Dendritic cells (DC) are migratory cells which exhibit complex trafficking properties in vivo, involving interaction with vascular and lymphatic endothelium and extracellular matrix (ECM). The underlying mechanisms involved in these processes are still ill defined. In the present study we have investigated the ability of DC to interact in vitro with human vascular endothelial cells (EC) and ECM. DC were differentiated from monocytes by in vitro exposure to granulocyte-macrophage colony-stimulating factor and interleukin-13 for 7 days. In adhesion assays a considerable proportion of DC bound to resting EC monolayers: (17% ± 4% mean ± SE of eight experiments). Adhesion to tumor necrosis factor (TNF)-activated EC was increased to 29% ± 5% (n = 8). Binding to resting EC was strongly inhibited by anti-CD11a and CD11b, but not by CD11c monoclonal antibodies (MoAbs); on TNF-activated EC, anti-INTA-4 in concert with anti-CD18 inhibited adhesion by more than 70%. Binding to a natural ECM, derived from cultured EC, or to purified fibronectin was high: 52% ± 6% (n = 8) involved VLA-4 and VLA-5 integrins. In a transmigration assay, 10% ± 2% (n = 6) of input cells were able to cross the EC monolayer. Unlike adhesion, transendothelial migration was significantly reduced by anti-CD31 MoAb. The amount of DC transmigrated through a monolayer of EC was increased twofold to threefold by a defined set of C-C chemokines including RANTES, MIP1α, MIP5, and, to a lesser extent, by MIP1β and MCP-3. Most importantly, in view of the trafficking pattern of these cells, a significant proportion of DC (13% ± 4% of input cells seeded) was able to migrate across the endothelial basement membrane and, subsequently, across the endothelial barrier (reverse transmigration). The adhesion molecules and chemoattractants characterized herein are likely to underlie the complex trafficking of DC in vivo.

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During migration from the tissue of residence to afferent lymphatics, DC interact with extracellular matrix (ECM) and with the endothelial lining of lymphatic vessels. DC interact also with blood endothelial cells (EC) during recruitment of precursors from the blood into tissues and during migration to spleen.8

No information is available on the ability of DC to adhere and to transmigrate through vascular EC. In the present study we have addressed the question of how DC interact with EC and ECM in vitro, which adhesion molecules are engaged, and which chemoattractant signals direct their transendothelial migration. Moreover, we devised a simple methodological approach to investigate the capacity of DC to enter vessels from tissues, which we named reverse transmigration.

MATERIALS AND METHODS

Culture media and reagents. The following reagents were used for separation of effector cells, cell culture, and experimental assays: pyrogen-free saline for clinical use and distilled water (BioWhittaker, Walkersville, MD); medium RPMI 1640 (10 × concentrated; Biochrom KG, Berlin, Germany); medium 199 (GIBCO, Paisley, UK); glutamine (GIBCO); penicillin and streptomycin (GIBCO); aseptically collected fetal calf serum (FCS; Hyclone, Logan, UT). The routinely used tissue culture medium was RPMI 1640 with 2 mmol/L glutamine, 50 µg/mL of gentamicin, 10% FCS.

Cytokines. Human recombinant GM-CSF was from Sandoz (Basel, Switzerland). Human recombinant (r) TNF-α was from BASF (Knoll, Germany). Human rIL-13 and MCP-3 were from Dr R. Todd, Ann Arbor, MI; L29 (IgG1 anti-CD11c; a kind gift from Dr L. Lanier, DNAX Palo Alto, CA); HP2/1 (IgG1 anti-CD1a; a kind gift from Dr F. Sanchez-Madrid, University of Madrid, Madrid, Spain); M89D3 [an F(ab′)2 fragment of anti CD31]; a kind gift from Dr R. Zocchi, Ist. S. Raffaele, Milan, Italy); SK11 (IgG2a anti-CD62L) and RUU-PL (IgG1 anti-CD61) were from Becton Dickinson (Mountain View, CA).

