

# The CD16<sup>+</sup> (FcγRIII<sup>+</sup>) Subset of Human Monocytes Preferentially Becomes Migratory Dendritic Cells in a Model Tissue Setting

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## Abstract

Much remains to be learned about the physiologic events that promote monocytes to become lymph-homing dendritic cells (DCs). In a model of transendothelial trafficking, some monocytes become DCs in response to endogenous signals. These DCs migrate across endothelium in the abluminal-to-luminal direction (reverse transmigration), reminiscent of the migration into lymphatic vessels. Here we show that the subpopulation of monocytes that expresses CD16 (Fcγ receptor III) is predisposed to become migratory DCs. The vast majority of cells derived from CD16<sup>+</sup> monocytes reverse transmigrated, and their presence was associated with migratory cells expressing high levels of CD86 and human histocompatibility leukocyte antigen (HLA)-DR, and robust capacity to induce allogeneic T cell proliferation. A minority of CD16<sup>-</sup> monocytes reverse transmigrated, and these cells stimulated T cell proliferation less efficiently. CD16 was not functionally required for reverse transmigration, but promoted cell survival when yeast particles (zymosan) were present as a maturation stimulus in the subendothelial matrix. The cell surface phenotype and migratory characteristics of CD16<sup>+</sup> monocytes were inducible in CD16<sup>-</sup> monocytes by preincubation with TGFβ1. We propose that CD16<sup>+</sup> monocytes may contribute significantly to precursors for DCs that transiently survey tissues and migrate to lymph nodes via afferent lymphatic vessels.

Key words: antigen presentation • cell survival • transforming growth factors • IgG receptors • cell differentiation

## Introduction

Antigen-presenting dendritic cells (DCs)\* initiate immune responses after they capture antigen from peripheral tissues and then migrate to lymph nodes where they efficiently interact with T cells to drive T cell activation and proliferation. Circulating HLA-DR<sup>+</sup> human DC precursors include CD14<sup>-</sup>CD64<sup>-</sup>CD11c<sup>+</sup> cells (1, 2), the IFN-α-producing CD123<sup>+</sup>CD11c<sup>-</sup> plasmacytoid cells (3), and CD14<sup>+</sup>CD64<sup>+</sup>CD11c<sup>+</sup> monocytes (4). The former cell types are committed DC precursors, but monocytes differentiate into macrophages unless redirected to a DC phenotype by cytokines. Empirical approaches have defined several cytokine

cocktails that promote differentiation of DCs from monocytes (5–8). However, whether these cytokines or other signals account for how monocytes differentiate into DCs in a tissue environment is unknown.

To study how monocytes become DCs in a tissue setting, we employed a previously characterized model in which some monocytes differentiate into DCs in response to cues that are endogenous to an endothelial cell/collagen culture (4). In this model, monocytes first migrate across an endothelial monolayer to enter a subendothelial collagen matrix. Those monocytes that will become DCs retrace the intact endothelium in the abluminal-to-luminal direction (reverse transmigration; references 4 and 9), a step that shares common features with the trafficking of DCs from tissues into the lumen of lymphatic vessels. These shared features include a key role for the ABC lipid transporter MDR-1 (ABCB1) in mediating both reverse transmigration in this model and

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\*Abbreviations used in this paper: CFSE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell.

the mobilization of skin DCs through authentic dermal lymphatic vessels (10, 11).

When phagocytic particulates like zymosan are incorporated in the underlying collagenous matrix, reverse-transmigrated cells acquire phenotypic and functional features of mature, terminally differentiated DCs (4). In the absence of an exogenous stimulus or inclusion of phagocytic particles in the subendothelial matrix, the reverse-transmigrated cells do not possess features of fully mature DCs, but bear characteristics more consistent with immature DCs (4). Instead of developing into DCs, monocyte-derived cells that do not reverse transmigrate but remain in the subendothelial collagen differentiate into macrophages (4).

In this study, we pursued whether differentiation of monocytes into reverse-transmigratory DCs or more sessile macrophages is stochastic or whether a population of monocytes is predisposed to become DCs in conjunction with their migration across endothelium. Our findings support the latter possibility. Here we show that the distinct CD16<sup>+</sup> subset of blood monocytes (12) have the greatest propensity among monocytes to develop into migratory DCs. CD16 participates in the survival of these cells in response to zymosan activation and can be induced by TGFβ1. TGFβ1 not only induces expression of CD16, as shown previously (13), but we find that it yields cells with an overall surface phenotype of CD16<sup>+</sup> blood monocytes, enhances migration, and promotes survival. In contrast to TGFβ1, other cytokines, including IL-10 and M-CSF, that have been reported to induce CD16 expression in monocytes did not recapitulate the phenotype of circulating CD16<sup>+</sup> monocytes. Overall, these data indicate that CD16<sup>+</sup> monocytes have the greatest capacity among monocytes to become trafficking DCs and highlights the possibility that this subset develops in response to TGFβ1.

## Materials and Methods

**Flow Cytometry.** Antibodies used for flow cytometric staining included purified mAbs to CD3, CD14, CD19, CD36, CD64, CD86, HLA-DR, HLA-DP (all from BD Biosciences), E-cadherin (Santa Cruz Biotechnology, Inc.), and IgM nonbinding control (BD Biosciences), CD83 (Serotec), control mAb UPC10 (Sigma-Aldrich), and CD16 (Mederex; and additional mAb given by Dr. Jay Unkeless, Mt. Sinai School of Medicine). Cell surface staining was detected with FITC-conjugated rabbit anti-mouse Ig (Dako). Multi-color analysis was conducted using PE-conjugates of anti-HLA-DR or anti-CD14 mAbs, FITC-conjugated CD64, and biotin-conjugates of anti-CD16 or anti-CD86 mAbs (BD Biosciences), followed by detection with streptavidin allophycocyanin (APC; Caltag).

**Leukocyte Isolation.** Blood from healthy donors was drawn according to guidelines approved by the Internal Review Board of Mt. Sinai School of Medicine. PBMCs were isolated by density gradient centrifugation on Ficoll. Magnetic depletion of NK cells or CD16<sup>+</sup> populations were performed using anti-CD56 and anti-CD16 miniMACS (Miltenyi Biotec). Depletion of M-DC8<sup>+</sup> cells was conducted by incubating PBMCs with diluted M-DC8 hybridoma supernatant, followed by incubation with FITC-conjugated anti-mouse Ig and removal by flow cyto-

metric cell sorting. The M-DC8 mAb was the gift of Dr. E. Peter Rieber, Institute for Immunology in Dresden, Germany. For carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, CD16<sup>+</sup> cells were isolated by flow cytometric cell sorting using 3G8 anti-CD16 mAb followed by detection with FITC-conjugated rabbit anti-mouse Ig. For allogeneic T cell proliferation studies, cell sorting was conducted by first depleting B and T cells from the PBMC population with anti-CD3, anti-CD19, and CD56 miniMACS, and then staining with PE-conjugated CD14. Cells with high forward scatter were separated according to level of expression of CD14, and CD14<sup>-</sup> cells were discarded.

