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CD14⁺CD34⁺ Peripheral Blood Mononuclear Cells Migrate Across Endothelium and Give Rise to Immunostimulatory Dendritic Cells

Elisabetta Ferrero,*, Attilio Bondanza,*, Biagio E. Leone,‡ Simona Manici,*, Alessandro Poggi,§ and Maria Raffaella Zocchi*

We describe a subset of peripheral CD14⁺ cells, coexpressing the CD34 progenitor marker and able to migrate across endothelial cell monolayers. On culture with granulocyte-macrophage-CSF, this population differentiated into dendritic cells expressing CD83, CD80, HLA-DRbright, CD86, and CD54. These dendritic cells were immunostimulatory, in that they induced proliferation of allogenic and tetanus toxoid-specific T lymphocytes. The CD14⁺CD34⁺ population expressed higher levels of platelet endothelial cell adhesion molecule-1 (PECAM-1) and α4β1 integrin than the CD14⁺CD34⁻ counterpart, being dull positive for other integrins. Using stably transfected PECAM-1⁺, VCAM-1⁺, or ICAM-1⁺ cells, we found that PECAM-1 and, to a lesser extent, VCAM-1, could support transmigration of CD14⁺CD34⁺ cells, whereas the αL-ICAM-1 interaction was involved in cell adhesion. PECAM-1-driven transmigration was conceivably dependent on a haptotactic gradient, as it was reduced by 80% across NIH/3T3 cells transfected with the PECAM-1Δcyto deletion mutant. This mutant lacks the cytoplasmic tail and displays a reduced tendency to localize at the intercellular junctions, thus failing to form a molecular junctional gradient. Once differentiated, dendritic cells derived from CD14⁺CD34⁺ precursors retained their transendothelial migratory capability, using both PECAM-1 and ICAM-1 for transmigration. We suggest that a subset of CD14⁺CD34⁺ circulating leukocytes can localize to peripheral tissues and differentiate into functional dendritic cells, thus representing a functional reservoir of potential APC. PECAM-1, constitutively expressed on vascular endothelium, is likely to play a relevant role in the egress of this population from the bloodstream. The Journal of Immunology, 1998, 160: 2675–2683.

Monocytes (Mo) are derived from bone marrow precursors that circulate in the blood and eventually traverse vascular endothelial lining to enter tissues, where they differentiate into macrophages or dendritic cells (DC) after inflammatory or immunologic stimuli (1, 2). Transmigration of mature Mo, expressing CD14, is a multistep process that follows the well-known stepwise model of rolling, mediated by selectins (3), cell adhesion, involving integrins (4, 5), and migration (3, 6). The last step is likely to depend on several junctional and nonjunctional molecules, expressed both by leukocytes and endothelial cells; among them, the platelet endothelial cell adhesion molecule-1 (PECAM-1) and the CD34 molecule itself and ligands of the selectin family is thought to be important for the initial binding of hemopoietic progenitors to endothelial cells (13). Likewise, members of the integrin family, such as the α4β1 integrin, proved to be important for progenitor cell trafficking both in vitro and in vivo (14, 15). Recently, PECAM-1/CD34 has been reported to enhance the adhesive sity of α4β1 integrin expressed by hemopoietic progenitor cells (16).

Mobilization of CD34⁺ cells from bone marrow is a rare event in the adult life, and it is conceivably due to changes in the expression, or affinity to their ligand, of different adhesion molecules during differentiation (17–19). Indeed, the adhesion molecules expressed on those progenitor cells that are committed to leave the bone marrow, should acquire a higher affinity for counterreceptors expressed by endothelial cells than by stromal cells and extracellular matrix (20–22). Likewise, the ability to localize to peripheral tissues depends, in mature leukocytes, on a sequence of molecular events that are tightly connected to each other (3–6). These events can also be regulated by local conditions, such as cytokine production during inflammation, Ag sensitization in the immune response, and secretion of chemokines (23, 24).

We show that a subset of CD14⁺ PBMC, coexpressing the CD34 precursor marker, is able to migrate across endothelial cells.
and differentiated into immunostimulatory DC. Migration is apparently mediated by the PECAM-1 molecule through a haptotactic gradient. Once differentiated, DC can use both PECAM-1 and vascular cell adhesion molecule-1 (VCAM-1) for transmigration.

