studies, DCs were preincubated with blocking Abs to LFA-1 (10 μg/ml; clone TS1/22) and Mac-1 (20 μg/ml; clone CBRM1/5) or mouse IgG1 (20 μg/ml) for 30 min on ice. To block ICAM-1, LECs were preincubated with anti-ICAM-1 Ab (10 μg/ml; clone P2A4 or 15.2) or mouse IgG1 (10 μg/ml) for 30 min before adding DCs. Cells were allowed to adhere for 30 min at 37°C; nonadherent cells were gently removed with PBS plus Ca2+ and Mg2+, and cell fluorescence was measured using a Synergy HT microplate reader. Alternatively, 1.5 × 106 LECs were seeded onto collagen-coated 8-well slides (LabTek II; Nalg Nunc), treated as described above, and 2 × 106 CFSE-labeled DCs were seeded on top in duplicates. Slides were gently washed, fixed with 1% formalin, covered with 50% glycerol in PBS, and examined with a Nikon E-600 microscope (Nikon). Images were captured with a SPOT digital camera (Diagnostic Instruments) at ×10 magnification (four images per well), and quantitative analysis was performed using IPLab software (Scanalytics).

Immunofluorescent staining of cells and tissues

Human neonatal foreskin and adult skin were collected and freshly frozen in OCT compound (Sakura Finetek) or cultured ex vivo with or without 100 ng/ml TNF-α in EBM containing 1% FBS for 4 and 24 h and then frozen. Foot pad or ear was injected with FITC-conjugated latex beads or with TNF-α (50 ng per ear, 200 ng per footpad; Peprotech) 20 h before harvesting of the skin or draining LN. Tissues were collected and freshly frozen in OCT compound. Tissues were sectioned (6 μm) using a Leica CM3050S cryostat (Leica Microsystems) and stained as described (27, 31). For staining of cells, LECs were grown to confluence on collagen-coated 8-well slides. Media was changed to EBM containing 1% FBS and some wells were treated with 2 ng/ml TNF-α for 24 h. Cells were stained with Abs to human ICAM-1, ICAM-2, ICAM-3, E-selectin, and podoplanin as described (27, 31). Specimens were examined with a Nikon E-600 microscope (Nikon) and images were captured with a SPOT digital camera (Diagnostic Instruments). For confocal analysis, images were captured with Leica SP5-DMI confocal microscope at an 0.118-μm interval. Three-dimensional reconstructions were performed using Velocity version 4.5 software (Improvision).

Flow cytometry

DCs were stained with allophycocyanin-conjugated Abs against CD14, CD83, CD86, and HLA-DR or with the appropriate allophycocyanin-conjugated isotype controls. To block unspecific binding, human DCs were incubated with 20% human serum and mouse DCs with mouse BD Fox block (BD Biosciences) for 15 min at 4°C before Ab incubation. Abs were incubated in 0.1% BSA-containing PBS for 30 min at 4°C and washed and fixed in 1% formalin. CFSE-labeled DCs were collected from cocultures with LECs as described in the MLR assay, washed with cold PBS without Ca2+ and Mg2+, and stained for FACS. LECs were released from the plate by mechanical scraping, centrifuged, resuspended in PBS containing 5% FBS, and incubated for 30 min at 4°C with primary Abs to ICAM-1, VCAM-1, or podoplanin. Following washes, appropriate secondary Abs conjugated with Cy5 were added for 30 min at 4°C and cells were washed and fixed in 1% formalin. Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star). The expression levels were reported as the difference between mean fluorescence intensity (MFI) with a specific Ab minus the isotype control MFI.

B2 Integrin activation analysis

Affinity modulation of LFA-1/CD11a was analyzed on DCs and Jurkat cells with the mAb AL-57 (gift from Drs. M. Shimaoaka and T. Springer, CBR Institute for Biomedical Research, Boston, MA), which selectively binds to the active conformation of human LFA-1 (32, 33). The activation
status of Mac-1/CD11b was examined using the mAb CBRM1/5 (eBio-science), which reacts with an activation-specific epitope of human Mac-1 (30). Abs that do not distinguish between activated and nonactivated forms of LFA-1 (MHHM24; GenTex) and Mac-1 (ICRF44; BD Biosciences) were used to quantify total surface expression of these integrins. Human IgG1 (Calbiochem) was used as a control. Epitope expression was examined on unstimulated cells and cells stimulated with EGTA/Mg2+ as described (32, 33), with some modifications. Cells were seeded into a 96-well plate at a density of 2 × 10⁵ cells per well. Following centrifugation, cell pellets were resuspended in 50 μl of activating buffer (HBSS with 5 mM MgCl₂ and 2 mM EGTA) or control buffer (HBSS without Ca2+ and Mg2+) and incubated for 20 min at 37°C in the presence of the Ab (AL-57, MHHM24, CBRM1/5, or ICRF44, all at 20 μg/ml). After washing, cells were stained with the Cy5-conjugated anti-mouse secondary Ab, fixed with 1% formalin, and analyzed by flow cytometry.

