initiation of the culture at 100 U/ml. On day 8, 6×10^6 to 10×10^6 responder cells were incubated with 1×10^7 autologous phytohemagglutinin (PHA) blasts infected with vCP182 and were cultured at 1×10^6 cells/ml in a 25-cm^2 flask in the presence of rIL-2 (20 U/ml) and rIL-7 (100 U/ml) for an additional 5 days. PHA blasts were generated by stimulating PBMCs with 0.2% (v/v) PHA (Sigma). Primary CTL assays were performed on day 7. Secondary CTL assays were performed on day 13 after restimulation on day 8. Target cells were autologous or HLA-mismatched PHA blasts that were sensitized overnight with synthetic peptides (10 μg/ml) representing previously identified CTL epitopes on the PfCSP (*19*). Targets were labeled with 100 μCi ^{51}Cr [sodium chromate solution (Dupont New England Nuclear, Boston, MA)] for 90 min at 37°C and washed three times before use. The CTL activity was assessed by a conventional 6-hour chromium release assay, in the presence of a peptide (10 μg/ml). The percent lysis was defined as (experimental release − medium control release)/(maximum release − medium control release) × 100. The percent specific lysis was determined by subtracting the percent lysis of the targets that were sensitized with control peptide 242 from the percent lysis of the targets that were incubated with the experimental peptide. The results were expressed as the mean of triplicate determinations. The CTL responses were considered to be positive only if the percent specific lysis after immunization was ≥10% for at least two effector:target (E:T) ratios in the same assay and if the percent specific lysis before immunization was <10%. Spontaneous release values were always <20%.