Materials and Methods

Monoclonal antibodies

The purified, or the FITC- or phycoerythrin (PE)-conjugated, anti-CD14, anti-CD80 (B7.1), anti-CD86 (B7.2), and the anti-surface Ig (sIg) mAbs were purchased from Becton Dickinson (San Jose, CA); the anti-ICAM-1 (CD54; 84H10), the anti-CD4 (HPCA-1)-Ab were purchased from Immunotech (Luminy, Marseille, France); the anti-CD106/VCAM-1 (BB6a) mAb were purchased from British Biotechnology (Oxford, U.K.). The anti-HLA-DR mAb (D1.12) was kindly provided by R. Accolla (ABC, Genoa, Italy), the anti-α4 integrin (CD49d) mAb (HP2/1) was provided by F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain), the anti-CD36 mAb (NL07) was a kind gift of M. Alessio (Dibit, Milan, Italy), and the anti-human mannose receptor (pan1) was a gift of A. Mantovani (Istituto Farmacologico M. Negri, Milan, Italy). The anti-CD11a/α4 integrin mAb 70H12 and the anti-CD31/PECAM-1 mAb M89D3 were obtained in our laboratory (25, 26), and the anti-CD1a (OKT6) mAb was obtained from the American Type Culture Collection (Rockville, MD). These mAbs were purified from ascites fluids by affinity chromatography. All the purified Abs were used at concentrations of 5 μg/ml in functional studies and at 1 μg/ml in immunofluorescence. When indicated, to avoid any unspecific effect due to FcγR binding, mAbs were pepsin digested. F(ab')2 fragments were prepared according to the method of Parham (27). PE-conjugated goat anti-isotype mouse IgGs (GAM) was from Zymed Laboratories Inc. (South San Francisco, CA).

Cell isolation and sorting

Peripheral blood CD14+ cells were isolated from healthy donors (buffy coats, kindly provided by the Blood Transfusion Department of our institute), after density gradient centrifugation, according to the method of Salustio and Lanzavecchia (28). CD34+ and CD34− cell subsets were fractionated using immunomagnetic beads (Dynal, Milan, Italy) according to the manufacturer's procedure. Purified T cells were obtained after two rounds of plastic adherence followed by immunodepletion of CD14+ and HLA-DR+ cells. When indicated, cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (Biochrom, Berlin, Germany), 10% heat-inactivated FCS (PAA Labor, Linz, Austria), and 20 ng/ml recombinant granulocyte-macrophage CSF (GM-CSF) as described (29). Media were endotoxin free (Worthington, Lake Kawana, NJ), antibiotics as above. For TT presentation, DC were loaded with TT for 12 h before addition of specific T cells. After 3 days, for the allogeneic reaction Antibiotics as above. For TT presentation, DC were loaded with TT for 12 h before addition of specific T cells. After 3 days, culture in T cell growth factor (Lymphocult, Biotest Diagnostics Inc., Morrisville, VA). Transfection was performed with the FITC- or PE-conjugated anti-CD14 and the PE-conjugated anti-CD34 mAbs or the FITC-anti-sIg antiserum. After washing, samples were run on a FACStar® equipped with an argon ion laser (Becton Dickinson, Mountain View, CA), gated to exclude cell debris and nonviable cells. At least 5000 events/sample were analyzed. Results are expressed as mean red fluorescence intensity vs mean green fluorescence intensity (arbitrary units, a.u.) or mean log fluorescence intensity (a.u.).

Analysis of Ig gene expression

Ig heavy chain gene rearrangement of TT-specific T cell lines alone was performed using the FITC- or PE-conjugated anti-CD14 and the PE-conjugated anti-CD34 mAbs or the FITC-anti-sIg antiserum. After washing, samples were run on a FACStar® equipped with an argon ion laser (Becton Dickinson, Mountain View, CA), gated to exclude cell debris and nonviable cells. At least 5000 events/sample were analyzed. Results are expressed as mean red fluorescence intensity vs mean green fluorescence intensity (arbitrary units, a.u.) or mean log fluorescence intensity (a.u.).

Adhesion and transmigration assay

HUVEC were isolated and cultured as described (30). Cells were within four passages. Adhesion or transmigration of radiolabeled 125I or DuPont NEN, Boston, MA), unsorted CD14+ or CD14+CD34+ and CD14+CD34− sorted cells, through HUVEC, ICAM-1, VCAM-1, PE CAM1, or PECAM-1-transfectants, was performed with Transwell cell culture chambers (polycarbonate filters, 3-μm pore size, Costar, Cambridge, MA) as described (9). In some samples, cells were precultivated for 15 min at 4°C with saturating amounts (5 μg/ml) of the anti-CD31 (M89D3), anti-CD11a (70H12), or anti-CD49d (HP1) (Fab')2 and washed twice before the onset of the transmigration assay. At different time points (4 or 12 h), migrated cells were recovered from the lower compartment and lysed with 100 mM Tris buffer containing 0.1% Triton X-100. The radioactivity of the samples was measured in a gamma counter (Packard, Sterling, VA). Results are expressed as percent migrating cells, calculated as described (9), i.e., (cpm of lysates from migrated cells/cpm of lysates from cells of the total input) x 100. In some experiments, both migrated and nonmigrated cells were recovered and cultured for 3, 7, or 14 days in the presence of GM-CSF and tested in the transmigration assay.