**Reverse Transmigration Assay.** Reverse-transmigrated and subendothelial leukocytes were studied using previously described methods (4). In brief, second passage human umbilical vein endothelial cells (HUVEC) were cultured on bovine type I collagen gels (Cohesion) in microtiter wells and used for experiments approximately three days after reaching confluence. Zymosan particles (0.0002%; wt/vol) were included during the polymerization of some collagen gels. For experiments, whole PBMCs were applied to the endothelium, incubated for 1.5 h, washed thoroughly in medium to remove nonmigrated cells from above the endothelium, and continued in culture for 48 h. Culture medium consisted of 20% heat-inactivated human serum in Medium 199 (M199). After 48 h, reverse-transmigrated cells were collected from above the endothelium by gentle pipetting in the presence of 1 mM EGTA. Subendothelial cells were analyzed after digestion of the collagen with Collagenase D (Boehringer) for 40 min.

In some experiments, anti-MDR-1 mAb MRK16 (gift of Dr. Isamu Sugawara, Research Institute for Tuberculosis, Tokyo, Japan), isotype-matched control IgG UPC10 (Sigma-Aldrich), or anti-CD16 mAb 3G8 was added to the cultures at 2–10 μg/ml after the initial 1.5 h incubation in which monocytes migrated into the subendothelial collagen as described previously (10). Antibodies added to the cultures have the capacity to cross the endothelium and gain access to subendothelial monocytes within 5 h of incubation.

Whole PBMCs or CD16<sup>+</sup> PBMCs isolated by flow cytometry were labeled with 2 μM CFSE (Molecular Probes) for 15 min at 37°C in protein-free Hank's buffered saline. Then these cells were remixed with CD16<sup>-</sup> monocytes and added to endothelial cultures for assessment of reverse transmigration.

**Mixed Leukocyte Reaction.** Allogeneic T cells were purified from PBMCs by depletion with anti-HLA II magnetic beads (Dyna). Live reverse-transmigrated cells to be evaluated as candidate APCs were irradiated with 3,000 rad, and triplicate cultures containing  $1.25 \times 10^3$  reverse-transmigrated cells were cultured with  $10^5$  T cells per well. After 5 d of culture, 1 μCi of [<sup>3</sup>H]thymidine was added per well for an additional 15 h.

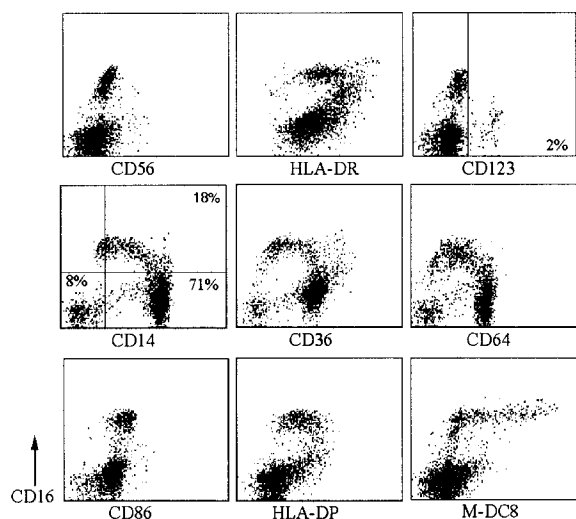
**Cytokine Treatments.** PBMCs were cultured under nonadherent conditions in Teflon beakers containing 10 ng/ml recombinant human TGFβ1, 20 ng/ml recombinant human IL-10, 10–50 ng/ml recombinant M-CSF, or rabbit pan anti-human TGFβ neutralizing antibody (10 μg/ml, added daily) in M199 containing 10% heat-inactivated human serum. All of these reagents were purchased from R&D Systems. After culture, cells were washed and prepared for reverse transmigration experiments, conducted as with freshly isolated PBMCs.

## Results

**CD16 Expression on Subpopulations of HLA-DR<sup>+</sup> Monocytes and DC Precursors.** The HLA-DR<sup>+</sup> fraction of PBMCs that lack characteristics of B lymphocytes represent ~20%

of PBMCs and contain several subpopulations that collectively comprise potential precursors for antigen-presenting DCs. These HLA-DR<sup>+</sup> cells can be subdivided into several distinct populations, as shown in Fig. 1: CD14<sup>-</sup> nonmonocytic DCs that contain a very small fraction of CD123<sup>+</sup> plasmacytoid cells, and CD14<sup>+</sup> monocytes that can be divided into CD16<sup>-</sup> and CD16<sup>+</sup> fractions. The CD14<sup>+</sup>CD16<sup>+</sup> monocytes can be further subdivided into cells that do or do not express the DC subset marker M-DC8 (14, 15). The CD16<sup>+</sup> subpopulation of monocytes expresses only an intermediate level of CD14 relative to CD16<sup>-</sup> monocytes (12; Fig. 1). These cells exhibit reduced levels of other markers associated with macrophage activities, including CD36 and the high affinity Fcγ receptor CD64 (Fig. 1). In contrast, CD16<sup>+</sup> monocytes exhibit somewhat elevated levels of accessory molecules, including CD86, HLA-DR (16), and HLA-DP (Fig. 1). These data indicate that CD16<sup>+</sup> monocytes more closely resemble DCs than do CD16<sup>-</sup> monocytes.

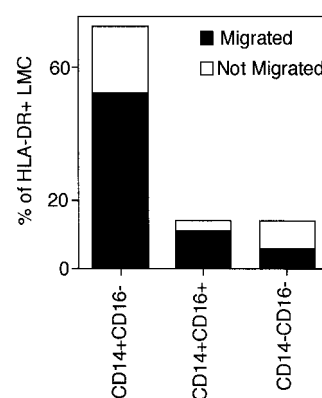
**CD16<sup>+</sup> Monocytes Traverse Endothelial Monolayers.** When the whole PBMC fraction is applied to the apical surface of cultured endothelial monolayers not pretreated with proinflammatory cytokines, very few B or T lymphocytes migrate beneath the endothelium, but a majority of monocytes (17, 18) and NK cells (19) will undergo transendothelial migration in less than 2 h of incubation. This step mimics the trafficking of these cells from the vascular



**Figure 1.** Cell surface markers distinguish CD16<sup>+</sup> and CD16<sup>-</sup>HLA-DR<sup>+</sup> PBMCs. Freshly isolated PBMCs were depleted of CD56<sup>+</sup> NK cells. Two-color flow cytometry was conducted with a gate set to exclude smaller PBMCs of the lymphocyte lineage. Expression of macrophage-associated markers CD14, CD36, and CD64 or DC-associated markers HLA-DR, HLA-DP, and CD86 (all x-axis) were examined. Cells were counterstained with a mAb to CD16 (y-axis). Some cells were stained with M-DC8 mAb to identify a subset of CD16<sup>+</sup> cells (reference 11). Quadrant markers (shown in CD14 and CD123 panels) are positioned according to the level of fluorescence observed in cells stained with nonbinding isotype-matched control mAbs (lower left quadrant, negative staining). The phenotype illustrated was similarly observed among four donors examined.