**MLR assay**

To assess the impact of LECs on DC functional status, DCs were cocultured with TNF-α-stimulated LECs (2 ng/ml) as described above and adherent and nonadherent cells were collected, washed in cold PBS, and assayed for their ability to stimulate allogeneic T cell proliferation. Nonadherent DCs were collected from LECs after 12 h or 2 h of coculture. To assay adherent cells, nonadherent DCs were removed after 2 h and adherent DCs were collected after a total of 12 h of coculture with LECs with cold PBS without Ca2+ and Mg2+. As an additional control, DCs were cultured in LEC-conditioned medium for 12 h, washed with cold PBS, and assayed in MLR. T cells were prepared by rosetting PBMCs with neuraminidase-treated sheep RBCs as described (34). DCs were irradiated and incubated with 1 × 10⁶ allogeneic T cells at different ratios (DC/T cell ratios, 1:5–1:50) for 5 days. At day 5, cells were pulsed with [3H]thymidine (1μCi/ well; Amersham), harvested after 18 h (PHD cell harvester; Cambridge Technologies), and measured with a beta counter (Beckman 3801; Beckman Coulter).

**Real-time PCR**

The expression levels of human ICAM-1 mRNA were quantified by SYBR Green-based real-time PCR using the Opticon 2 detection system (Bio-Rad Laboratories). Total RNA was extracted from cells using RNeasy mini kit (Qiagen), and cDNA was generated using SuperScript II reverse transcriptase (Invitrogen). Triplicate reactions containing SYBR Green Jump Start Taq ready mix (Sigma-Aldrich), cDNA, and forward and reverse primers were amplified with 40 cycles at 94°C for 40 s, 59°C for 30 s, and 72°C for 30 s with fluorescent data recorded at the end of each cycle in a single step. Data were normalized based on the expression levels of β-actin in each sample. Primers used were as follows: ICAM-1, 5′-TCCGTGCTGTTGA CATGCCAG-3′ (forward) and 5′-AGGCAACGGGCGCTCTATGC-3′ (reverse); and β-actin, 5′-TACACCCACGTGCCCACCTCAGA-3′ (forward) and 5′-CAGCGGAAACCGCCTATGGAATG-3′ (reverse).

**DC migration assays**

FITC-labeled latex microspheres (1 μm diameter; Polysciences) were injected into the footpads of C57BL/6 wild type (WT) and ICAM-1−/− mice (purchased from The Jackson Laboratory as described (35). Two days after microsphere injection, the mice were sacrificed and draining LNs were collected, processed, and stained with an allophycocyanin-conjugated anti-mouse CD86 Ab (BD Biosciences) for FACs analysis. For adoptive transfer, bone marrow-derived DCs (BMDCs) from C57BL/6 WT mice were prepared as described (36) and on day 8 stimulated with 100 ng/ml TNF-α for 24 h. At day 9, CFSE-labeled BMDCs (1 × 10⁵) were injected into each side of the scapular skin of C57BL/6 WT and ICAM-1−/− mice. Two days later, draining brachial LNs were collected and processed and the whole LN sample was analyzed by FACs.

**Statistical analysis**

Statistical significance was determined using the paired Student t test.

**Results**

**Immature DCs adhere to LECs more than mature DCs or macrophages**

APCs traverse LECs en route to the LNs, but the nature of their interactions with the lymphatic endothelium is poorly understood. We sought to examine the ability of LECs to support the adhesion of monocyte-derived APCs under steady-state and inflammatory conditions. Because lymphatic capillaries are points of entry for APCs, we used human LECs derived from the skin microvasculature (27). These cells have been characterized previously and have been shown to express LYVE-1, Prox-1, and podoplanin, typical markers of lymphatic endothelial lineage, and to respond to the lymphangiogenic vascular endothelial growth factor (VEGF)-C by increased growth and tube formation in vitro. Under steady-state conditions, immature DCs (iDCs) generated from CD14+ human monocytes adhered to LECs to a moderate extent (Fig. 1). Adhesion was markedly increased upon the stimulation of LECs with TNF-α (32 vs 79% adherent cells). In contrast, LPS-matured DCs showed very little adhesion to LECs under basal conditions (4.2% adherent cells), and although TNF-α slightly increased binding (6.9% adherent cells), the overall adhesion levels were much lower in comparison with those of iDCs (Fig. 1). To exclude the possibility that the increase in adhesion was due to direct effects of TNF-α on DCs, we compared the binding of iDCs to that of TNF-α-activated LECs in the presence or absence of TNF-α in the medium. As shown in Fig. 1H, the presence of TNF-α in the medium did not change the number of adherent cells, indicating that TNF-α promoted the adhesion of DCs by altering the adhesive properties of LECs. Activated macrophages generated from monocytes with IFN-γ and LPS (37) were even less adhesive than mature DCs (mDCs), whereas PBMCs exhibited higher levels of adhesion to TNF-α/LECs than iDCs (data not shown). These results indicate selectivity in the ability of LECs to support the adhesion of different monocyte-derived cell subsets.