Mixed lymphocyte reaction and Ag-specific stimulation

To test the immunostimulatory potential of migrated and nonmigrated cells cultured with GM-CSF, after 7 days of culture cell derived from either CD14+CD34+ or CD14+CD34− cell precursors were used as stimulators for allogeneic or tetanus toxoid (TT, kindly provided by S. Burastero, HSR-Dibit, Milan, Italy)-specific T lymphocytes at different T:DC ratios. TT-specific T cell lines were obtained by stimulation of syngeneic PBMC with TT (10 μg/ml), Percoll gradient separation of T cell blasts after 7 days, culture in T cell growth factor (Lymphocult, Biotest Diagnostics Inc., Dreieich, Germany), and weekly restimulation with TT. About 105 allogeneic or 2 x 104 TT-specific syngeneic T cells, washed, and maintained without lymphocult for 48 h. were added to irradiated (4000 rad) DC (from 2 x 104 to 1 x 105) in 96-well round bottom microwell plates in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS and antibiotics as above. For TT presentation, DC were loaded with TT for 12 h before addition of specific T cells. After 3 days, for the allogeneic reaction (MLR), or 48 h for TT-specific response, cells were pulsed with 1 μCi of [3H]TdR (DuPont NEN) per well for the last 18 h of culture, harvested, and counted in a beta counter (Packard). Tests were conducted in triplicate, and results are expressed as mean cpm ± SD. [3H]TdR uptake by stimulatory DC or responder allogeneic T cells alone was <100 cpm; [3H]TdR uptake by TT-specific T cell lines alone was <1000 cpm (not shown).
Results

**CD14+ PBMC coexpressing CD34 transmigrate across HUVEC monolayers**

CD14+ cells collected from peripheral blood of healthy donors usually represent mature Mo; however, we found that a small proportion of these cells, ranging from 5 to 10%, coexpress the CD34 molecule (Fig. 1A shows a representative experiment of 10), which is a marker of hemopoietic progenitors (31). CD34 expression in this cell population was also confirmed by Western blot (not shown). Since CD34 is usually lost during leukocyte maturation, the CD14+CD34+ cell population might represent an intermediate stage of differentiation toward mature Mo. This subset of double positive cells can transmigrate across endothelial cell monolayers: indeed, when purified CD14+ cells were used in a transmigration assay, almost all the cells (95%) recovered from the lower chamber after 12 h were CD34+ (Fig. 1B). This transendothelial migration apparently occurs in the absence of chemokines, because we did not find detectable levels of monocyte chemoattractant protein 1 (MCP1), MCP2, or IL-8 in the supernatants of HUVEC after 3 days of culture (not shown). The migration ability of sorted CD14+CD34+ cells was compared with that of CD14+CD34- cells. As shown in Figure 1C, only the former subset transmigrated without the addition of exogenous chemokines, whereas the latter could migrate only after addition of 50 nM MCP1 (not shown), in keeping with reported data (32). Along this line, Table I shows that CD14+CD34- cells displayed a higher tendency to adhere to HUVEC, compared with CD14+CD34+ cells, and this adhesion is blocked by anti-CD11a mAbs.

**CD14+CD34+ cells express high levels of CD31/PECAM-1 driving transmigration**

Both peripheral blood and bone marrow CD34+ precursors express β1 or β2 integrins and PECAM-1 (15, 16). We found (Fig. 2 shows a representative experiment) that the CD14+CD34+ cell subset expresses higher levels of PECAM-1 (mean fluorescence intensity (MFI) ± SD from 10 independent experiments, 110 ± 4 a.u.) than the CD14+CD34- counterpart (MFI 28 ± 3 a.u.). Likewise, the CD34+ cell population was brightly positive for the α4 integrin (MFI 80 ± 5 a.u.), whereas the CD34- cells were weakly stained with the anti-α4 integrin mAb (MFI 25 ± 3 a.u.). Conversely, the α4 integrin was highly expressed on CD14+CD34+ cells (MFI 66 ± 7 a.u.), but not on the CD14+CD34- cell subset (MFI 22 ± 5 a.u.).