compartment into subendothelial connective tissue, and in the absence of exogenous cytokine stimulation, may reflect populations of leukocytes that leave the blood in the steady-state. We conducted experiments to evaluate whether the CD16<sup>+</sup> monocytes were among those that efficiently traversed the endothelium. We also examined the transmigration of the previously defined CD14<sup>-</sup>CD64<sup>-</sup>HLA-DR<sup>+</sup> nonmonocytic DCs (1, 2), as their interaction with endothelium directly after isolation has not been reported. Flow cytometric cell sorting of this fraction of HLA-DR<sup>+</sup>CD14<sup>-</sup> cells confirmed the differentiation of these cells into mature DCs after cultured in macrophage conditioned medium (unpublished data) as reported previously (1), indicating that the CD14 cells we tracked were indeed DC precursors. Both CD16<sup>-</sup> and CD16<sup>+</sup> monocytes migrated efficiently across unstimulated endothelium (Fig. 2), with more than two-thirds of each population entering the subendothelial collagen. On a relative basis, CD16<sup>+</sup> monocytes migrated somewhat more efficiently than CD16<sup>-</sup> monocytes. Among the CD16<sup>+</sup> cells that entered the collagen gel, 15 to 25% were M-DC8<sup>+</sup> (unpublished data). Similar extents of transendothelial migration occurred in endothelial cultures in which zymosan, a preparation of yeast cell membranes, was embedded in the type I collagen matrix (unpublished data). In contrast, less than half of CD14<sup>-</sup>CD64<sup>-</sup>CD11c<sup>+</sup>HLA-DR<sup>+</sup> DC precursors traversed the endothelium (Fig. 2).

**CD16<sup>+</sup> Monocytes Preferentially Reverse Transmigrate.** Next we followed the expression of CD16 by monocyte-derived cells present in endothelial/collagen cultures for 48 h, during which time nearly half of the originally transmigrated cells retransverse the endothelium in the abluminal-to-luminal direction (reverse transmigration). In endothelial cultures receiving no exogenous stimulus such that the reverse-transmigrated population fails to differentiate into



**Figure 2.** Transendothelial migration of blood DC precursors across unstimulated endothelium. The entire fraction of freshly isolated PBMCs were incubated with endothelial cell/collagen cultures for 1.5 h to permit transmigration. The apical surface of the cultures was washed to collect nonmigrated cells, and the migrated population was recovered from the subendothelial collagen using collagenase D. Cells considered for quantitative evaluation were large mononuclear cells (LMC) uniformly positive for HLA-DR

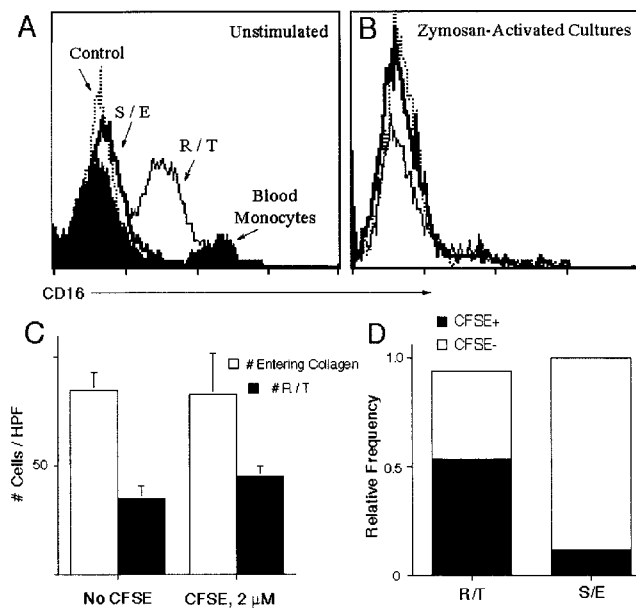
and negative for the B cell marker CD19 or T cell marker CD3. The plot shows the percent distribution of three distinct populations: CD14<sup>+</sup>CD16<sup>-</sup>, CD14<sup>+</sup>CD16<sup>+</sup>, and CD14<sup>-</sup>CD16<sup>-</sup> cells. The total height of each bar represents the relative distribution of these populations in freshly isolated PBMCs. The filled portion of each bar indicates the fraction of each population that emigrated beneath the endothelium, and the open portion of each bar represents the portion of the population that was recovered in the nonmigrated fraction. These data are representative of results obtained using PBMCs from three different blood donors.

fully mature DCs, most or all of the reverse-transmigrated cells expressed CD16 (Fig. 3 A), and as many as 20% of these cells were M-DC8<sup>+</sup> (unpublished data). However, the cell surface density of CD16 declined by about half during the 1.5 h incubation when monocytes entered the subendothelial collagen (unpublished data), relative to the level expressed in blood. This reduced level of CD16 was maintained throughout the culture period (Fig. 3, compare level of CD16 in blood monocyte to that expressed by reverse-transmigrated cells). Overt activation of the cultures with proinflammatory cytokines (unpublished data) or inclusion of zymosan in the collagen promoted complete loss of CD16 in the reverse-transmigrated population (Fig. 3 B), consistent with its documented downregulation during maturation of DCs.

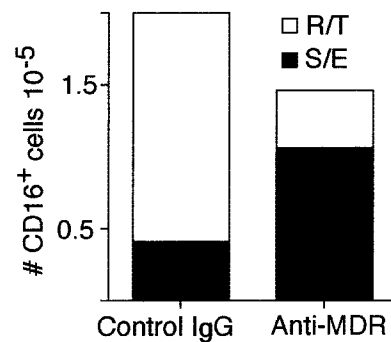
In contrast to the reverse-transmigrated cells recovered from unstimulated cultures, the vast majority of monocyte-derived cells remaining in the subendothelium were CD16<sup>-</sup> (Fig. 3) and M-DC8<sup>-</sup> (unpublished data). That CD16<sup>+</sup> cells could be recovered from the subendothelial

collagen after only 1.5 h of incubation (Fig. 2) argues that our failure to detect CD16 on the surface of monocyte-derived cells that remained in the subendothelium was not due to cleavage of the relevant CD16 epitope during recovery of these cells from the collagen using collagenase D. Furthermore, CD16<sup>+</sup> cells were recovered from the subendothelial matrix after 48 h when we prevented reverse transmigration using an antagonist to the p-glycoprotein lipid transporter ABCB1 (10; Fig. 4).