Interestingly, upon prolonged contact with TNF-α-activated LECs (14 h) the morphology of iDCs was dramatically altered (compare Fig. 1, A and B). At early time points, 30 min to 1 h, iDCs that bound to LECs were rounded with very few thin projections. After 14 h of direct contact with TNF-α-activated LECs but in the absence of TNF-α in the medium, the shape of mDCs changed from round to elongated with many projections, assuming a dendritic appearance (Fig. 1, B and C). In contrast, the appearance of iDCs bound to the control unstimulated LECs did not change upon extended contact. After 14 h of adhesion to control LECs, iDCs remained rounded and the number of cells adhering was lower than after 1 h. Together, these data demonstrate that TNF-α-activated LECs strongly support the adhesion of iDCs and affect their phenotypic characteristics.

**LECs express high levels of ICAM-1 upon TNF-α stimulation**

To investigate the mechanism of DC adhesion to the lymphatic endothelium, we next analyzed the expression of cell adhesion molecules that mediate leukocyte adhesion to blood endothelium in the skin lymphatic vasculature. TNF-α stimulation of human skin explants (adult skin and foreskin) strongly induced ICAM-1 expression in lymphatic vessels 24 h after treatment, as determined by immunostaining (supplemental Fig. 1). Low levels of the ICAM-1 protein were detected at 4 h in a subset of small lymphatic vessels, whereas at 24 h many small and large lymphatic vessels strongly expressed ICAM-1. The pattern of ICAM-1 expression on a single lymphatic vessel was heterogenous, as reported previously for the blood vasculature (38). Lymphatic vasculature in control, untreated skin was consistently devoid of ICAM-1 expression. In contrast, ICAM-1 was constitutively expressed by a subset of blood vessels, and its expression was further increased with TNF-α in accordance with previous reports (38). A similar pattern of ICAM-1 expression was observed on lymphatic vessels in mouse skin upon the injection of TNF-α (supplemental Fig. 2). ICAM-2

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and ICAM-3 were not expressed by lymphatic vessels under the conditions examined. Likewise, E-selectin, which is strongly induced with TNF-α in the skin blood vessels, was not expressed by the lymphatic vasculature (data not shown).

This pattern of cell adhesion molecule expression was recapitulated in primary cultures and early passage LECs in vitro. ICAM-1 was most robustly expressed cell adhesion molecule in LECs treated with TNF-α, whereas ICAM-2, ICAM-3 and E-selectin were not expressed (Fig. 2 and data not shown). Immunostaining of TNF-α-treated cultured LECs revealed high levels of ICAM-1 expression that was barely detectable on unstimulated cells (Fig. 2, A–D). Kinetic studies with real-time quantitative PCR demonstrated the onset of ICAM-1 expression at 2 h and maximum expression at 12 h, followed by a sharp decline 48 h later (Fig. 2, E and F). FACS analysis showed that surface expression of ICAM-1 was highly inducible in LECs and that ICAM-1 expression levels were much higher than those of VCAM (Fig. 2G). It is important to note that upon prolonged culture LECs began to express low levels of ICAM-1, ICAM-2, and E-selectin (Fig. 2G and data not shown), which was apparently a tissue culture artifact as this was never observed on lymphatic capillaries in vivo. In summary, these results demonstrate that ICAM-1 is a highly expressed inducible adhesion molecule on LECs.

Adhesion of imDCs to ICAM-1 is mediated by Mac-1

To examine whether ICAM-1 may mediate the adhesion of imDCs to LECs, we next investigated the ability of imDCs to adhere to recombinant ICAM-1-Fc immobilized on a plate. The imDCs adhered to immobilized ICAM-1-Fc to a much greater extent than mDCs (3.8-fold increase), which exhibited minimal binding (Fig. 3). Activation with Mg²⁺/EGTA significantly increased the adhesiveness of both mature DCs and imDCs. For comparison, adhesion levels of nonactivated imDCs were comparable to those of Jurkat cells, whereas adhesion of Mg²⁺/EGTA-activated imDCs was lower than the adhesion of activated Jurkat cells and THP-1 cells (Fig. 3A). Binding of imDCs to ICAM-1-Fc was also greatly augmented by stimulation with PMA or Mn²⁺, and the combination of PMA and Mg²⁺/EGTA showed additive effects (data not shown). Nevertheless, the adhesion of imDCs to recombinant ICAM-1 was much greater than the adhesion of mDCs in all conditions tested, consistent with the data showing higher levels of adhesion of imDCs to LECs. These data demonstrate that ICAM-1 is a ligand for integrins expressed by imDCs.