To investigate whether the higher expression of PECAM-1 and α4 integrin by CD14+CD34+ cells was responsible for transendothelial migration, transmigration assays were performed using purified CD14+CD34+ cells and NIH/3T3 cell monolayers stably expressing PECAM-1, VCAM-1, or ICAM-1. Both PECAM-1 (Fig. 3A) and, to a lesser extent, VCAM-1 (Fig. 3B) transfectants proved to support CD14+CD34+ cell migration, at variance with ICAM-1+ cell monolayers (Fig. 3C). Migrated cells were detectable in the lower Transwell compartment after 4 h (not shown), reaching the maximum after 12 h (Fig. 3), in particular when transmigration was evaluated across PECAM-1-transfected cell monolayers (Fig. 3A). Pretreatment of migrating cells with the F(ab')2 of

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<tr>
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<td>25 ± 5</td>
<td>50 ± 4</td>
</tr>
<tr>
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<td>Anti-CD31</td>
<td>18 ± 4</td>
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* Sorted CD14+CD34+ or CD14+CD34- cells were radiolabeled (¹⁵⁸Cr, NEN Dupont) and used in adhesion assays to HUVEC. In some samples, cells were preincubated for 15 min at 4°C with saturating amounts (5 µg/ml) of the anti-CD31 (M89D3), anti-CD11a (70H12), or anti-CD49d (BP2/1) Fab', and washed twice, before the onset of the adhesion assay. After 2 h, nonadherent cells were washed out, and adhered cells were lysed with 100 mM Tris buffer containing 0.1% Triton X-100. The radioactivity of the samples was measured in a gamma counter (Packard). Results are expressed as percent migrating cells (mean ± SD from six independent experiments), calculated as described (9); i.e., (cpm of lysates from migrated cells/cpm of lysates from cells of the total input) × 100.

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**FIGURE 1.** Transendothelial migration of CD14+CD34+ peripheral blood precursors. A. Adherent cells from peripheral blood stained with the PE-anti-CD34 and the FITC-anti-CD14 mAbs. B. Migrated cells collected from the lower chamber after 12 h and stained with the same mAbs as in A. Results are expressed as mean red fluorescence intensity vs mean green fluorescence intensity and are representative of 20 donors analyzed. C. Radiolabeled CD14+ or sorted CD14+CD34+ or CD14+CD34- cells were used in the transendothelial migration assay. At different time points (4 or 12 h), migrated cells were recovered from the lower compartment and lysed. The radioactivity of the samples was measured in a gamma counter. Results are expressed as percent migrating cells (mean ± SD from 10 independent experiments).
mAbs directed against either PECAM-1 (Fig. 3A) or α4 integrin (Fig. 3B) prevented cell migration. Likewise, preincubation of PECAM1transfectants with the (Fab')2 of M89D3 mAb abrogated cell migration (not shown), suggesting that a CD31/PECAM-1-mediated homophilic adhesion was operating. This was also supported by the observation that the αvβ3 integrin, another reported ligand for PECAM-1 (33), was virtually absent from both CD14+CD34+ cells and PECAM-1+ transfectants (not shown). Background migration across mock-transfected NIH/3T3 or CHO cells was always 10% (9).

We have recently reported that, unlike ICAM-1, PECAM-1 can drive transmigration of a subset of lymphocytes by creating a haptotactic gradient because of preferential intercellular localization of the molecule in endothelial and transfected fibroblast monolayers (8, 9). To investigate whether a similar mechanism is involved in CD14+CD34+ cell migration, we analyzed their capacity of migrating across monolayers of NIH/3T3 cells transfected with the PECAM-1-Dcyto deletion mutant. This mutant lacks the cytoplasmic tail and displays a reduced tendency to localize at the intercellular junctions, thus being homogeneously distributed along the plasma membrane (9). Transmigration was reduced by 80% using monolayers of NIH/3T3 cells transfected with the PECAM-1-Dcyto mutant (Fig. 3D), indicating that a PECAM-1 haptotactic gradient can drive transmigration of CD14+CD34+ cells.

**FIGURE 3.** PECAM-1-driven transmigration of CD14+CD34+ cells. Transmigration assay was performed in a Transwell system through confluent PECAM-1 (A), VCAM-1 (B), ICAM-1 (C), or PECAM-1-Dcyto (D) transfectants. In some samples, CD14+CD34+ cells were preincubated for 15 min at 4°C with saturating amounts (5 μg/ml) of the anti-CD31 (M89D3) or the anti-CD49d (HP2/1) or the anti-CD11a (70H12) Fab’2, and washed twice, before the onset of the transmigration assay. After 12 h, migrated cells were recovered from the lower compartment and lysed. The radioactivity of the samples was measured in a gamma counter. Results are expressed as percent migrating cells (mean ± SD from 10 independent experiments).