In adhesion or transmigration assays, optimally diluted MoAbs were preincubated with the cells for 20 minutes before plating; medium control contained an isotype-matched irrelevant MoAb. For fluorescence-activated cell sorter (FACS) analysis, cell staining with primary MoAb was followed by fluorescein isothiocyanate (FITC)-conjugated affinity purified, isotype-specific goat anti-mouse antibodies (Valter Occhiena, Torino, Italy). Results are expressed as percent of positive cells and as relative fluorescence intensity (RFI), calculated according to the formula: RFI = Mean Fluorescence (sample) − Mean Fluorescence (control)/Mean Fluorescence (control).

Preparation of EC. Human EC were obtained from umbilical vein (HUVEC) and cultured as previously described.32 Routinely we used confluent cells (10^3/cm^2 culture well) between the first and fourth passage maintained in 199 medium with 20% bovine serum (Hyclone) supplemented with endothelial cell growth supplement (50 µg/mL; Collaborative Research Inc, Lexington, MA) and heparin (100 µg/mL; Sigma Chemical Co, St Louis, MO). The purity of EC cultures was checked by expression of von Willebrand factor and found to be greater than 99% positive.

Adhesion assay. Adhesion of DC to EC was studied as described previously.33 EC were grown to confluence in flat-bottomed 48-well trays.34Cr-labeled DC (Amersham, UK) were coincubated with EC monolayers at 37°C for 1 hour. At the end nonadherent cells were washed away and adherent cells were solubilized with 0.1 mL of 0.1% sodium dodecyl sulfate and radioactivity was counted in a gamma counter. Results represent the percent of adherent cells ± SD of three replicates/group.

Transmigration and reverse transmigration assay. This assay was performed as previously described.35 In brief, EC were grown to confluence on polycylinipyrrolidone (PVP)-free polycarbonate filters (5-µm pore) and mounted on modified Boyden chambers over a nitrocellulose filter.36Cr-labeled DC were seeded in the upper compartment and coincubated with EC monolayers for 1 hour at 37°C. Nonadherent cells were gently washed away, the radioactivity in the double filter and in the lower compartment referred to transmigrated cells. The adherent cells were considered to comprise both cells bound to EC as well as those that had transmigrated.

In the reverse transmigration assay an upper polycarbonate filter was coated with ECM, and the lower polycarbonate filter was coated by a monolayer of EC, placed upside down, and mounted in the Boyden chamber. The ECM was prepared by growing a monolayer of EC on filters for at least 5 days; EC were then stripped away by a short treatment with a 20 mmol/L NH4 OH solution for 30 seconds. In this assay the radioactivity present in the lower compartment only accounted for the transmigrated cells.

In some experiments polycarbonate transwell inserts (5-µm pore; Coming, Costar, Cambridge, MA) were used. Inserts were coated with EC monolayers; chemoattractants, usually 100 ng/mL, were seeded in the lower compartment.

RESULTS

In vitro adhesion of DC to EC monolayers and ECM. DC were differentiated in vitro from highly purified human monocytes in the presence of GM-CSF + IL-13 for 7 days. As previously reported these cultured DC expressed high levels of CD1a and MHC class II and had low levels of CD14. As APC,
they were strong stimulators of allogeneic T-lymphocyte proliferation in MLR.20,26

In preliminary experiments we evaluated the role of IL-13 on the adhesive ability of cultured DC. There was a concern in performing adhesion assays with cells cultured in the presence of IL-13, as we reported that IL-4, a functionally related cytokine, strongly inhibits leukocyte adhesion to vascular endothelium.35 To verify this possibility, DC were washed at day 5 of culture to remove IL-13 and then incubated for 2 additional days in the presence of GM-CSF only. These cells (termed DC w/o IL-13) were compared with conventional DC cultures exposed to IL-13 for 7 days (DC + IL-13). In vitro adhesion to monolayers of resting or TNF-activated EC indeed showed that adhesion of DC w/o IL-13 was higher compared with DC + IL-13 (Fig 1).