Next, we investigated the relative contribution of the β₂ integrin receptors LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) for the binding of imDCs to ICAM-1. A function-blocking Ab to LFA-1 (29) did not influence the binding of imDCs to ICAM-1. FIGURE 1. DC adhesion to LECs is increased with TNF-α. A–F, Adhesion of imDCs and LPS/mDCs to control (A and D) and TNF-α-treated LECs (B, C, E, and F) was examined in a static adhesion assay. imDCs showed higher affinity for LEC in steady state (A) and in inflammatory condition (B) than mDC (D and E). Upon contact with TNF-α/LEC (1 ng/ml for 24 h), imDCs changed phenotype from round to dendritic (B and C). In C, arrowheads point to protrusions. In contrast, mDCs did not change morphology upon contact with TNF-α/LEC (E). In F, nuclear staining with Hoechst overlaid on Nomarsky image shows DCs (arrowheads) adhering to confluent LEC (bar, 100 μm). DIC, Differential interference contrast. G, DC adhesion was quantified by FACS and expressed as a percentage of input cells. H, Binding of imDCs to TNF-α/LECs in presence or absence of TNF-α in the medium. Note that there is no difference in the adhesion levels. I, Phenotype of DCs used in the adhesion assays was determined by FACS just before the experiment. Data shown are representative of four independent experiments. Statistical significance was determined with Student’s t test. **, p < 0.01; ***, p < 0.001.
regardless of the DC activation status (Fig. 3B), although it potently inhibited the adhesion of PMA-activated Jurkat cells (data not shown). FACS analysis showed that LFA-1 was expressed constitutively and at high levels by imDCs (Fig. 3C). However, labeling with the mAb AL-57, which specifically recognizes the active high-affinity conformation of the LFA-1 domain (32, 33), revealed that on imDCs LFA-1 was present in the latent form. Affinity up-regulation was not observed upon stimulation with Mg\(^{2+}/\)H\(_{11001}/\)EGTA (Fig. 3C) or when imDCs were cultured in LEC-conditioned medium in the presence or absence of TNF-\(\alpha/H9251\) (data not shown). Jurkat cells served as a positive control and, as expected, demonstrated an increase in LFA-1 affinity upon stimulation with Mg\(^{2+}/\)H\(_{11001}/\)EGTA.

In contrast, a function blocking Ab to Mac-1 (30) strongly diminished the adhesion of imDCs to ICAM-1 in the presence or absence of Mg\(^{2+}/\)EGTA (Fig. 3C) or when imDCs were cultured in LEC-conditioned medium in the presence or absence of TNF-\(\alpha\) (data not shown). Jurkat cells served as a positive control and, as expected, demonstrated an increase in LFA-1 affinity upon stimulation with Mg\(^{2+}/\)EGTA.

Contact of imDCs with LECs impairs their ability to activate T cells
To examine whether interactions between DCs and LECs have functional implications, we next investigated the capacity of DCs
cocultured with the lymphatic endothelium to stimulate the proliferation of allogeneic T cells. DCs were cultured on top of the confluent lymphatic endothelial monolayer, which was pretreated with TNF-α, and the adherent as well as nonadherent cells were assayed in MLR. Coculture of LPS-matured DCs (LPS/DC) with LECs did not affect their ability to activate T cells. As shown in Fig. 4A, the extent of T cell proliferation was not significantly different between the control, adherent, and nonadherent LPS/DCs. Furthermore, LPS/DCs cultured in the LEC-conditioned medium were equally effective in activating T cells as the nonadherent LPS/DCs. However, we found that when immature DCs were cocultured with LECs, their ability to stimulate T cells was significantly enhanced.