**FIGURE 2.** CD14+CD34+ cells express high levels of CD31/PECAM-1. CD14+CD34+ or CD14+CD34+ cells (10⁵/sample) were stained with the various mAbs followed by the anti-isotype-specific FITC-conjugated GAM. After washing, samples were run (5000 events/sample) on a FACStar™ equipped with an argon ion laser (Becton Dickinson), gated to exclude cell debris and nonviable cells. Results are expressed as log green fluorescence intensity vs number of cells. One representative experiment of 10.

**Migrated CD14+CD34+ cells can differentiate into functional DC**

Migrated CD14+CD34+ cells (Fig. 4, a and b) expressed the CD80 Ag (Fig. 4c), which is a DC hallmark (34), beside CD86 (not shown), and very low levels of CD36 (Fig. 4d), a marker of mature Mo or macrophages (35). This would suggest that CD14+CD34+ cells might represent a subset of precursors capable of differentiating into DC, rather than Mo. Indeed, after 7 days of culture in the presence of GM-CSF, migrated cells lost the CD14 (Figs. 4 and 4f), the CD34 (Fig. 4g), and the CD36 (Fig. 4i) molecules and up-regulated HLA-DR (Fig. 4l), remaining positive for both CD80 (Fig. 4h) and CD86 (not shown) costimulatory (34) molecules; furthermore, they were not stained with dyes for the
nonspecific esterases (not shown). On the contrary, nonmigrating CD14<sup>1</sup>CD34<sup>2</sup>CD36<sup>1</sup> cells (Fig. 4, m–q), cultured in GM-CSF, gave rise to CD14<sup>1</sup>CD34<sup>2</sup>CD80<sup>2</sup>CD36<sup>1</sup> cells (Fig. 4, r–v), positive for the nonspecific esterase staining (not shown), which conceivably represent macrophages. However, activated B cells have been reported to share some phenotypic features of monocyte/macrophages (36); indeed, we found that about 2% of purified peripheral blood CD14<sup>1</sup> leukocytes coexpressed sIg (Fig. 5A), at variance with CD14<sup>1</sup>CD34<sup>1</sup>migrated cells, which were sIg negative (not shown). Thus, we analyzed for Ig heavy chain gene rearrangement both CD14<sup>1</sup>CD34<sup>1</sup> and CD14<sup>1</sup>CD34<sup>2</sup> subsets, before and after culture with GM-CSF. The electrophoresis of PCR products revealed the presence of a smear, suggestive of polyclonal rearrangement, in the CD14<sup>1</sup>CD34<sup>2</sup> population (Fig. 5B, lane 2), while samples run in the remaining lanes, from the same subset after culture in GM-CSF (lane 4) or from CD14<sup>1</sup>CD34<sup>1</sup> cells, before (lane 3) and after culture (lane 5), were essentially devoid of amplified DNA fragments. That cells derived from migrating CD14<sup>1</sup>CD34<sup>1</sup> leukocytes were actually DC was confirmed by electron microscopy. Figure 6A shows that CD14<sup>1</sup>CD34<sup>1</sup> cells have few cytoplasmic projections and high nucleo-cytoplasmic ratio, are roundish, and are larger than CD14<sup>1</sup>CD34<sup>1</sup> leukocytes. Nuclei are smooth-countered with finely dispersed chromatin and usually one small nucleolus is usually present; the cytoplasmic organelles are mainly represented by mitochondria, rare rough reticulum cisternae, and small Golgi complexes (Fig. 6A), CD14<sup>1</sup>CD34<sup>1</sup> cells show, in turn, slender cytoplasmic projections, irregular nuclei with margined chromatin, and abundant cytoplasm containing some electron-dense lysosome-like granules (Fig. 6B). Cells derived from CD14<sup>1</sup>CD34<sup>1</sup> precursors display many dendritic-like cytoplasmic protrusions (filopodia) and a cytoplasm poor of granules and lysosomes (Fig. 6C), thus being bona fide DC, whereas cells derived from CD14<sup>1</sup>CD34<sup>2</sup> leukocytes have ultrastructural characteristics consistent with monocyte/macrophage lineage such as short and thick membrane projections and a cytoplasm filled with dense lysosome-like granules (Fig. 6D). We failed to demonstrate Birbeck’s granules (usually found in Langerhans cells) in our DC; however, unlike cells from CD14<sup>1</sup>CD34<sup>2</sup> leukocytes (not shown), DC acquired CD1a (Fig. 7d) in ~50% of the donors examined, and CD83, which is a DC marker (38), in all of the 10 donors analyzed (Fig. 7e) and expressed low levels of mannose receptor (Fig. 7f), as reported in mature DC (39). Because activation of naive T lymphocytes in response to allogenic Ags is one of the functional property of DC (39, 40), the two cell populations derived from migrating CD14<sup>1</sup>CD34<sup>1</sup> or nonmigrated CD14<sup>1</sup>CD34<sup>2</sup> precursors were tested for their ability to induce allogenic or TT-specific T cell proliferation. As shown in Figure 8, DC obtained from CD14<sup>1</sup>CD34<sup>1</sup>Mo were
presenting capacity is the ability to become veiled cells and recirculate (2, 40). Thus, we addressed the question of whether DC derived from CD14\(^{+}\)CD34\(^{+}\) cells displayed any migratory activity. To this aim, transmigration assays were performed using both HUVEC, and the various transfectants expressing either ICAM-1, VCAM-1, or PECAM-1. We found that DC obtained from CD14\(^{+}\)CD34\(^{+}\) precursors displayed a higher migratory capacity through HUVEC monolayers than cells derived from the CD14\(^{+}\)CD34\(^{-}\) counterpart (Fig. 9). This was confirmed by the finding that DC obtained from CD14\(^{+}\)CD34\(^{+}\) precursors could efficiently migrate across PECAM-1 and VCAM-1 transfectants as well, at variance with cells from CD14\(^{+}\)CD34\(^{-}\) Mo (Fig. 9). Moreover, GM-CSF-cultured CD14\(^{+}\)CD34\(^{-}\) cells could adhere to ICAM-1-transfected cell monolayers more efficiently (35 \pm 4\%) than the CD14\(^{+}\)CD34\(^{-}\)-derived DC (10 \pm 3\%), whereas adhesion of CD34\(^{-}\) or CD34\(^{+}\)-derived populations to PECAM-1 or VCAM-1 transfectants was superimposable (not shown). These data support the hypothesis that DC derived from CD14\(^{+}\)CD34\(^{+}\) precursors show peculiar migratory properties.