16. PBMCs were resuspended at a concentration of 10×10^6 cells/ml in 20% fetal calf serum (Sigma) in RPMI 1640, and an equal volume of ice-cold 15% dimethyl sulfoxide in RPMI 1640 was added dropwise, with shaking. All procedures were performed on ice. The cells were transferred to cryotubes at a final concentration of 5×10^6 cells/ml in each vial, and the tubes were placed in a plastic foam container at −80°C overnight before being transferred to liquid nitrogen.
17. The CTL assays were conducted with four E:T combinations: (i) ALVAC PfCSP effectors and Western Reserve (WR) vaccinia PfCSP targets, (ii) ALVAC PfCSP effectors and experimental or control peptide–pulsed targets, (iii) experimental peptide–induced effectors and WR vaccinia PfCSP targets, and (iv) experimental peptide–induced effectors and experimental or control peptide–pulsed targets. All assays with WR vaccinia PfCSP-infected targets were excluded from the analysis because a simultaneous assay of PBMCs from control-naïve volunteers demonstrated an unacceptably high level of positivity (*21*). Assays that were conducted with PfCSP peptide-stimulated effectors against peptide-sensitized targets were not positive.
18. Recombinant pox viruses were produced in collaboration with Virogenetics (Troy, NY) [J. A. Tine *et al.*, *Infect. Immun.* **64**, 3833 (1996); D. E. Lanar *et al.*, *ibid.*, p. 1666]. The ALVAC virus expressing PfCSP (vCP182) was used for the stimulation of CTL effectors. Recombinant vaccinia viruses (WR) encoding PfCSP (vP1255) or PfLSA-1 (vP1253) (control) were used for the infection of target cells.
19. The following peptide sequences and residue numbers are based on the complete PfCSP 3D7 amino acid sequence (residues 1 through 397), and variant residues are indicated in bold and underlined: (i) $A1_{310}$, HLA-A1 restricted, residues 310 through 319, sequence EPSDKHI**KE**Y (*28, 29*); (ii) $A2_{386}$, HLA-A2 supertype, residues 386 through 394, GLIMVLSFL (*24*); (iii) $A2_{7}$, HLA-A2 supertype, residues 7 to 16, ILSVSSFLFV (*24*); (iv) $A2_{1}$, HLA-A2.1, residues 1 through 10, MMRKLAILSV (*30*); (v) $A2_{319}$, HLA-A2.1, residues 319 through 327, YL**N**KI**Q**NSL (*30*); (vi) $A3/11_{336}$, HLA-A3/11 supertype, residues 336 through 345, VTCGNGIQVR (*24*); (vii) $B7_{285}$, HLA-B7, residues 285 through 293, MPN**D**PNRNV (*25*); (viii) $B8_{86}$, HLA-B8, residues 86 through 94, LRKPKHKKL (*25*); (ix) $B35_{353}$, HLA-B35, residues 353 through 360, K**P**-KDELDY (*26*); (x) $B35+_{353}$, HLA-B35 plus (contains a HLA-B35 restricted epitope but also contains one or two additional epitopes of undefined genetic restriction), residues 353 through 375, K**P**KDELDYANDIEK-KICKKMEKCS (*27*); (xi) Pep242, random sequence, RALMSMVLIK. PfCSP-derived synthetic peptides were synthesized by Pasteur-Merieux Connaught Laboratories and were purified through high-performance liquid chromatography. Control peptide 242 was generated by a random scrambling of the HLA-A2 binding peptide, $A2_1$. Lyophilized peptides were reconstituted at 20 mg/ml with 100% dimethyl sulfoxide (Sigma) and stored at −80°C until use. The peptide was diluted to 2 mg/ml with RPMI 1640 without serum before use.
20. A comparison of the primary assay (one in vitro restimulation) versus the secondary assay (two in vitro restimulations) gave the following results: The number of positive individuals out of the total number of tested individuals was 10 of 20 versus 6 of 20; the number of positive assays out of the total number of assays was 52 of 218 (22.8%) versus 28 of 123 (23.9%); the range of the percent specific lysis was 10.2 to 67.7% versus 10.1 to 37.91%. Volunteer 33, who was not positive in the primary assay, was only studied at one time point (*13*).
21. R. Wang *et al.*, data not shown.
22. The ALVAC PfCSP–stimulated effector cell populations were depleted in vitro of $CD8^+$ or $CD4^+$ T cells immediately before the assay with anti-$CD4^+$– or anti-$CD8^+$–coated Dynabeads M-450, according to the manufacturer's instructions (Dynal, Great Neck, NY). Flow cytometric analysis confirmed that the cell subset depletion was >95% in all cases (*21*).
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29. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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31. We are indebted to P. De la Vega, A. Figer, T. D. Bangura, S. Doria, V. Fallarame, and S. Abot for excellent technical assistance; W. O. Rogers for advice and support; J. Tine (Virogenetics), for providing the canary pox (ALVAC) and vaccinia (WR) viruses; A. Pedyczak (Pasteur-Merieux Connaught) for synthesis of PfCSP peptides; the many individuals at Vical who contributed to the project, especially S. Kradjian, R. Zaugg, and J. Meek; and Pasteur-Merieux Connaught for financial support. Our thanks extend to the volunteers, without whom this study would not have been possible. The research protocol for human participants in this study was approved by the Naval Medical Research Center's Committee for the Protection of Human Subjects, the U.S. Army Medical Research Institute of Infectious Diseases Human Use Committee, and the Surgeon General's Human Subjects Research Review Board, in accordance with the U.S. Navy regulation (SECNAVINST 3900.39B) governing the use of human participants in medical research. This work was supported in part by Naval Medical Research and Development Command Work Unit STO F 6.3a63002AA0101HFX, Office of Naval Research ATD PEN0603792 project R1889, and 060357OD.R357.6FDP9500-00.1532 Federal Defense Laboratories Diversification Program.

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Differentiation of Monocytes into Dendritic Cells in a Model of Transendothelial Trafficking

Gwendalyn J. Randolph,* Sylvie Beaulieu, Serge Lebecque, Ralph M. Steinman, William A. Muller

Essential to the dendritic cell system of antigen-presenting cells are the veiled dendritic cells that traverse afferent lymph to enter lymph nodes, where they initiate immune responses. The origin of veiled cells, which were discovered 20 years ago, is unclear. Monocytes cultured with endothelium differentiated into dendritic cells within 2 days, particularly after phagocytosing particles in subendothelial collagen. These nascent dendritic cells migrated across the endothelium in the ablumenal-to-lumenal direction, as would occur during entry into lymphatics. Monocytes that remained in the subendothelial matrix became macrophages. Therefore, monocytes have two potential fates associated with distinct patterns of migration.