**Fig. 3.** imDCs bind ICAM-1 via Mac-1. A, Cell adhesion to ICAM-1-Fc immobilized on the plate with and without Mg<sup>2+</sup>/EGTA stimulation. Note that imDCs adhere to ICAM-1 more than LPS/mDCs. B, imDCs were preincubated with blocking Abs to LFA-1 or Mac-1 and assayed for adhesion to ICAM-1-Fc (20 µg/ml). Binding of control and Mg<sup>2+</sup>/EGTA-activated imDCs is inhibited by blocking Mac-1 but not LFA-1. Data shown are representative of three experiments performed in triplicate. C, To examine the activation state of β<sub>2</sub> integrins on imDCs, cells were incubated with Abs recognizing active conformation of LFA-1 (AL-57 Ab) or Mac-1 (CBRM1/5 Ab) or control Abs (MHM24 and ICRF44, respectively) and analyzed by FACS. Jurkat cells were used as a positive control for Mac-1 activation and negative control for Mac-1. Data are expressed as MFI and are representative of two independent experiments. D, Adhesion of imDCs to TNF-α-treated LECs preblocked with function-blocking Abs to LFA-1 and Mac-1. Note that Mac-1 Ab inhibits adhesion of imDCs, but LFA-1 Ab does not. E, Adhesion of THP-1 cells, which express activated LFA-1 but not Mac-1, was inhibited when blocking LFA-1. Data shown are representative of three experiments performed in triplicate. Appropriate isotype controls were used in all experiments (see Materials and Methods). Statistical significance was determined with Student’s t test. *, p < 0.05; **, p < 0.01.
impaired (Fig. 4B). We excluded any potential direct effects of LECs on T cell proliferation, as LECs alone did not exhibit any effects on T cells in MLR assay (supplemental Fig. 4A). Importantly, the effect of LECs on imDCs was dependent on the direct cell contact, because LEC-conditioned medium did not inhibit the capacity of imDCs to activate T cells. Nonadherent imDCs and imDCs incubated in the LEC-conditioned medium exhibited comparable effects (Fig. 4B), indicating that the soluble factors produced by LECs were not responsible for the inhibition. Thus, we conclude that the direct contact between imDCs and inflamed LECs is essential for restricting the ability of imDCs to stimulate the proliferation of T cells.

Functional impairment of immature DCs was accompanied with the change in expression of the costimulatory molecule CD86. FACS analysis of CFSE-labeled DCs following coculture with LECs showed that the surface expression of CD86 was reduced following adhesion of imDCs to activated LECs (Fig. 4C and supplemental Fig. 4B). In contrast, CD86 expression did not change on nonadherent cells and was comparable to that of control DCs, indicating that the soluble factors produced by LECs are not responsible for the observed change of phenotype. Expression of the maturation marker CD83 was not altered upon coculture of imDCs with LECs under the conditions tested (Fig. 4C). Hence, these findings indicate that TNF-α-activated LECs selectively instruct immature DCs to down-regulate CD86 expression and impair their ability to stimulate T cells.

Effects of LECs on DC maturation are dependent on ICAM-1 and the presence of pathogen-derived signals

In addition to their role in inducing immune responses, DCs have been more recently shown to play an important role in maintaining peripheral tolerance in the steady state (17–19, 22). Our data showing that TNF-α-stimulated LECs suppress the maturation and costimulatory activity of imDCs led us to hypothesize that the interactions of DCs with the lymphatic endothelium during the course of an inflammatory response represent a mechanism for preventing undesired immune reactions. Because in inflammation not only LECs but also DCs will be exposed to inflammatory cytokines, we next asked the question of whether LECs can exert suppressive effects on DCs exposed to TNF-α (TNF-α/DCs). To address this, monocyte-derived DCs were matured with TNF-α, allowed to adhere to TNF-α-activated LECs overnight, and the expression levels of the DC maturation markers CD86 and MHC-II were determined by FACS (Fig. 5). Contact with LECs dramatically decreased surface expression of CD86 on TNF-α-matured DCs (Fig. 5A). Down-regulation of CD86 was ICAM-1-dependent, because preincubation of LECs with the blocking Ab to ICAM-1 completely abrogated this effect. In contrast, when LPS-matured DCs were cocultured with activated LECs, CD86 expression was diminished only marginally (Fig. 5A). The minor decrease in CD86 expression, however, was also dependent on ICAM-1-mediated adhesion to LECs, as blocking Ab to ICAM-1 abolished this effect. In addition, inhibition of ICAM-1-mediated adhesion to LECs increased the...
surface expression of CD83 by TNF-α/DCs, but when DCs matured with LPS were used, CD83 expression was not significantly altered (data not shown). MHCII expression on both TNF-α/DCs and LPS/DCs was slightly lowered upon adhesion to LECs, and this effect was abrogated when adhesion was blocked with the anti-ICAM-1 blocking Ab (Fig. 5B). Blocking ICAM-1 or Mac-1