Analysis of adhesion molecules, relevant for interaction with the vascular endothelium, showed that DC + IL-13 expressed lower levels of CD11a and VLA-4 than DC w/o IL-13 (Fig 2A).

It was important to verify that removal of IL-13 in the last 2 days of culture did not prevent DC differentiation. Fig 2A shows that CD1a and MHC class II were expressed to the same extent in both populations. Figure 2B shows that DC w/o IL-13 were more potent APC for allogeneic T cells, compared with DC + IL-13 for the whole period of culture. At 1% APC stimulation index (SI) was 28 and 48 for DC + IL-13 and DC w/o IL-13, respectively. Based on these results all subsequent experiments were performed with DC cultured in the absence of IL-13 for the last 48 hours.

Analysis of expression of β1 and β2 integrins is reported in Fig 3. DC expressed high levels of CD11b and CD11c and intermediate levels of CD11a. Among β1 integrins, CD49d and CD49e (VLA-4 and VLA-5) were expressed at high levels. VLA-4 levels were variable among donors (26.3% ± 13%; range, 3.4 to 77.3; mean of five experiments). All the cells stained positive for CD61 (β3) and CD31 (PECAM-1), and were negative for CD62L (L-selectin).

The identification of adhesion molecules involved in the binding to resting EC was performed by using functional blocking MoAb. Anti-CD11a, anti-CD11b, and anti-CD18

Fig 1. Adhesion of cultured DC to monolayers of resting or TNF-activated EC (10 ng/mL for 24 hours). DC were differentiated from blood monocytes by 5-day culture with 50 ng/mL GM-CSF and 10 ng/mL IL-13. (○) DC were cultured for 2 additional days in the presence of GM-CSF and IL-13. (□) Cells were washed and cultured for 2 additional days without IL-13.3H-Tdr-labeled DC were coincubated for 1 hour at 37°C with EC monolayers. Results are expressed as percent of adherent cells, mean ± SEM of three replicates. Three distinct experiments are shown.

Fig 2. (A) Characterization of adhesion molecules expressed by DC. After 5 days in vitro with GM-CSF and IL-13, DC were cultured for 2 additional days in the presence (○) or absence (□) of IL-13. Cells were labeled with the designed MoAb and then with FITC-labeled goat anti-mouse Ig. (B) Mixed leukocyte reaction (MLR).Responder cells were allogeneic T lymphocytes depleted of autologous monocytes and B lymphocytes; they were plated at 1 x 10^5 cells/well. DC cultured for last 2 days with (○) or without (□) of IL-13 were mixed at the indicated concentrations. 3H-Tdr was added in the last 18 hours of a 5-day experiment.
MoAbs partially inhibited adhesion (52%, 64%, and 68%, respectively, in the representative experiments shown in Fig 4A). It is of interest that CD11c, although expressed to a very high extent on DC, is not involved in binding to endothelium. These results were confirmed in a larger series of experiments (mean inhibition with anti-CD18, 61% ± 4%; mean SE of seven experiments).

On TNF-activated EC expressing high levels of ICAM-1 and VCAM-1, basal adhesion of DC was increased from 17.3% ± 4.4% to 28.7% ± 5.6% (mean ± SE of eight experiments). Anti–VLA-4 alone did not inhibit adhesion (not shown) but the combination of anti-CD18 + anti–VLA-4 was more inhibitory compared with anti-CD18 alone (Fig 4B), a phenomenon already observed with other leukocytes.33 These results show the important role of VLA-4 in leukocyte adhesion, although it is secondary to β2 integrins.

Binding of DC to a natural ECM, derived from cultured EC, or to purified fibronectin, was higher than to EC monolayers: 52% ± 6% (mean ± SE of eight experiments) (not shown).