We conducted cell tracking experiments to evaluate whether cells in the reverse-transmigrated population indeed arose from circulating CD16<sup>+</sup> monocytes. Labeling monocytes with CFSE at 2  $\mu$ M did not alter the migratory patterns of monocyte-derived cells within the cultures (Fig. 3 C). To focus on the migratory behavior of CD16<sup>+</sup> monocytes, these cells were isolated by flow cytometric cell sorting, labeled with CFSE and then mixed with unlabeled CD16<sup>-</sup> PBMCs to match their original frequency in the population (15% of total monocytes). We then traced the distribution of CFSE-containing cells in the reverse-transmigrated and subendothelial monocyte-derived fractions after 48 h of culture with endothelial cell/collagen matrices. CFSE<sup>+</sup> cells comprised approximately half of the reverse-transmigrated population, which represents a great enrichment of these cells in the migratory fraction (Fig. 3), relative to their frequency in the overall population. In total, 85% of all CFSE<sup>+</sup>CD16<sup>+</sup> cells were in the reverse transmigrated fraction, whereas only 34% of CD16<sup>-</sup> cells reverse transmigrated. Given the strong association of CD16 expression with reverse transmigration, we assessed whether CD16 was functionally required for reverse transmigration. Inclusion of the neutralizing anti-CD16 mAb 3G8 in these cultures (no zymosan) did not block reverse transmigration.



**Figure 3.** Distribution of CD16<sup>+</sup> monocyte-derived cells after coculture with endothelial cells grown on collagen. (A) Expression of CD16 was monitored in monocyte/endothelial cocultures at 48 h, when the majority of cells that will reverse transmigrate have done so. Assessments were made in reverse transmigrated (R/T, thin-lined profile) and subendothelial (S/E, bolded line profile) monocyte-derived cells from cultures that received no activation stimuli such as exogenous cytokines or phagocytic particles. Dotted line demarcates the staining intensity of cells incubated with isotype-matched mAbs to an irrelevant antigen. Filled profile represents the expression of CD16 in freshly isolated monocytes. (B) In other cultures, zymosan was included in the collagen to promote DC maturation. In some unstimulated endothelial cultures, the whole fraction of PBMCs (C) or CD16<sup>+</sup> monocytes sorted using flow cytometry (D), were labeled with CFSE. Transendothelial migration into the collagen and subsequent reverse transmigration was evaluated to assess the distribution of CFSE<sup>+</sup> cells. CFSE-labeled CD16<sup>+</sup> monocytes were remixed with CD16<sup>-</sup> PBMCs so that CD16<sup>+</sup> CFSE<sup>+</sup> cells represented 15% of the total population (D).

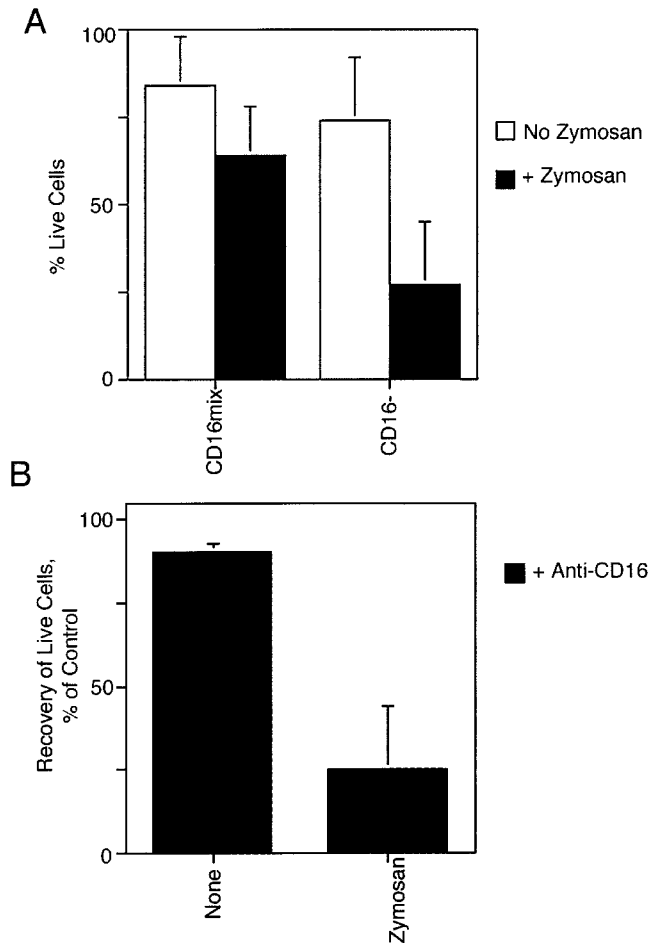


**Figure 4.** Effect of a reverse transmigration antagonist on the distribution of CD16<sup>+</sup> cells in endothelial cell cultures. PBMCs were incubated with endothelial/collagen cultures for 1.5 h, then washed to remove non-adherent, nonmigrated cells. Cultures were fed with medium containing mAb to MDR-1 or isotype-matched control mAb UPC10, and incubation was continued for 48 h to allow reverse transmigration. The presence of CD16<sup>+</sup> cells in reverse-transmigrated and subendothelial leukocytes was monitored after 48 h by flow cytometry. The total number of CD16<sup>+</sup> cells recovered from cultures in the presence and absence of anti-MDR-1 is shown. The fraction of such cells that had reverse transmigrated (R/T) is shown by the open portion of the bars, whereas the fraction that remained in the subendothelium (S/E) is shown by the filled portion of the bars.

*Expression of CD16 Promotes Survival after Activation by Zymosan.* Next we compared the effects on the yield of DCs recovered from endothelial cultures that received PBMCs that included or lacked CD16<sup>+</sup> monocytes. CD56<sup>+</sup> NK cells, which also express CD16 and can migrate beneath the endothelium (19), were always first depleted from the PBMC preparation before addition of mononuclear cells to the endothelium. When these cells remained present, they had a deleterious effect on the viability of activated DCs in the presence of zymosan, consistent with the recently described role of NK cells in killing immature DCs (20). After depletion of CD56<sup>+</sup> cells, the remaining CD16<sup>+</sup> PBMCs were CD16<sup>+</sup>CD14<sup>+</sup> monocytes and these were present together with CD16<sup>-</sup> monocytes in control cultures (referred to as CD16<sup>mix</sup> monocytes). For comparison, the total fraction of CD16<sup>+</sup> cells were removed by magnetic depletion, leaving only CD16<sup>-</sup> monocytes. The depletions of the desired population were greater than 95%.

Although the above studies failed to show a functional role for CD16 in migration, we observed that zymosan stimulation after magnetic depletion of CD16<sup>+</sup> monocytes led to a decreased viability in the reverse-transmigrated population (Fig. 5 A). However, viability was uniformly high in the absence of this maturation stimulus. To determine whether survival was functionally related to expression of CD16, we conducted experiments in which CD16<sup>mix</sup> monocytes were added to endothelial cultures with or without inclusion of zymosan in the collagen matrix. In some wells, monocytes were treated with neutralizing anti-CD16 mAb. In zymosan-containing cultures, the yield of live reverse-transmigrated DCs was reduced by 75% in the presence of anti-CD16 mAb (Fig. 5 B). These data suggest that stimulation of monocytes with this microbial particulate leads to cell death in the absence of functional CD16. Anti-CD16 mAb did not prevent phagocytic uptake of zymosan (unpublished data).