FIGURE 5. Interaction of TNF-α-matured DCs with ICAM-1 expressed by LECs decreases the expression of CD86 in the absence of Ag. A and B, DCs matured with LPS (DC/LPS) or with TNF-α (DC/TNF-α) were cocultured for 12 h with TNF-α/LECs or TNF-α/LECs preincubated with the anti-ICAM-1 blocking Ab. DCs incubated in the same culture medium for 12 h were used as a control. All DCs from cocultures (adherent and nonadherent) were analyzed by FACS. A, Histograms show that CD86 is down-regulated on DC/TNF-α upon contact with LECs but not on DC/LPS. This effect was reversed by adding a blocking Ab to ICAM-1. B, MHC-II expression on DC/TNF-α or DC/LPS in cocultures with TNF-α/LECs. Note the modest change of MHC-II expression when compared with CD86. Data are expressed as MFI; the percentage of positive cells is indicated in brackets. C, Adhesion of DC/TNF-α to TNF-α-treated LECs preblocked with function-blocking Abs to ICAM-1, Mac-1, or both. Data shown are representative of three independent experiments. Statistical significance was determined with Student’s t test. **, p < 0.01; ***, p < 0.001.
decreased the adhesion of TNF/DCs to LECs to a comparable extent, and the effects of blocking Mac-1 and ICAM-1 together were less than additive, indicating that Mac-1 mediates binding to ICAM-1 on LECs (Fig. 5C). In summary, these data demonstrate that the suppressive effects of LECs on DCs are independent of the DC maturation status but instead depend on the presence or absence of pathogen-associated molecular patterns (PAMPs).

ICAM-1 deficiency correlates with increased DC maturation in vivo

To determine whether ICAM-1 influences the maturation status of DCs as they migrate to the LNs in vivo, we used ICAM-1 knockout (KO) mice (39). Latex beads were injected into the mouse footpads and the migration of monocyte-derived DCs into the LNs was traced 2 days later to remain consistent with our in vitro model that used monocyte-derived DCs. The number of migrated DCs was not significantly different between the WT and ICAM-1-deficient mice (data not shown). However, FACS analysis showed that the expression of CD86 was increased on DCs that migrated in ICAM-1 KO mice upon the injection of FITC-latex beads as compared with WT mice (Fig. 6, A and B). To examine the effects of ICAM-1 deficiency selectively by host cells and to exclude the direct effects of ICAM-1-deficiency on DC maturation, we performed adoptive transfer of WT BMDCs into the ICAM-1 KO mice (Fig. 6, C–E). TNF-α-matured BMDCs down-regulated CD86 expression upon migration to the LNs in WT mice (Fig. 6C). Furthermore, TNF/DCs, which migrated to the LNs in ICAM-1-deficient mice, showed higher levels of CD86 surface expression compared with TNF/DCs that migrated into the LNs of WT mice (Fig. 6, D and E). These results further indicate a role for ICAM-1 in DC maturation and are consistent with our in vitro data showing that the suppression of DC maturation status by LECs is ICAM-1 dependent.

LN sinuses and collecting lymphatics are major sites of ICAM-1 expression

En route to the LNs, DCs could interact with the endothelium of lymphatic capillaries, collecting vessels, and lymphatic sinuses. We examined the pattern of ICAM-1 expression within the lymphatic system to define the most likely site of DC interaction with ICAM-1. Upon inflammation, ICAM-1 was strongly up-regulated on collecting lymphatics leading to the mouse LN (Fig. 7) and on collecting vessels in the cremaster muscle (not shown). Confocal analysis demonstrated ICAM-1 expression on the apical side and the basal side of the collecting vessels (Fig. 7, A–F). Furthermore, ICAM-1 was highly expressed on LN lymphatic sinuses (Fig. 7,

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**FIGURE 6.** The effect of ICAM-1 deficiency on CD86 expression by DCs migrated into regional LNs. A and B, FITC-latex microspheres were injected into the footpads of WT and ICAM-1 KO mice (n = 5). After 2 days, draining LNs were collected (three per mouse) and pooled and the phenotype of migrated DCs with beads was analyzed by FACS. A, Quantitative comparison of CD86 surface expression by migrated DCs. B, Representative plots depicting the entire population of microsphere-bearing cells recovered from the LNs. C–E, Adoptive transfer of TNF-α-matured BMDCs into the back skin of WT and ICAM-1 KO mice (n = 5). C, FACS analysis of CD86 expression on DCs immediately before injection and on DCs that migrated into the LNs of WT mice. D, Comparison of CD86 surface expression on migrated DCs in WT and ICAM-1 KO mice. E, Representative plots depicting the entire population of CFSE-labeled DCs recovered from the LNs. Data shown are representative of at least two experiments. Statistical significance was determined with Student’s t test. *, p < 0.05; **, p < 0.01.
G–L). Surprisingly, LN sinuses expressed ICAM-1 already in a steady state, and the expression was further increased upon TNF-α or latex bead injection. This pattern of expression was very different from that observed in lymphatic capillaries, which were negative for ICAM-1 in a steady state and showed a partial expression upon TNF-α treatment of mouse and human skin (supplemental Figs. 1 and 2). These data suggest that the main sites of DC interaction with ICAM-1 on LECs are collecting vessels and LN sinuses.