Direct and reverse transendothelial migration. We previously described an in vitro method to measure transmigration through monolayers of EC cultured on porous polycarbonate filters.34 In this assay, freshly isolated monocytes showed to have high adhesive (40% ± 13%) and transmigrating ability (16.8 ± 2; mean ± SE of three experiments) compared with other leukocytes.34 In vitro cultured DC, differentiated from monocytes, effectively transmigrated through resting EC monolayers. The proportion of adhesive and transmigrated cells compared with input cells in a 1-hour assay was 35.8% ± 2% and 10.6% ± 1.6%, respectively (mean ± SE; n = 6). Because migration is preceded by adhesion, anti-CD18 inhibited both adhesion (79%) and transmigration (75%) in the representative experiment shown in Fig 5. Treatment of EC with anti-CD31 MoAb did not affect adhesion, but did inhibit transmigration by 38% (Fig 5).

GM-CSF augments the adhesive properties of monocytes.36 Therefore, experiments were performed with DC washed away of GM-CSF and rested for 24 hours. Adhesion and transmigration values were not different from conventional cultures: eg, 28% ± 1% (adhesion) and 7.5% ± 1% (transmigration) for DC with GM-CSF; 27% ± 4% and 8.6% ± 1% for DC rested without GM-CSF (mean ± SE of two experiments).

Circulating DC precursors migrate from the blood compartment into tissues. From there, DC reach lymphoid organs via lymph or blood.4 Therefore, we established an assay to mimic the tissue-to-lymph/blood part of the natural history of DC (reverse transmigration). In this assay the upper filter of the two-filter system is coated with ECM, and the lower filter is coated by a monolayer of EC, placed upside down. As shown in Fig 6A, this natural matrix was highly adhesive for DC. Anti–VLA-4 + VLA-5 MoAbs or anti-CD29 inhibited most of the binding (>75% inhibition) while (as expected) anti-CD18 had no effect. Figure 6B shows the results of two experiments of reverse transmigration. The mean amount of transmigrated cells relative to input was 13% ± 4%, which corresponded to 20.8% of adherent cells.

Response of DC to chemokines. We and others have previously reported that cultured DC migrate in vitro in response to chemoattractants including formyl peptides and C5a and a distinct set of CC and CXC chemokines.22-26 Therefore, it was important to assess whether CC chemokines affected the capacity of DC to interact with and migrate across EC monolayers. Polycarbonate Transwell inserts were coated with a monolayer of EC and chemoattractants were seeded in the lower compartment. As shown in Fig 7, increased migration was induced in response to MIP-1α, RANTES, HCC2/MIP-5, and to a lesser extent to MIP-1β and MCP-3. These results are in line with our previous findings of chemotactic response in a classical chemotaxis assay.22,26

DISCUSSION

The present study is the first analysis of the interaction of DC with EC and their ECM. DC obtained from monocytes by culture with GM-CSF and IL-13 expressed CD31, the β2 integrins LFA-1, Mac-1, and p150,95, the β1 integrins VLA-4 and VLA-5, but were negative for other β1 integrins and for L-selectin. They bound resting and activated EC via LFA-1, Mac-1, and VLA-4, and EC-derived ECM via VLA-4 and VLA-5. DC were able to migrate across EC monolayers; unlike adhesion, transendothelial migration involved engagement of...
CD31. Most importantly, in view of the trafficking of these cells, DC were able to perform reverse transmigration.

We and others have reported that DC express receptors for chemokines and respond to chemotactic signals. Active chemoattractants included formyl peptides and C5a and a distinct set of CC and CXC chemokines, which overlaps with but is distinct from those active on other leukocytes. In the present study we assessed the response of DC to CC chemokines in a transendothelial migration assay. RANTES, MIP-1α, and HCC2/MIP-5 augmented the transmigration of DC. Intriguingly MCP-3, a strong attractant in a conventional assay, had little effect in this assay. The reason for the dissociation of the capacity of this chemokine to attract DC in the presence and absence of an EC monolayer is elusive. One could speculate that at least the EC population used here does not efficiently present MCP-3 to migrating DC.