Discussion

Herein we describe a subset of CD14\(^{+}\) circulating leukocytes, co-expressing the CD34 progenitor marker, which are able to migrate across endothelial monolayers, unlike the CD14\(^{+}\)CD34\(^{-}\) counterpart. Moreover, CD14\(^{+}\)CD34\(^{+}\) cells differentiate into immunostimulatory DC on culture with GM-CSF.

This population possibly represents an intermediate stage of differentiation from bone marrow precursors to mature Mo or DC. Although DC maturation has been described as a single pathway, the heterogeneity of DC within peripheral blood points to the existence of two separate pathways in DC development, one committed directly from an early precursor and the other from a CD14\(^{+}\) monocyte-like stage (41). Our data would indicate that such a bipotent precursor is present in peripheral blood, in keeping with a recent interpretation of DC origin and maturation (41, 42). Unlike mature CD14\(^{+}\) leukocytes, the CD14\(^{+}\)CD34\(^{+}\) subset shows high expression of PECAM-1 and \(\alpha_4\) integrin, being dull positive for all the other integrins. Both \(\beta_1\) and \(\beta_2\) integrins are expressed in lower levels by circulating CD34\(^{+}\) cells, compared with noncirculating bone marrow CD34\(^{+}\) progenitors (43). It is conceivable that the preferential expression of certain molecules accounts for the different adhesive/migratory behavior of the two cell populations. Indeed, CD14\(^{+}\)CD34\(^{+}\) precursors use both PECA1-dependent homophilic interaction and an \(\alpha_5\)\(-\)VCAM-1 adhesion system for transmigration, at variance with CD14\(^{+}\)CD34\(^{-}\) cells which adhere, rather than migrate, through the \(\alpha_4\)\(\beta_1\) receptor-ligand pair.

These findings are in agreement with data from other authors showing that human CD34\(^{+}\) progenitor cells use both PECA1 and VCAM-1 adhesion systems (16). However, although they describe an indirect involvement of PECA1 in cell adhesion, as PECA1 engagement leads to up-regulation of integrin affinity for their ligands, we found that PECA1 can drive directly the migration of CD34\(^{+}\) precursors through a homophilic haptotactic gradient. It is tempting to speculate that in addition to modulation of integrin avidity state, PECA1 can regulate the adhesive and migratory properties of CD34\(^{+}\) precursors by two additional mechanisms: 1) up- or down-regulation of PECA1 surface expression during leukocyte maturation; 2) modulation of PECA1 surface distribution on endothelial cells, thus favoring either migration (haptotactic gradient) or adhesion (homogeneous distribution).