One of the important features of dendritic cells (DCs) is their capacity to migrate from peripheral tissues to lymphoid organs and initiate immunity. DCs gain access to the spleen from the bloodstream and enter lymph nodes by migration through afferent lymphatic vessels (*1*). Bearing soluble proteins (*2*) and particulates (*3*) that they acquire before entry into lymph nodes, DCs localize to the T cell areas where they are ideally positioned to select and activate clones of antigen-reactive T lymphocytes. When afferent lymphatic conduits are severed, immunity to peripherally administered antigen does not develop (*4*). Lymph DCs may also induce peripheral tolerance to self-antigens acquired during the

G. J. Randolph and W. A. Muller, Department of Pathology, Cornell University Medical College, 1300 York Avenue, Room C-420, New York, NY 10021, USA. S. Beaulieu and R. M. Steinman, Laboratory of Cellular Physiology and Immunology, Rockefeller University, New York, NY 10021, USA. S. Lebecque, Laboratory for Immunological Research, Schering-Plough, 69571 Dardilly, France.

*To whom correspondence should be addressed. E-mail: GJRandol@mail.med.cornell.edu

capture of apoptotic cells (*5*, *6*). After interacting with T cells, DCs probably die within the lymph nodes, because they do not enter efferent lymph (*1*).

The origin of lymph-borne DCs, often called veiled cells because of their many sheetlike cellular processes, is unclear. Large-scale migration of DCs from immunologically perturbed tissues can be attributed in part to the mobilization of interstitial and epidermal DCs (*7*, *8*). Yet even in the absence of overt stimuli, numerous DCs traffic through lymph (*9*) at an estimated rate of 10^4 cells per hour (*10*, *11*), suggesting that veiled DCs are constitutively generated from a precursor population. When rats are pulsed with [^{3}H]thymidine, it is evident that veiled cells, which are not in cell cycle, arise from a precursor that underwent cell division 1 to 4 days (mode = 3 days) previously (*11*).

Monocytes continuously emigrate from the blood into peripheral tissues with a half-life in the blood of about 1 day in mice (*12*). Nondividing monocytes can differentiate into DCs in culture. However, this conversion requires treatment of monocytes with combinations of cytokines, typically interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor, and takes 7 to 11 days in culture (*13*, *14*). We now describe a culture system in which monocytes differentiate into DCs in only 2 days as they traverse endothelial monolayers in the basal-to-apical direction (reverse transmigration), a migratory process that is reminiscent of the movement of cells from tissues into lymphatic vessels. Monocytes that do not reverse transmigrate become macrophages, exhibiting a phenotype entirely different from DCs.

In this culture system, freshly isolated blood mononuclear cells were incubated with unstimulated monolayers of human umbilical vein endothelial cells grown on an endotoxin-free collagenous matrix (*15*). Within 1 hour, monocytes bound to and diapedesed into the subendothelial collagen, by use of β_2 integrins (CD18) (*15*) and CD31 (*16*), respectively. Because few lymphocytes transmigrated under these conditions, subendothelial cells were greatly enriched for CD14$^+$ cells (about 90%). During 2 days of further culture, half of the subendothelial monocytes subsequently reverse transmigrated across the overlying, intact endothelium (*17*, *18*). Reverse transmigration does not require CD18 or CD31 but uses functional P-glycoprotein (MDR-1), a transmembrane transporter of the adenosine triphosphatase–binding cassette superfamily (*18*). Similarly, the migration of DCs from skin through authentic lymphatic channels does not require CD18 or CD31 but is blocked by antagonists of P-glycoprotein (*18*).