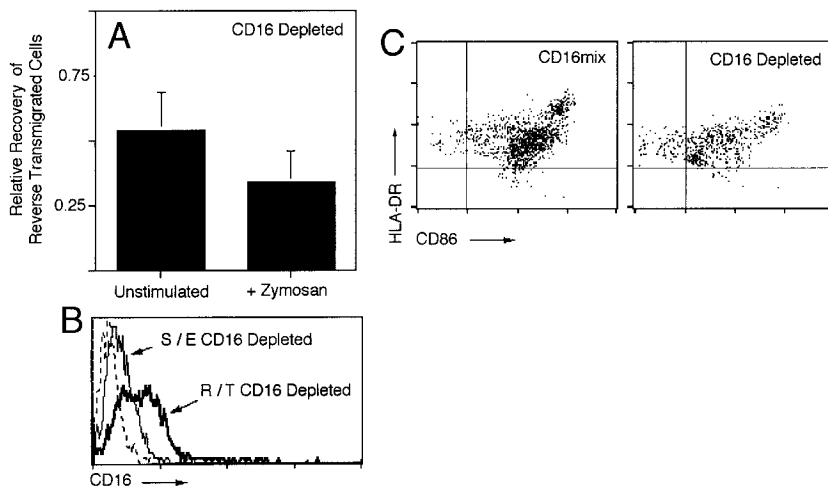
*Differentiation and Migration Analysis after Depletion of CD16<sup>+</sup> Monocytes.* In some depletion experiments, only the M-DC8<sup>+</sup> subpopulation of CD14<sup>+</sup>CD16<sup>+</sup> monocytes was removed. Removal of M-DC8<sup>+</sup> cells only had no marked effect on the yield or phenotype of reverse-transmigrated cells (unpublished data), but when the depletion scheme eliminated all CD16<sup>+</sup> monocytes (CD16 depleted), 47 ± 22% (P < 0.05; four experiments) to 66 ± 13% (P < 0.005; four experiments) fewer reverse transmigrated cells were recovered from unstimulated and zymosan-stimulated cultures, respectively (Fig. 6 A). These results are in agreement with the CFSE experiments in Fig. 3. Even after thorough depletion of CD16<sup>-</sup> monocytes, many reverse-transmigrated cells recovered from cultures receiving only the CD16<sup>+</sup> monocytes expressed CD16 upon reverse transmigration, in contrast to the subendothelial monocyte-derived cells from the same cultures (Fig. 6 B). Thus, these cells appear to upregulate CD16 expression during reverse transmigration. When flow cytometry was conducted to analyze the maturation status of cells in the reverse-transmigrated fraction, the number of HLA-DR<sup>+</sup>CD86<sup>+</sup> cells was 63 ± 14% (average of three experiments; P < 0.005)



**Figure 5.** Viability of reverse-transmigrated cells in the presence or absence of functional CD16. The viability of reverse-transmigrated cells was assessed by trypan blue staining after recovery from cultures lacking or containing zymosan. (A) Viability assessment from cultures in which CD16-depleted monocytes (CD16<sup>-</sup>) or the full fraction of monocytes containing both CD16<sup>+</sup> and CD16<sup>-</sup> subpopulations (CD16<sup>mix</sup>) were applied to the endothelium. (B) Recovery of live CD16<sup>mix</sup> monocytes in the presence of neutralizing anti-CD16 mAb 3G8 was calculated in relation to the number of live cells recovered in the presence of isotype-matched control antibody UPC10 under each condition.

decreased per unit area of endothelial cell surface when blood CD16<sup>+</sup> monocytes were depleted from the starting population (Fig. 6 C; cells shown in each group were recovered from an equivalent area of endothelial surface). Moreover, the residual DCs recovered after depletion of CD16<sup>+</sup> monocytes expressed an order of magnitude less CD86 on the cell surface, indicating that these reverse-transmigrated, CD16<sup>-</sup> monocyte-derived cells were less mature than the reverse-transmigrated cells that develop in cultures that contained CD16<sup>+</sup> blood monocytes.

*Role of Monocyte Subsets in Giving Rise to Antigen-presenting Cells that Stimulate Proliferation of Allogeneic T Cells.* The negative effects of anti-CD16 mAb on cell survival obviated experiments in which we sorted CD16<sup>+</sup> monocytes using anti-CD16 mAb and then added them to endothelial cultures to assess differentiation and antigen-presenting capacity after encounter with a potent maturation



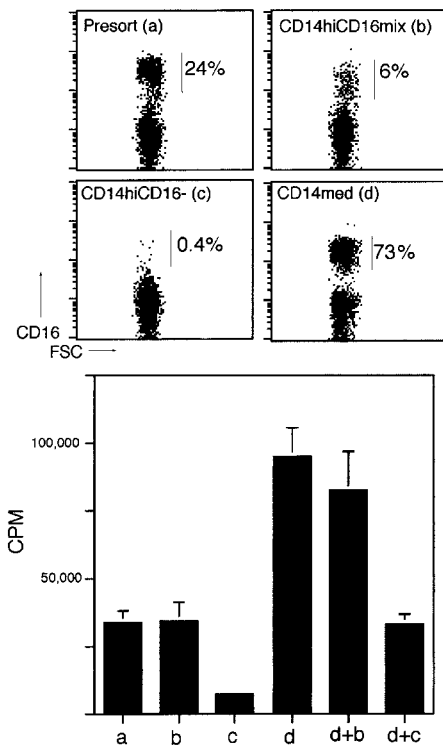
**Figure 6.** Evaluation of reverse transmigration and expression of CD16 after depletion of peripheral blood CD16<sup>+</sup> monocytes. CD56<sup>+</sup> NK cells were depleted from the starting PBMC fraction, leaving a fraction of PBMCs that included both CD16<sup>-</sup> and CD16<sup>+</sup>(CD16<sup>mix</sup>) CD14<sup>+</sup> monocytes. In some samples, the remaining CD16<sup>+</sup> cells were depleted, leaving CD56<sup>-</sup>CD16<sup>-</sup> PBMCs. CD16<sup>mix</sup> or CD16<sup>-</sup> PBMCs were applied to endothelial/collagen cultures at the same starting density. The number of reverse transmigrated cells in the CD16<sup>-</sup> fraction was evaluated after 2 d and compared in five independent experiments to the number of reverse transmigrated cells in the control CD16<sup>mix</sup> population of PBMCs (A). The relative recovery was calculated by setting equal to 1.0 the number of reverse transmigrated cells recovered per well of cultured endothelium after application of CD56<sup>-</sup>CD16<sup>mix</sup> PBMCs and then determining the fractional recovery in each experiment when CD16-depleted PBMCs were applied. (B) The

possibility that CD16 might be upregulated on peripheral blood cells that originally lacked CD16 was tested by examining the expression of CD16 in reverse-transmigrated and subendothelial populations after full depletion of CD16<sup>+</sup> blood cells. Flow cytometric evaluation of CD16 expression in reverse transmigrated (R/T) and subendothelial (S/E) cells that arose from CD16-depleted PBMCs is plotted as a histogram. (C) Phenotyping for CD86 and HLA-DR expression was conducted in reverse-transmigrated populations derived in the presence or absence of CD16<sup>+</sup> monocytes. The dot plots shown in panel C represent the number and phenotype of cells recovered from equal numbers of zymosan-containing endothelial cell microtiter cultures to which equivalent densities of CD16<sup>mix</sup> and CD16<sup>-</sup> monocytes were applied.