Discussion

The lymphatic system is critical for generating efficient immune responses by serving as a conduit for DC traffic from the periphery to the LNs (1, 6). The lymphatic system also plays an important role in dampening inflammation by removing extravasated fluids and inflammatory mediators and cells from tissues. Traditionally, lymphatics have been assigned a passive role in performing these functions and have been viewed primarily as a transportation system. In this study, we demonstrate an active role of the lymphatic endothelium in the modulation of immune response. Our findings show that the direct contact of DCs with the inflamed lymphatic endothelium in the modulation of immune response. Our findings show that the direct contact of DCs with the inflamed lymphatic endothelium results in the down-regulation of the maturation marker CD86 on DCs and the reduction of the ability of DCs to stimulate T cell proliferation. These effects were dependent on adhesive interactions between DCs and LECs, which were mediated by the binding of Mac-1 on DCs to ICAM-1 on LECs.

By using cocultures with human primary LECs (27), we found that immature DCs and DCs exposed to TNF-α (TNF/DC) were significantly more adhesive to LECs than LPS-matured DCs (LPS/DC) and macrophages. This was observed in normal and in inflammatory conditions and indicated the selectivity of LECs in their ability to support the adhesion of different monocyte-derived cell subsets. Only DC subtypes that were highly adhesive to LECs were instructed to down-regulate CD86 expression. These findings identify for the first time the lymphatic endothelium as a source of signals that regulate DC maturation and aid in shaping the emerging concept that the tissue microenvironment plays an important role in the regulation of DC function not only by presenting soluble stimuli but also via direct cell contact. Previously, intestinal epithelial cells were shown to inhibit DC maturation upon contact, resulting in DCs with poor T cell stimulatory capacity (40). Ligation of E-cadherin on immature Langerhans cells inhibited their maturation (41), and it was recently demonstrated that the disruption of E-cadherin-mediated adhesion leads to the generation of tolerogenic DCs (42). These data suggest that the peripheral tissues such as skin and intestine, which face continuous environmental provocation and are therefore particularly prone to aberrant immune system activation, share similar cell contact-dependent regulatory mechanisms that control the maturation of DCs.

We demonstrated that the suppression of DC maturation by LECs was ICAM-1 dependent by using function-blocking Abs. In contrast, ICAM-1 expressed by dermal fibroblasts was shown to promote DC maturation and its ability to activate T cells (43). Similarly, ICAM-1-mediated interaction of DCs with bronchial epithelial cells was shown to induce DC maturation, although it did not alter its capacity to activate T cells (44). Together, these data...
underscore the importance of ICAM-1 signaling beyond its traditional role as an adhesion molecule involved in leukocyte trafficking and indicate the significance of the cellular and tissue context of ICAM-1 in determining its function. The ability of ICAM-1 to mediate such opposite functions could also be explained by its binding to different ligands on DCs, such as CD11a, CD11b, or CD11c. The exact mechanism by which binding of DCs to ICAM-1 modulate DC functional status remains to be investigated. Ligation of ICAM-1 could initiate signaling events that lead to the production of immunomodulatory cytokines by LECs. For example, IL-10 has been implicated in the suppression of DC maturation and function (45, 46). IL-6 plays a major role in maintaining immature, functionally impaired DCs, and its expression can be up-regulated by TNF-α (47, 48). Growth factors such as VEGF can inhibit the functional maturation of DCs (49), and we have shown previously that VEGF is indeed expressed by LECs (27). Because LEC-conditioned medium did not suppress DC maturation, indicating that soluble factors were not involved, these inhibitory signals must remain bound to the cell surface. Regulatory cytokines may also be induced in LECs by TNF-α. In this case, adhesion to ICAM-1 may regulate the bioavailability of the cytokine, allowing for its presentation to DCs only when DCs are in close proximity to LECs, i.e., adherent to ICAM-1. Another intriguing possibility is that the adhesion to ICAM-1 leads to down-regulation of the maturation signals produced by LECs. It has been shown recently that the chemokines CCL19 and CCL21, which are produced by LECs, promote DC maturation (50). Down-regulation of such signals in LECs upon contact with DCs may halt DC maturation.