Using this model (*19*), we investigated whether monocyte-derived, reverse-transmigrated cells could develop into DCs by monitoring the phenotype of the cells at different stages in culture. The cells that initially transmigrated into the collagen (lumenal-to-ablumenal transendothelial migration) after a 1.5-hour incubation of endothelium with bulk peripheral blood mononuclear cells (PBMCs) were typical monocytes. These cells expressed CD14 and the Fcγ receptors CD32 and CD64 (Fig. 1A). Human leukocyte antigen–DR (HLA-DR) was present but at relatively low levels. The T cell costimulatory molecules CD80 and CD86 were absent or very low, respectively, and the DC maturation marker CD83 (*20*) was undetectable. A few CD3$^+$ T cells were also retained in the cultures (about 10% of total); CD3$^-$CD14$^-$CD19$^-$HLA-DR$^+$ nonmonocytic DC precursors (*21*) were present at 2 to 5% relative to CD14$^+$ monocytes.

Reverse-transmigrated cells were collected after 48 hours (*22*), when most cells that will reverse transmigrate have done so

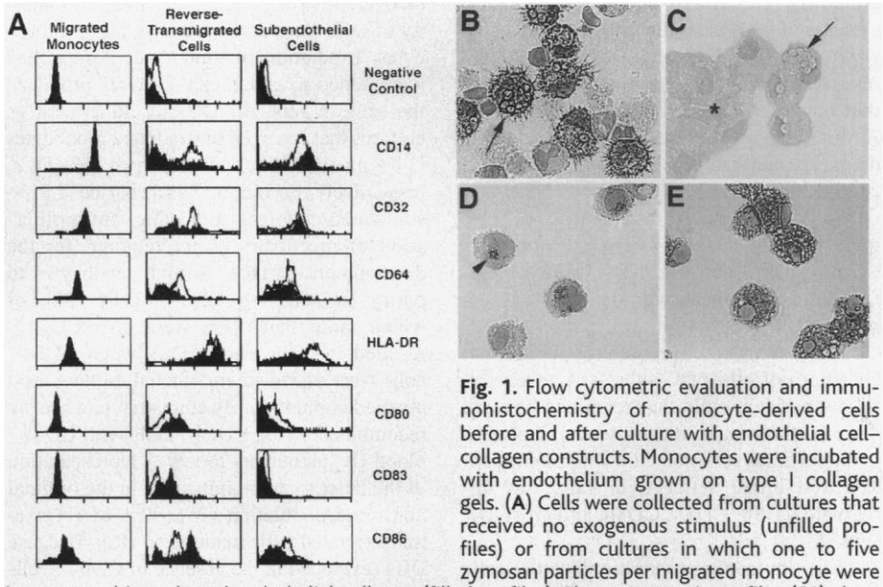

Fig. 1. Flow cytometric evaluation and immunohistochemistry of monocyte-derived cells before and after culture with endothelial cell–collagen constructs. Monocytes were incubated with endothelium grown on type I collagen gels. (**A**) Cells were collected from cultures that received no exogenous stimulus (unfilled profiles) or from cultures in which one to five zymosan particles per migrated monocyte were incorporated into the subendothelial collagen (filled profiles). Flow cytometric profiles (*30*) show monocytes (left) collected immediately after their transmigration into the collagen (profiles from unstimulated and zymosan-containing cultures overlap), reverse-transmigrated cells (middle) collected after 48 hours, and monocyte-derived cells remaining in the subendothelial matrix (right) at 48 hours. Tick marks on the *x* axis designate logarithmic increments in fluorescence intensity. (**B** to **E**) Immunostaining (*31*) for p55 (dark cytoplasmic staining indicates positive staining) is shown for reverse-transmigrated (B) and subendothelial (C) cells derived from cultures containing zymosan. Arrows illustrate cells with ingested zymosan particles. Digests to obtain cells residing in the collagen contain some clusters of endothelial cells (asterisk). Lysosomal markers were examined with cells derived from IL-1–stimulated cultures. The presence of DC-LAMP in perinuclear lysosomes (arrowhead) was observed in reverse-transmigrated cells (D). Subendothelial monocyte-derived cells expressed another lysosomal marker, CD68, throughout the cytoplasm (E).