stimulus such as subendothelial zymosan. Instead, we approached comparing the antigen-presenting cell function of these subsets after reverse transmigration using cell isolation techniques that did not leave residual anti-CD16 mAb on CD16<sup>+</sup> cells. We reasoned that if CD16<sup>+</sup> monocytes contributed significantly to the majority of mature antigen-presenting, reverse-transmigrated cells that their absence would result in reduced T cell proliferative responses. However, it was also important to be sure that any T cell proliferation observed did not arise from stimulation by the relatively rare CD14<sup>-</sup>CD11c<sup>+</sup> DCs (1, 2). Thus, we separated monocyte subsets based on their level of CD14 staining (CD14<sup>-</sup> cells were discarded), as most CD16<sup>+</sup> monocytes express lower levels of CD14 (CD14<sup>med</sup>) than CD16<sup>-</sup> monocytes (CD14<sup>hi</sup>). As there is some breadth in the spectrum of CD14 that is expressed by CD16<sup>+</sup> monocytes, this approach did not achieve absolute purity of the two subsets, but did result in populations that were greatly enriched for one or the other subset (Fig. 7). Some of the sorted CD14<sup>hi</sup> cells were finally depleted completely of CD16<sup>+</sup> monocytes using anti-CD16 magnetic beads. All populations were then applied separately to endothelial monolayers containing zymosan in the subendothelial matrix. The presence of CD16<sup>+</sup> monocytes in the population applied was correlated with recovery of reverse-transmigrated cells that exhibited robust capacity to induce T cell proliferation (Fig. 7). Mixing reverse-transmigrated cells from CD16-depleted cultures with reverse-transmigrated cells derived from a population highly enriched in CD16<sup>+</sup> monocytes led to an overall inhibitory effect, consistent with the possibility that reverse-transmigrated cells derived from CD16<sup>-</sup> monocytes differentiated into presentation-suppressive macrophages (21) in the absence of CD16<sup>+</sup> monocytes (Fig. 7). The presence of relatively few CD16<sup>+</sup> monocytes

amongst total monocytes added to endothelial cultures curbed this inhibitory effect (Fig. 7, compare d and b to d and c), raising the possibility that CD16<sup>+</sup> cells may not only become DCs themselves but that they may also promote differentiation of reverse-transmigrated cells derived from CD16<sup>-</sup> monocytes toward a DC-like phenotype (Figs. 6 and 7).

*Treatment with TGFβ1 Induces CD16<sup>-</sup> Monocytes to Acquire Similar Properties of Circulating CD16<sup>+</sup> Monocytes.* The origin of CD16<sup>+</sup> monocytes and their relationship to CD16<sup>-</sup> monocytes are unknown. TGFβ1, IL-10, and M-CSF have been previously shown to induce expression of CD16 on monocytes (13, 22–24), and neutralizing endogenous TGFβ with anti-TGFβ antibody prevents “spontaneous” up-regulation of CD16 by cultured monocytes (13). However, whether this cytokine induced other characteristic features of CD16<sup>+</sup> monocytes was not studied. We depleted PBMCs of CD16<sup>+</sup> cells and then cultured the remaining CD16<sup>-</sup> monocytes under nonadherent conditions in TGFβ1 or neutralizing anti-TGFβ antibody (to prevent spontaneous induction) for up to 3 d. Within an overnight period of culture, TGFβ1 treatment induced CD16 (Fig. 8 A), as expected from previous reports (13). During this time period, some other features of blood CD16<sup>+</sup> monocytes (as shown in Fig. 1) became apparent, such as the slightly higher expression of CD86 and increased HLA-DP that typify CD16<sup>+</sup> monocytes (Fig. 8A, compare with Fig. 1). Other markers that distinguish CD16<sup>+</sup> monocytes from their CD16<sup>-</sup> counterparts took longer to become apparent. After 3 d of culture in TGFβ1, levels of CD14 were reduced by one-half to one log, just as observed on circulating CD16<sup>+</sup> monocytes. Moreover, the slight increase in HLA-DR and somewhat decreased levels of CD64 that are observed in CD16<sup>+</sup> blood monocytes were also acquired during culture with TGFβ1.



**Figure 7.** Capacity of reverse-transmigrated cells derived from monocyte subsets to stimulate allogeneic T cell proliferation. Monocytes were sorted according to their level of expression of CD14. First lymphocytes were removed from the PBMC fraction to generate a “presort” population of monocytes (a). Monocytes were then sorted into CD14<sup>hi</sup> (b, c) and CD14<sup>med</sup> (d) expressing cells. Some of the CD14<sup>hi</sup> monocytes were then treated with anti-CD16 miniMACs beads to remove any remaining CD16<sup>+</sup> cells (c). These populations were added to endothelial cultures in which zymosan was present in the subendothelial matrix, and then live reverse transmigrated cells collected 2 d later were tested for their ability to induce allogeneic T cell proliferation at a ratio of 1 antigen-presenting cell: 80 T cells. In some conditions, a 1:1 mix of some reverse-transmigrated populations (d + b, or d + c) were used as APCs. Background counts for T cells cultured alone were <300. This pattern of responses was observed in 2 independent analyses.

Although occasionally observed, the M-DC8 antigen was not consistently up-regulated in response to TGFβ1. The Langerhans cell marker E-cadherin was not expressed on these TGFβ1-treated monocytes (unpublished data), even though TGFβ1 is known to induce E-cadherin on monocytes that are additionally cocultured in GM-CSF and IL-4. In contrast to our results with TGFβ1, culturing CD16<sup>-</sup> monocytes with IL-10 was accompanied by down-regulation of HLA-DR and CD86, and culturing in M-CSF failed to induce CD16 within the 3 d period tested (unpublished data).