We report here that Mac-1 on DCs was the main ligand for ICAM-1, as blocking LFA-1 did not diminish DC adhesion to LECs. Adhesion could also be partially mediated by CD11c (α,β2) on DCs, which has been reported to bind ICAM-1 (51), although the physiological relevance of this interaction remains unclear. Expression of Mac-1 by DCs was reported previously (52, 53), and its function has been related to the phagocytic properties of immature DCs. Mac-1 (CD11b/CR3) was shown to play a role in complement-mediated phagocytosis of bacteria and apoptotic cells (54–56). Our data showing that Mac-1 is involved in regulating the functional status of DCs by ligating ICAM-1 on LECs demonstrate a novel function for Mac-1 on DCs. Mac-1 ligation could directly impact cytokine production by DCs. In support of this idea are the data showing that cross-linking of Mac-1 on DCs suppressed the production of immunomodulatory cytokines (55). Recent reports indicate a function of Mac-1 in preventing immune activation upon binding to several different ligands in the absence of PAMPs. Ligation of Mac-1 to complement the C3 fragment iC3b mediated peripheral tolerance to self-Ags (57), and Mac-1 rendered DCs tolerant upon the capture of apoptotic cells (58). It was also reported that upon activation with divergent cations, Mac-1 on DCs can directly inhibit T cell activation (58). These data are consistent with our model showing that Mac-1/ICAM-1 interaction results in immunosuppression.

ICAM-1–Mac-1 interaction may occur as DCs enter lymphatic capillaries at the tissue periphery and travel within the lymphatic system or as they exit lymphatic sinuses in the LNs. We show that ICAM-1 is up-regulated by lymphatic capillaries in inflammation in accordance with the data by Johnson et al. (23). In addition, we demonstrate that LN sinuses and collecting lymphatics are major sites of ICAM-1 expression, suggesting that DCs may interact with ICAM-1 on LECs upon their arrival into the LN. In further support of this concept, our data show ICAM-1 expression on the luminal side and on the basolateral side of the lymphatic endothelium of the collecting vessels. This pattern of ICAM-1 expression has also been reported on capillary LECs in vitro (23) and on IL-1-stimulated HUVECs (59), and it has been proposed to contribute to the bidirectional movement of immune cells across the endothelium (60). Although some studies suggested that ICAM-1 is involved in DC migration to the LNs (23–25), others reported that β2 integrin-mediated adhesion is not required (61). In agreement with the latter, our data did not show a reduced rate of DC migration to the LNs in ICAM-1 KO mice, suggesting that the effects of ICAM-1-mediated DC adhesion to lymphatics are not necessarily linked to migration. The discrepancy of these findings could be explained by the difference in the subset of DCs used in the experiments. Whereas we used immature and semimature TNF-α/DCs, others used mature DCs exposed to Ag in their studies (23, 24). Interestingly, the effects of the lymphatic endothelium on DCs were observed only in the absence of PAMPs (LPS), suggesting that TLR activation renders DCs unresponsive to the signals from LECs. Alternatively, because LPS/DCs adhered to LECs to a much lesser extent than imDCs or TNF/DCs, it is conceivable that by escaping close contact with LECs, LPS/DCs avoid inhibitory signals produced by LECs. These data suggest that signals induced by LPS in DCs override regulation by LECs, because in the presence of a pathogen full immunogenic potential is required and down-regulation of DC maturation is therefore not desirable. It has been recognized more recently that, during steady state, upon the capture of self-Ags imDCs constitutively migrate to the LNs where they induce and maintain peripheral tolerance (17, 18, 20–22). Migration of DCs into draining LNs is greatly increased during inflammation (2, 3, 6), and in this condition DCs can be exposed to proinflammatory cytokines in the absence of a pathogen. Semimature induced by proinflammatory cytokines alone seems to represent a unique developmental, tolerogenic stage for DCs that is characterized by the absence of proinflammatory cytokine production despite high expression of MHC-II and costimulatory molecules (62, 63). In inflammatory conditions, DCs carrying self-Ags and environmental Ags are exposed to inflammatory stimuli while migrating to the LNs, which could result in their maturation and consequently an immune response to self-Ags or nondangerous environmental Ags. Hence, this condition would pose a risk for the induction of autoimmunity. We found that both imDCs and TNF/DCs were responsive to signals from TNF-α-stimulated LECs that lead to suppression of maturation. Based on our results, we propose that the interactions of DCs with the lymphatic endothelium during the course of an inflammatory response might represent one of the control mechanisms for preventing an undesired immune response. In addition to the phenotypic characteristics, the functional status of DCs is determined by their capacity to produce proinflammatory cytokines in the absence of a pathogen. Semi-mature induced by proinflammatory cytokines alone seems to represent a unique developmental, tolerogenic stage for DCs that is characterized by the absence of proinflammatory cytokine production despite high expression of MHC-II and costimulatory molecules (62, 63). In inflammatory conditions, DCs carrying self-Ags and environmental Ags are exposed to inflammatory stimuli while migrating to the LNs, which could result in their maturation and consequently an immune response to self-Ags or nondangerous environmental Ags. Hence, this condition would pose a risk for the induction of autoimmunity.
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Disclosures

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References


Lymphatic Endothelium regulates Dendritic Cell Function

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