Table 1. Total cells recovered and extent of DC maturation in reverse-transmigrated (RT) and subendothelial (SE) monocyte-derived populations.

Stimulus	Experiments (*N*)	Total cells recovered from 20 ml of blood × 10^{-5}*		Mature DC (%)‡	
		RT†	SE†	RT	SE
None	9	12	13	7 ± 7	0.2 ± 0.4
IL-1	5	9	9	15 ± 7	0.1 ± 0.5
LPS	4	13	14	14 ± 2	2 ± 2
MCM	3	9	9	19 ± 3	0.7 ± 1.2
Latex	4	8	9	43 ± 15	0 ± 0.5
Zymosan	9	9	10	47 ± 13	2 ± 3

*Data are derived from 48-hour cultures. An average of 23 × 10^5 monocytes from 20 ml of blood were present in the subendothelial collagen after 2 hours of coculture with endothelial cells. †Differences in recovery between treatments are not significant. ‡Maturity defined by two-color flow cytometry to identify HLA-DR^{high}/CD83$^+$ cells.

(*17*, *18*). The monocyte-derived cells that did not reverse transmigrate but remained in the collagen (*23*) were also analyzed. In the absence of additional stimuli, most reverse-transmigrated cells and subendothelial mononuclear phagocytes had a similar phenotype (Fig. 1A). CD32 was maintained at levels similar to monocytes, but CD14 and CD64 typically decreased by 50%. CD80 and CD86 remained low. Other T cell stimulatory molecules, including HLA-DR (Fig. 1A) and CD54 (*24*), increased by an order of magnitude.

Incorporation of phagocytic particles, including latex microspheres and zymosan (yeast cell walls), in the collagen gel had a striking differential effect on the cells that reverse transmigrated compared with those that remained in the subendothelial matrix. About half of reverse-transmigrated cells displayed characteristics of mature DCs (Fig. 1A and Table 1). CD14, CD32, and CD64 (Fig. 1A) were lost, as was the integrin CD11b (*24*). T cell costimulators CD80, CD86, and HLA-DR were all upregulated, and there was de novo expression of the DC maturation marker CD83. CD1a, a marker expressed by epidermal DCs but not other DC subsets, was absent. In comparison, cells that remained beneath the endothelium in the presence of phagocytic particles retained CD14 and Fc receptors and either reduced or failed to upregulate CD80, CD83, CD86, and HLA-DR (Fig. 1).

When cytospin preparations of the cells were examined for intracellular markers, many reverse-transmigrated cells had the features of DCs. These cells were large, with extended dendritic processes. They were positive for the DC marker actin bundling protein p55 (*25*) (Fig. 1B). In contrast, the subendothelial cells had the appearance of typical phagocytes, lacking p55 (Fig. 1C). Reverse-transmigrated cells were also positive for the newly identified DC-specific lysosomal marker DC-LAMP (*26*) (Fig. 1D), whereas subendothelial cells were strongly positive throughout the cytoplasm for a different lysosomal marker, CD68, expressed by macrophages (Fig. 1E) (*27*). Both reverse-transmigrated and subendothelial cells phagocytosed the zymosan when it was present in the collagen (Fig. 1, B and C).