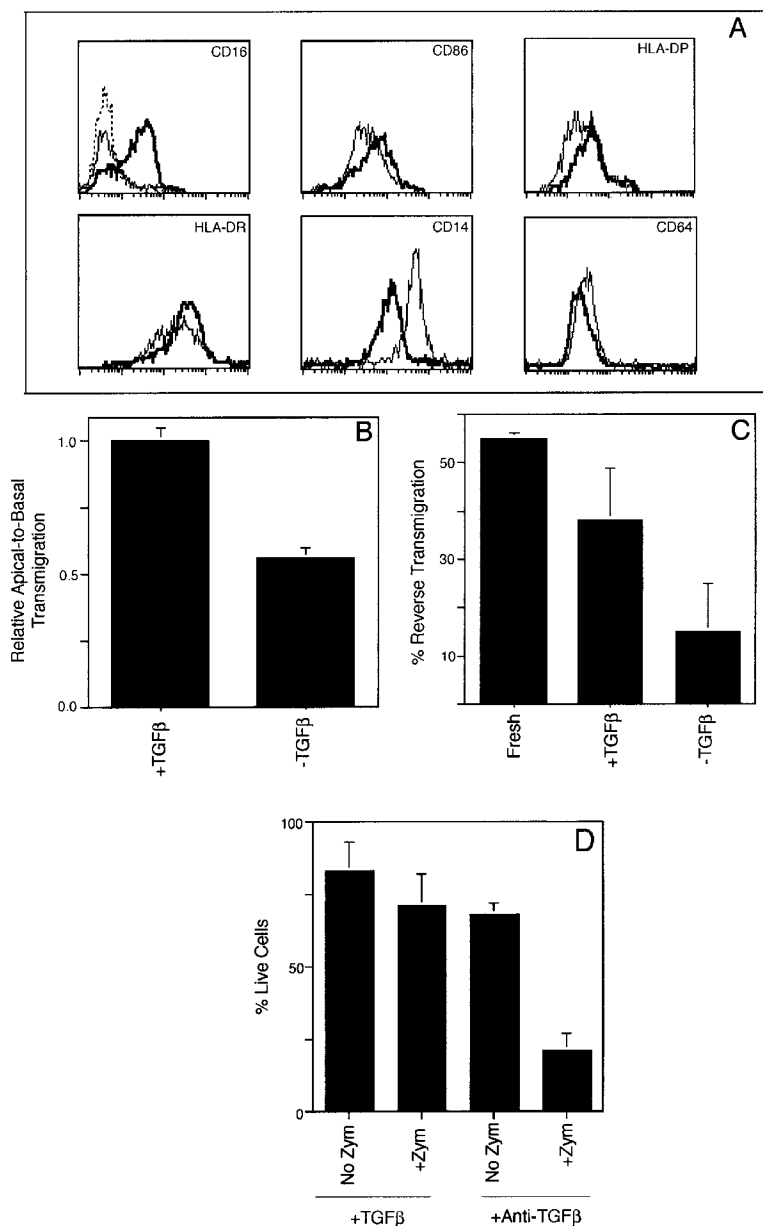
When TGFβ1 and anti-TGFβ treated cultured monocytes were applied to unstimulated endothelial monolayers, the TGFβ1-treated cells were much more migratory and nearly twice as many entered the subendothelial collagen (Fig. 8 B). The extent of reverse transmigration was also greater in the TGFβ1-treated cells, even when we corrected for the different levels at which the two populations initially crossed the endothelium (Fig. 8 C). These data suggest that TGFβ1 can regulate potential for reverse transmigration.

We also added TGFβ1 and anti-TGFβ treated monocytes to zymosan-containing cultures. In contrast to TGFβ1-treated cells, the majority of reverse-transmigrated cells from anti-TGFβ-treated cultures died rapidly after reverse transmigration, as the viability of these cells was low (Fig. 8 D). This finding is in agreement with the previous experiments in which expression of CD16 promoted survival of maturing DCs after phagocytic uptake of zymosan (Fig. 6). Overall, culture of CD16<sup>-</sup> monocytes with TGFβ1 invokes expression of a number of phenotypic and functional features that mimic CD16<sup>+</sup> blood monocytes, consistent with the possibility that TGFβ1 participates in the development of this subset in vivo.

## Discussion

A subpopulation of CD14<sup>+</sup> monocytes that expresses the Fcγ receptor CD16 (FcγRIII) has been previously defined (12). This population represents ~15% of circulating monocytes in a normal individual. The origin, biologic activity, and fate of CD16<sup>+</sup> monocytes has been enigmatic. Although early reports concerning the functional capacity of CD16<sup>+</sup> monocytes suggested that these cells had low capacity to stimulate autologous T cell proliferation (25), a recent study concluded that CD16<sup>+</sup> monocytes possess the highest capacity among freshly isolated blood leukocytes, including CD14<sup>-</sup> DC precursors, to promote allogeneic T cell proliferation (16). Here we provide evidence that this subpopulation of monocytes is predisposed to become migratory DCs, and they may also impact development of an antigen-presenting cell phenotype in CD16<sup>-</sup> monocyte-derived cells. Depletion of CD16<sup>+</sup> monocytes or monitoring their migration after CFSE labeling indicates that blood CD16<sup>+</sup> monocytes are much more likely to join the reverse-transmigrating DC population than monocytes derived from the CD16<sup>-</sup> fraction. Furthermore, as DCs derived from CD16<sup>+</sup> monocytes show increased potential for survival after phagocytic stimulation, DCs derived from these precursors become even more enriched in the migratory fraction when phagocytic antigens are present in the subendothelial matrix.

It is unlikely that expression of CD16 by monocyte-derived cells is invariably associated with development of DCs. Whereas our data argue that CD16 expression by human monocytes is associated with an increased potential to become DCs and that induction of CD16 expression by TGFβ1 induces a similar phenotype, the expression of CD16 may also occur under other circumstances in response to stimuli that are not otherwise associated with development of migratory DCs. The expression of CD16 by tissue macrophages (26) has led to the idea CD16<sup>+</sup> monocytes bear a close relationship with tissue macrophages. In contrast to our observations herein, the kinetics of CD16 upregulation by monocytes that become tissue macrophages is quite delayed and does not occur until after about 6 d in culture (27). At this stage, the developing macrophage may have diminished capacity to differentiate into a DC. Indeed, there appears to be heterogeneity of CD16<sup>+</sup>



**Figure 8.** Effect of TGFβ1 on the phenotype, migratory ability, and survival of CD16<sup>-</sup> monocytes. PBMCs were depleted of CD16<sup>+</sup> cells using miniMACS magnetic selection. Then remaining cells were cultured for up to 3 d in TGFβ1 (bold lines) or anti-TGFβ (thin lines) to block activity of endogenous TGFβ. (A) The cell surface phenotype of these cultured cells was assessed by flow cytometry. Control mAb staining is depicted as a dashed line in top left histogram. (B) Monocytes cultured overnight in TGFβ1 or anti-TGFβ were applied to endothelial monolayers grown on collagen gels lacking zymosan and apical-to-basal transendothelial migration was quantified after a 1.5 incubation. (C) Reverse transmigration was quantified at 48 h as the percent of cells that originally migrated across the endothelium in each condition and then later retransversed the endothelium in abluminal-to-luminal direction. (D) Percentage of live cells in the reverse transmigrated populations derived from TGFβ1 or anti-TGFβ treated monocytes incubated with endothelial cultures lacking or containing zymosan within the subendothelium was assessed by trypan blue exclusion.

cells found within tissues *in vivo*, with some positive cells illustrating typical features of macrophages lacking expression of HLA-DR, and others coexpressing HLA-DR (26). It is important to note that although CD16 expression appears to signify potential to become migratory DCs, we observe that CD16 is rapidly lost upon stimulation. Thus, CD16<sup>+</sup> monocytes with the capacity to become migratory DCs may not be readily detected *in situ* due to the transient nature of its expression.