**DC derived from CD14\(^{+}\)CD34\(^{+}\) precursors retain their transendothelial migratory ability**

One of the predictable characteristics of DC with potential Ag presenting capacity is the ability to become veiled cells and recirculate (2, 40). Thus, we addressed the question of whether DC derived from CD14\(^{+}\)CD34\(^{+}\) cells displayed any migratory activity. To this aim, transmigration assays were performed using both HUVEC, and the various transfectants expressing either ICAM-1, VCAM-1, or PECAM-1. We found that DC obtained from CD14\(^{+}\)CD34\(^{+}\) precursors displayed a higher migratory capacity through HUVEC monolayers than cells derived from the CD14\(^{+}\)CD34\(^{-}\) counterpart (Fig. 9). This was confirmed by the finding that DC obtained from CD14\(^{+}\)CD34\(^{+}\) precursors could efficiently migrate across PECAM-1 and VCAM-1 transfectants as well, at variance with cells from CD14\(^{+}\)CD34\(^{-}\) Mo (Fig. 9). Moreover, GM-CSF-cultured CD14\(^{+}\)CD34\(^{-}\) cells could adhere to ICAM-1-transfected cell monolayers more efficiently (35 \pm 4\%) than the CD14\(^{+}\)CD34\(^{-}\)-derived DC (10 \pm 3\%), whereas adhesion of CD34\(^{-}\) or CD34\(^{+}\)-derived populations to PECAM-1 or VCAM-1 transfectants was superimposable (not shown). These data support the hypothesis that DC derived from CD14\(^{+}\)CD34\(^{+}\) precursors show peculiar migratory properties.

**Discussion**

Herein we describe a subset of CD14\(^{+}\) circulating leukocytes, co-expressing the CD34 progenitor marker, which are able to migrate across endothelial monolayers, unlike the CD14\(^{+}\)CD34\(^{-}\) counterpart. Moreover, CD14\(^{+}\)CD34\(^{+}\) cells differentiate into immunostimulatory DC on culture with GM-CSF.

This population possibly represents an intermediate stage of differentiation from bone marrow precursors to mature Mo or DC. Although DC maturation has been described as a single pathway, the heterogeneity of DC within peripheral blood points to the existence of two separate pathways in DC development, one committed directly from an early precursor and the other from a CD14\(^{+}\) monocyte-like stage (41). Our data would indicate that such a bipotent precursor is present in peripheral blood, in keeping with a recent interpretation of DC origin and maturation (41, 42). Unlike mature CD14\(^{+}\) leukocytes, the CD14\(^{+}\)CD34\(^{+}\) subset shows high expression of PECAM-1 and \(\alpha_4\) integrin, being dull positive for all the other integrins. Both \(\beta_1\) and \(\beta_2\) integrins are expressed in lower levels by circulating CD34\(^{+}\) cells, compared with noncirculating bone marrow CD34\(^{+}\) progenitors (43). It is conceivable that the preferential expression of certain molecules accounts for the different adhesive/migratory behavior of the two cell populations. Indeed, CD14\(^{+}\)CD34\(^{+}\) precursors use both PECA1-dependent homophilic interaction and an \(\alpha_5\)\(-\)VCAM-1 adhesion system for transmigration, at variance with CD14\(^{+}\)CD34\(^{-}\) cells which adhere, rather than migrate, through the \(\alpha_4\)\(\beta_1\) receptor-ligand pair.

These findings are in agreement with data from other authors showing that human CD34\(^{+}\) progenitor cells use both PECA1 and VCAM-1 adhesion systems (16). However, although they describe an indirect involvement of PECA1 in cell adhesion, as PECA1 engagement leads to up-regulation of integrin affinity for their ligands, we found that PECA1 can drive directly the migration of CD34\(^{+}\) precursors through a homophilic haptotactic gradient. It is tempting to speculate that in addition to modulation of integrin avidity state, PECA1 can regulate the adhesive and migratory properties of CD34\(^{+}\) precursors by two additional mechanisms: 1) up- or down-regulation of PECA1 surface expression during leukocyte maturation; 2) modulation of PECA1 surface distribution on endothelial cells, thus favoring either migration (haptotactic gradient) or adhesion (homogeneous distribution).