After 48 hours of culture, total cell yields were similar in all conditions tested, so that only the extent to which reverse-transmigrated cells had become mature DCs varied depending on the stimulus (Table 1). The number of nonmonocytic $CD14^-$ large mononuclear cells retained in the cultures was too low to account for the abundance of DCs recovered, strongly implying that the majority of DCs were derived from $CD14^+$ monocytes. Irradiation of monocytes before they were added to endothelial cultures produced the same number of DCs by all criteria as cultures that received unirradiated monocytes [14% mature DCs compared with 13% DCs, respectively, from lipopolysaccharide (LPS)–stimulated cultures], indicating that proliferation of precursors is not required for the development of DCs. Sorting monocytes to purity by selecting $CD64^+$ cells (*28*), of which more than 97% were also $CD14^+$, resulted in 29% mature DCs when $CD64^+$ cells were added to endothelial cultures as a purified population. By comparison, when we recombined sorted $CD64^+$ cells with $CD64^-$ blood DC precursors to match representation of the latter to their abundance in the original nonsorted population (*28*), 30% of reverse-transmigrated cells matured to DCs. Because DCs developed in the absence of $CD64^-$ cells to a similar extent as in their presence, these data indicate that reverse-transmigrated DCs originated predominantly from $CD14^+CD64^+$ monocytes.

Reverse-transmigrated cells from unstimulated endothelial cell cultures were studied further to examine whether they would differentiate to DCs. Removed from the endothelium after 48 hours, these cells were subsequently cultured in medium lacking additional stimuli or in medium containing LPS or five zymosan particles per cell. After 3 days of incubation in these conditions, 16, 24, and 81% were mature DCs, respectively (Fig. 2, B to D). Thus, although only 6% of reverse-transmigrated cells from unstimulated cultures resembled mature DCs after collection from endothelial cultures (Fig. 2A), these data indicate that such cells can mature to DCs upon further stimulation. However, cells that did not mature fully to DCs could also revert to macrophages when continued in culture on plastic dishes, as previously documented for immature DCs (*14*). Fully mature $CD83^+$ DCs did not revert to a

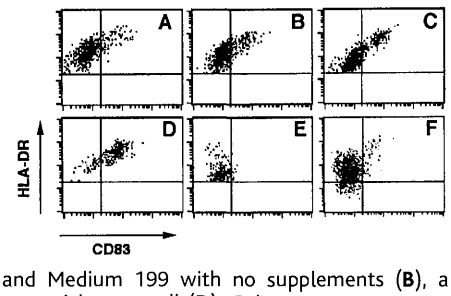

Fig. 2. Maturation of unstimulated reverse-transmigrated cells to DCs after collection from endothelium and analysis of the requirement for endothelium. Maturation was monitored by expression of CD83 and high levels of HLA-DR. Quadrants are marked on the basis of the amount of fluorescence intensity observed in samples stained with control mAbs. Cells were analyzed just after collection from unstimulated endothelial cultures at 48 hours (**A**) or after 3 days of further culture in Teflon beakers containing 20% heat-inactivated human serum and Medium 199 with no supplements (**B**), a supplement of LPS (10 ng/ml) (**C**), or five zymosan particles per cell (**D**). Other monocytes were never added to endothelial cell cultures but instead were incubated in Teflon beakers in the same medium containing LPS (10 ng/ml) (**E**) or were incubated with fibronectin-coated collagen gels impregnated with zymosan particles but lacking an endothelial monolayer (**F**). Data are representative of three experiments.