The interaction of leukocytes with the luminal surface of vascular endothelium and their passage into tissues has been extensively investigated in vitro in adhesion and transmigration assays. Among leukocytes, lymphocytes and DC have the ability to undergo the reverse process of migrating from tissue into the lumen of blood or lymphatic vessels. In an effort to model this tissue-to-lymph/blood component of the natural history of certain leukocytes we have set up a simple reverse transmigration assay. As expected, DC were able to migrate across the basal membrane and then through the endothelial monolayer to reach the luminal surface. The reverse transmigra-
tion assay may represent a new useful tool to investigate this aspect of leukocyte trafficking.

DC are heterogeneous populations of BM-derived elements. One pathway of DC differentiation is closely related to the monocyte-macrophage lineage. The population used in the present study was obtained by culturing monocytes with GM-CSF and IL-13 and therefore differentiated from monocytic precursors. It is well established that CD34+ precursors cultured with stem cell factor, GM-CSF, and TNF give rise to a population containing different types of DC. At early time points of culture two subsets have been phenotypically identified: one CD1a+, CD14-, the other CD1a-, CD14+. Both subsets eventually mature into typical DC: the CD1a precursors generate cells with the typical features of epidermal Langerhans cells, while the CD14+ precursors, related to the monocytic lineage, express markers of dermal DC. In a limited series of experiments (n = 4) we tested DC obtained from CD34+ cord blood cells, cultured as described above. The CD34+-derived DC behaved similarly to monocyte-derived DC in terms of expression of adhesion molecules and binding ability. Adhesion to activated EC was 22% ± 3% of input cells and anti-CD18 used in concert with anti–VLA-4 inhibited up to 80%. Hence, at least some of the results reported here with monocyte-derived DC may also apply to CD34+-derived DC.

EC are heterogeneous in terms of morphology and function, both within the same vascular bed and among different organs. Lymphatic EC in particular differ considerably from blood-vessel EC. In the present study we used conventional HUVEC as a model EC population. Further work and the development of appropriate reliable culture technique will be required to

**Fig 6.** Adhesion to ECM and reverse transmigration of DC. (A) Molecules involved in the adhesion of DC to EC-derived matrix (ECM). ECM were prepared by culturing a monolayer of EC on polycarbonate filters for at least 5 days; EC were then stripped by an NH4OH + Triton solution for 30 seconds. Results are expressed as percent of adherent cells, mean ± SEM of three replicates. **Statistically significant at P < .01 versus medium control. (B) Reverse transmigration of DC. A double-filter system was used. The upper filter was coated with an ECM and the lower filter was coated with a monolayer of EC placed upside down. 51Cr-labeled DC were seeded in the upper compartment of the chamber and coincubated with EC monolayer for 3 hours at 37°C. Results are expressed as percent of adherent and transmigrated cells (see Materials and Methods), mean ± SEM of three replicates. Two independent experiments are shown.

**Fig 7.** Effect of chemokines on DC transendothelial migration. Polycarbonate Transwell inserts were coated with a monolayer of EC and chemoattractants (100 ng/mL) were seeded in the lower compartment. 51Cr labeled DC were placed in the upper compartment and incubated for 1 hour at 37°C. **Statistically significant at P < .01 and *P < .05 versus medium control.
analyze the interaction of DC with more relevant EC populations such as those that line lymphatics. DC undergo complex trafficking patterns.\(^6\) Circulating precursors localize in tissues and this process can be increased by local inflammation. At epithelial lining surfaces, after antigen capture, DC traffic to lymph nodes via lymphatics. The same route is followed by DC present in the interstitium of organs such as heart and kidney; however, these cells can also reach the spleen via the blood stream. A subset of DC present in the liver can capture circulating antigen and travel along the lymph/lymph nodes as well as the blood/spleen route. The molecular pathways of interaction with EC and ECM proteins described herein are likely to be important in at least some of the trafficking patterns of DC.

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Adhesion, Transendothelial Migration, and Reverse Transmigration of In Vitro Cultured Dendritic Cells

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