**DC derived from CD14\(^{+}\)CD34\(^{+}\) precursors retain their transendothelial migratory ability**

One of the predictable characteristics of DC with potential Ag presenting capacity is the ability to become veiled cells and recirculate (2, 40). Thus, we addressed the question of whether DC derived from CD14\(^{+}\)CD34\(^{+}\) cells displayed any migratory activity. To this aim, transmigration assays were performed using both HUVEC, and the various transfectants expressing either ICAM-1, VCAM-1, or PECAM-1. We found that DC obtained from CD14\(^{+}\)CD34\(^{+}\) precursors displayed a higher migratory capacity through HUVEC monolayers than cells derived from the CD14\(^{+}\)CD34\(^{-}\) counterpart (Fig. 9). This was confirmed by the finding that DC obtained from CD14\(^{+}\)CD34\(^{+}\) precursors could efficiently migrate across PECAM-1 and VCAM-1 transfectants as well, at variance with cells from CD14\(^{+}\)CD34\(^{-}\) Mo (Fig. 9). Moreover, GM-CSF-cultured CD14\(^{+}\)CD34\(^{-}\) cells could adhere to ICAM-1-transfected cell monolayers more efficiently (35 \pm 4\%) than the CD14\(^{+}\)CD34\(^{-}\)-derived DC (10 \pm 3\%), whereas adhesion of CD34\(^{-}\) or CD34\(^{+}\)-derived populations to PECAM-1 or VCAM-1 transfectants was superimposable (not shown). These data support the hypothesis that DC derived from CD14\(^{+}\)CD34\(^{+}\) precursors show peculiar migratory properties.
We have reported that PECAM-1 localization at the intercellular junctions in endothelial cells depends on the connection with cytoskeletal proteins and is tightly regulated by PECAM-1 phosphorylation (30). The balance between phosphorylated and dephosphorylated endothelial PECAM-1, can be perturbed by TNF-α, through the activation of cellular kinases, both in vitro and in vivo (30), thus implying that tissue microenvironment can influence both PECAM1 distribution and function. Nevertheless, the finding that PECAM1-driven transmigration of CD14⁺CD34⁺ cells is apparently chemokine-independent would favor the hypothesis that this population is committed to localize to peripheral tissues, whatever the microenvironment, to complete their maturation and exert their function. This is further supported by the observation that both migrated and sorted CD14⁺CD34⁺ cells can differentiate into immunostimulatory DC during culture with GM-CSF, which is known to enhance DC maturation (36, 44, 45).

**FIGURE 6.** Migrated CD14⁺CD34⁺ cells can differentiate into DC (second evidence). Ultrastructure of CD14⁺CD34⁺ (A) or CD14⁺CD34⁻ (B) precursors and cell derived from CD14⁺CD34⁺ (C) or CD14⁺CD34⁻ (D) subsets. Cells were fixed for EM in 2.5% glutaraldehyde. Post fixation was performed in 1% osmium tetroxide, and samples were embedded in Epon-Araldite resin. Thin sections (80 nm) were then stained with uranyl acetate and lead citrate and analyzed under a Zeiss CEM 902 electron microscope. A–D. Original magnification, ×3000. One representative experiment of six.

**FIGURE 7.** Migrated CD14⁺CD34⁺ cells can differentiate into DC (third evidence). CD14⁺CD34⁺ precursors before (a–c) or after culture with 20 ng/ml GM-CSF for 7 days (d–f), were stained (10⁵ cells/sample) with the various mAbs followed by the anti-isotype-specific PE-conjugated GAM. After a washing, samples were run (5000 events/sample) on a FACStar® Plus equipped with an argon ion laser (Becton Dickinson), gated to exclude cell debris and nonviable cells. Results are expressed as log green fluorescence intensity vs number of cells. One representative experiment of 10. ManR, mannose receptor.
DC precursors originating from the bone marrow migrate to peripheral tissues and primary lymphoid organs, where they become professional APC (46). After processing, Ag-carried DC migrate to the lymph nodes for the induction of Ag-specific T lymphocytes at different T:DC ratios. T cells, 10⁵ allogeneic or 2 × 10⁶ TT-specific syngeneic, were added to irradiated DC (from 2 × 10⁵ to 10⁶). For TT presentation, DC were loaded with TT (10⁻⁴ M) for 12 h before addition of specific T cells. After 3 days for MLR or 48 h for TT stimulation (proliferation of TT-specific T cell lines). After 7 days of culture, harvested, and counted in a beta counter. Results are mean ± SD from 10 independent experiments. [³H]TdT uptake by stimulatory DC or responder allogenic T cells alone was <100 cpm. [³H]TdT uptake by TT-specific T cell lines alone was 1000 cpm.

References