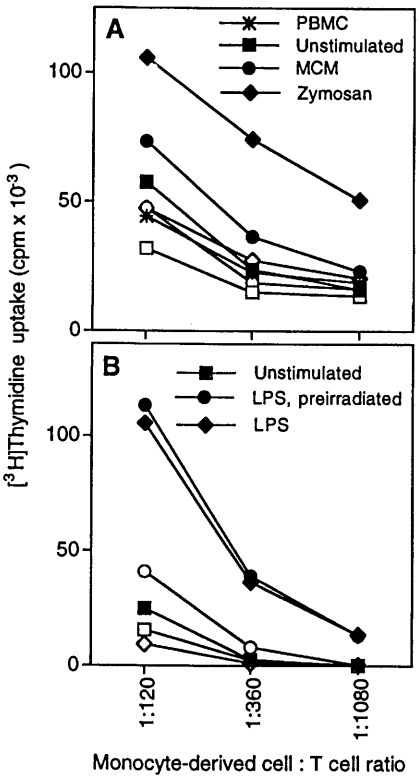

Fig. 3. Capacity of reverse-transmigrated and subendothelial cells to stimulate allogeneic T cell proliferation. After monocytes entered collagen gels (1.5 hours), cultures were treated with LPS (1 ng/ml) and 30% MCM or incubated in medium without additional additives. Zymosan particles were incorporated into the collagen of some cultures. Monocyte-derived cells taken from reverse-transmigrated (filled symbols) or subendothelial populations (open symbols) were collected from endothelial cell cultures at 48 hours of incubation and irradiated with 30 grays before being combined with allogeneic T cells (**A**) (*32*). Alternatively, monocytes were irradiated just after their isolation and subsequently added to endothelium (**B**) (preirradiated samples) for a 48-hour incubation, and then reverse-transmigrated and subendothelial populations were collected. For all conditions, monocyte-derived cells were mixed with T cells at indicated ratios and cultured for 5 days and then finally in [^{3}H]thymidine (4 μCi/ml) for 18 hours. Counts per minute for T cells alone was <300. Standard errors were not greater than 10% of the mean value. (A) and (B) show two independent analyses that are representative of five experiments.

macrophage phenotype.

Exposure to the endothelium was essential for this rapid differentiation of monocytes to DCs. When monocytes were incubated in the presence of LPS or zymosan particles but were not added to endothelial-collagen cultures (Fig. 2E) or were incubated with the collagen matrix containing zymosan particles but lacking an endothelial monolayer (Fig. 2F), they expressed low HLA-DR and had little to no detectable expression of CD83 or p55.

To verify that the reverse-transmigrated cells had acquired the functional features of DCs, we carried out mixed lymphocyte reactions. The reverse-transmigrated cells were potent stimulators of allogeneic T cell proliferation and much more efficacious than their subendothelial counterparts (Fig. 3). The stimulatory capacity of reverse-transmigrated cells was increased upon addition of a macrophage-conditioned medium (MCM) (Fig. 3A) that has been described as a potent maturation cocktail for DCs (*14*) or by treatment with LPS (Fig. 3B) or IL-1. Reverse-transmigrated cells derived from monocytes that were irradiated before they were added to endothelial cultures promoted proliferation of T cells as well as their unirradiated counterparts, underscoring the conclusion that DCs from these cultures derived from a pool of nonproliferating mononuclear cells (Fig. 3B). In agreement with the potentiating effects of phagocytic stimuli on surface markers of DC maturation in the vessel wall cultures (Table 1), reverse-transmigrated cells derived from cultures containing latex (*24*) or zymosan (Fig. 3A) were the most potent stimulators of T cell proliferation.

Consistent with our observations, previous studies have observed that the composition of large mononuclear cells in lymph (excluding lymphocytes) is heterogeneous with respect to the extent to which they have matured to DCs. Some of these cells exhibit features characteristic of monocytes (*7, 9, 29*), including residual phagocytic activity. A few studies have noted that lymph DCs have phagolysosomes containing cellular debris (*7, 11*) and other particles (*3*). Thus, phagocytic stimuli, possibly including the ingestion of apoptotic cells (*6*), may well be a maturation stimulus for DCs in vivo. However, development of monocytes to fully differentiated DCs may not always predominate in vivo. In one study of lymph collected from superficial leg lymphatics of humans (*29*), cells that resembled monocytes were three to four times more abundant than those that resembled mature DCs. These monocyte-like cells may be analogous to reverse-transmigrated cells from our unstimulated cultures.

Our studies indicate that, in the presence of endothelial cells grown on an extracellular matrix, the differentiation of monocytes diverges along two distinct pathways toward DCs or macrophages. DCs arise from monocytes that migrate across endothelium in an ablumenal-to-lumenal direction, whereas macrophages develop from monocytes that remain in the subendothelial matrix. We suggest that endothelial tissues initiate differentiation of monocytes to DCs and that full differentiation requires an additional stimulus such as the uptake of particles or exposure to microorganisms.