Besides TGFβ1, IL-10 has been reported to rapidly up-regulate CD16 expression on monocytes (22). However, we found that, in contrast to TGFβ1, IL-10 promoted the downregulation of CD86 and HLA-DR. Thus, stimulation by IL-10 may lead to the differentiation of CD16<sup>+</sup> macrophages, rather than DCs. It is also possible that IL-10 may promote the development of a CD16<sup>+</sup> DC precursor in

particular cytokine environments that we did not study. Ancuta and colleagues induced CD16 expression on monocyte-derived cells by culturing them in the simultaneous presence of three cytokines: IL-10, GM-CSF, and IL-4 (28). These cells developed key features of typical DCs (28), although their migratory properties were not studied. It will be important to determine whether this combination of cytokines fully recapitulates the onset of the CD16<sup>+</sup> monocyte phenotype. Although we know little about the expression of combinations of particular cytokines *in vivo*, it may be more likely that a single cytokine, endogenous TGFβ1, mediates the differentiation of CD16<sup>+</sup> monocytes *in vivo*. The kinetics of induction of CD16<sup>+</sup> monocyte-like cells from CD16<sup>-</sup> monocytes *in vitro* is also consistent with the circulating half-life of human monocytes *in vivo* (29). However, the *in vivo* environment in which mono-



cytes would encounter TGF $\beta$ 1 is uncertain; it is possible that this differentiation occurs in the bone marrow before release of monocytes into the circulation.

Understanding the biological role of the CD16<sup>+</sup> subset of monocytes may shed light on the mechanisms of disease and host responses in some clinical settings, because the frequency of CD16<sup>+</sup> monocytes in the blood varies dramatically in association with disease states, inflammatory conditions, and during immune-modulating therapy (30). For example, clinical administration of GM-CSF (23) or glucocorticoids (31) reduces the number of circulating CD16<sup>+</sup> monocytes to nearly undetectable levels, whereas M-CSF treatment augments their frequency to as high as 80% of total monocytes (23, 24). The effect of M-CSF is likely indirect, as the kinetics of CD16 upregulation in response to this cytokine are quite delayed, and we were unable to observe onset of the CD16<sup>+</sup> monocyte phenotype during 3 d of culture with this cytokine. The fraction of monocytes that express CD16<sup>+</sup> is also increased, for example, in patients burdened with metastatic carcinoma (24), and in persons infected with HIV (32, 33). Patients suffering from AIDS-related dementia manifest a particularly dramatic increase in CD16<sup>+</sup> monocytes, and these cells may have a key role in the neurodegenerative pathology (33, 34). It is of interest to determine whether the increased levels of CD16<sup>+</sup> monocytes that are observed during inflammatory diseases may be related to an increased production of TGF $\beta$ 1.

One of the distinctive features of CD16<sup>+</sup> monocytes is their superior capacity to produce TNF $\alpha$  (32). Inasmuch as their trafficking capacity is consistent with the possibility that CD14<sup>+</sup>CD16<sup>+</sup> monocytes give rise to DCs that may survey tissues constitutively and thereby possibly participate in peripheral tolerance, it is interesting to note that TNF $\alpha$  stimulation of immature DCs renders a population that promotes tolerance *in vivo* rather than immune priming (35). The steady-state trafficking of self antigens, such as melanin, to lymph nodes may be mediated by a TGF $\beta$ 1-dependent antigen-presenting cell (36). TGF $\beta$ 1-dependent DCs other than Langerhans cells (37) have not been reported, but our data suggest that migratory monocyte-derived DCs may depend upon TGF $\beta$ 1 as well. It was concluded that the carriage of melanin to lymph nodes in the steady-state was not mediated by monocyte-derived cells (36), based on the prolonged *in vivo* use of a neutralizing anti-M-CSF antibody. However, considering that it is likely that an immune response to the foreign antibody developed and thereby attenuated its effectiveness after several weeks of administration, this conclusion may require reevaluation.

If monocytes give rise to a population of constitutively trafficking DCs, a concept that is consistent with the conditions and kinetics of their migration *in vivo*, they may contribute significantly to peripheral tolerance. CD16<sup>+</sup> monocytes have also been suggested to participate preferentially in Th<sub>2</sub> immunity. Sanchez-Torres and coworkers evaluated the role of the two blood subsets, CD16<sup>+</sup> and CD16<sup>-</sup> monocytes, by first separating them and then monitoring their acquisition of DC features after incubation in IL-4/

GM-CSF and TNF- $\alpha$ . Whereas both subsets exhibited the capacity to differentiate into DCs in this model, T cells cocultured with DCs derived from CD16<sup>+</sup> monocytes secreted IL-4 upon stimulation, whereas DCs from CD16<sup>-</sup> monocytes produced IL-12 and promoted Th<sub>1</sub>-like cytokine production in T cells (38). DCs derived from CD16<sup>+</sup> monocytes may have been biased toward Th<sub>2</sub> responses due to their purification using anti-CD16 mAb-coated minibeads, as a recent report indicates that Th<sub>1</sub> responses can be converted to Th<sub>2</sub> responses by targeting Fc $\gamma$  receptors (39). However, the possibility that CD16<sup>+</sup> DC precursors may participate in Th<sub>2</sub> polarization is interesting considering that we have also uncovered a key role for CCR8 in mediating reverse transmigration (unpublished data), and *in vivo* studies indicate that CCR8<sup>-/-</sup> mice manifest greatly diminished Th<sub>2</sub> responses without diminution of Th<sub>1</sub> immunity (40). Future studies to determine whether a subpopulation of monocytes exists in mice that are analogous to the CD16<sup>+</sup> blood monocyte population and the role of CCR8 in their migration are needed.

The biologic function of CD16 expressed by monocytes that later develop into migratory DCs appears to include promoting survival in response to a phagocytic stimulus. CD16 is specialized for recognition of immune complexes, but it is also likely that CD16 is directly involved in binding to zymosan via serum amyloid proteins that opsonize zymosan and are ligands for Fc receptors (FcRs; reference 41). Although other FcRs (41), integrins (42), and toll-like receptors (43) are involved in the uptake and/or response to zymosan in the absence of available CD16, CD16 nevertheless appears to have an important role in signaling survival in the presence of this stimulus. Engagement of CD16 has been previously shown to prevent apoptosis of monocytes in response to IL-10 (44). This mechanism operating *in vivo* might lead to the death and clearance of CD16<sup>-</sup> phagocytes that have performed their duties at sites of inflammation, but preserve the viability of phagocytes with the capacity to migrate to lymph nodes as antigen-presenting DCs. On the other hand, CD16<sup>+</sup> monocyte-derived cells may promote not only their own survival, but also the survival and differentiation of CD16<sup>-</sup> cells *in trans*. Finally, observations that FcRs critically participate in the generation of CTLs after antibody-mediated opsonization and cross-presentation highlight the importance of FcR function in DCs during immune surveillance (45). The present findings suggest that CD16<sup>+</sup> monocytes may serve as a major pool of DC precursors with optimal capacity for FcR-facilitated presentation *in vivo*.

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