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19. Endothelial cultures were established in 96-well plates and incubated at all times in Medium 199 supplemented with 20% heat-inactivated human serum. Transendothelial migration assays were conducted as described in (*15–18*). For some samples, during the preparation of polymerized collagen gels and before addition of endothelial cells, latex microspheres (0.65-μm diameter; Bangs Laboratories, Carmel, IN) or zymosan (ICN, Costa Mesa, CA; autoclaved in phosphate-buffered saline) was mixed with monomeric collagen at concentrations of 0.05% (w/v) or 0.0025% (w/v), respectively. LPS was undetectable in the collagen preparations, as tested with a limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). PBMCs were incubated with confluent, unstimulated endothelial monolayers for 1.5 hours and then washed to remove nonmigrated lymphocytes and monocytes (only about one-half of monocytes transmigrate into the collagen). Then Medium 199 containing 20% heat-inactivated human serum with or without soluble stimuli [recombinant human IL-1 (500 pg/ml), 30% MCM (*14*), or LPS (1 ng/ml)], when used, was added for 2 days.
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22. Reverse-transmigrated cells accumulated in the medium overlying endothelium and were pipetted off for collection. To obtain reverse-transmigrated cells that were loosely adherent to the apical surface of the endothelium, we also collected two subsequent washes in Hanks' buffered saline solution containing 1 mM EGTA.
23. After collection of reverse-transmigrated cells, Hanks' buffered saline solution containing 1 mM EGTA and collagenase D (2 mg/ml) (Boehringer Mannheim) was added to each well and incubated at 37°C for 40 min. Solubilized cultures were transferred to a cell strainer and washed with medium to obtain cell suspensions devoid of collagenous debris. Digests contained a mixture of monocyte-derived and endothelial cells. Cell viability was > 95%. In control experiments, treatment of reverse-transmigrated cells with collagenase by means of this procedure did not alter the phenotypic properties of these cells.
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28. For isolation of $CD64^+$ cells to purity, PBMCs were incubated with antibody to CD3– and antibody to CD19–conjugated magnetic beads (Dynal) to remove lymphocytes by use of a magnet. Remaining cells were immunolabeled with fluorescein isothiocyanate (FITC)–conjugated monoclonal antibody (mAb) to CD64 (Ancell, Bayport, MN) and sorted (FACS Vantage; Becton-Dickinson) to greater than 98% purity. $CD64^-$ large mononuclear cells were separately retained from the sort, and their maturation to $CD83^+$ DC was verified after 2 days of culture in 30% MCM (*21*). For some control experiments, these $CD64^-$ cells were added back to $CD64^+$ sorted cells to a level of 10% of the total population to match their abundance in nonsorted cells and used in reverse transmigration assays. Lymphocytes that were not depleted by magnetic beads were discarded during the cell sorting by setting a gate to remove cells with the forward and side scatter properties of lymphocytes.
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30. Portions of cells were incubated on ice with mAbs (1 μg/ml) to the following antigens: CD14 (3C10), CD32 (IV.3; Mederex, Annandale, NJ), CD64 (10.1; Ancell), CD80 (P1.H5.A1; Ancell), CD83 (HB15a; Immunotech, Miami, FL), and CD86 (IT2.2; Pharmingen). Nonbinding control mAbs included immunoglobulin G1 (IgG1) and IgG2a control from Immunotech, UPC10 (Sigma), and MOPC 21 (Sigma). These mAbs were detected with FITC-conjugated goat antibody to mouse IgG (Dako). R-phycoerythrin–conjugated antibody to HLA-DR (Becton-Dickinson) was used for two-color flow cytometry. Samples were analyzed in a FACSCalibur instrument, with CellQuest software.
31. Cytospins of reverse-transmigrated cells and subendothelial cells were stained with isotype-matched control mAb UPC10 (Sigma), p55 mAb, or CD68 mAb followed by horseradish peroxidase–conjugated antibody and diaminobenzamidine substrate. Nuclei were counterstained with hematoxylin.
32. E-rosette–positive T cells were prepared from human blood and further purified by panning with HLA-DR mAb.

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