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Differentiation of Phagocytic Monocytes into Lymph Node Dendritic Cells In Vivo

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Summary

We investigated the differentiation and trafficking of inflammatory monocytes that phagocytosed subcutaneously injected fluorescent microspheres. As expected, most of the monocytes became microsphere$^+$ macrophages, which remained in subcutaneous tissue. However, about 25% of latex$^+$ cells migrated to the T cell area of draining lymph nodes, where they expressed dendritic cell (DC)-restricted markers and high levels of costimulatory molecules. Microsphere-transporting cells were distinct from resident skin DCs, and this transport was reduced by more than 85% in monocyte-deficient osteopetrotic mice. Thus, a substantial minority of inflammatory monocytes carry phagocytosed particles to lymph nodes and differentiate into DCs.

Introduction

Monocytes continuously exit the bloodstream and enter body tissues, where many differentiate to macrophages (van Furth, 1988). Since there is no net accumulation of macrophages in tissues during the steady state, monocyte entry into tissues may be just sufficient to replace dying macrophages, or monocytes may only transiently traverse tissues (van Furth, 1988). During resolution of inflammation, at least some monocytes leave tissues by migrating to draining lymph nodes (Bellingan et al., 1996). However, the state of differentiation of monocyte-derived cells that arrive in lymph nodes has not been characterized.

Human monocytes can be directed to develop into potent immunostimulatory dendritic cells (DCs) when cultured in the presence of GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994; Bender et al., 1996; Kiertscher and Roth, 1996; Romani et al., 1996; Zhou and Tedder, 1996), but it is not known whether monocytes encounter physiologic conditions that induce their differentiation to DCs in vivo. Recently, we observed that monocytes differentiate into DCs in an in vitro model of transendothelial trafficking without addition of exogenous cytokines (Randolph et al., 1998), supporting the idea that monocytes can become DCs under physiologic conditions. Monocytes that mature to DCs in this model do so most efficiently when they receive a phagocytic stimulus during their transient residence in subendothelial matrix. These nascent DCs then traffic across the endothelium in the ablumenal-to-lumenal direction (Randolph et al., 1998), a movement that may mimic the migration of DCs from the periphery into the lumen of an afferent lymphatic vessel. Prompted by these in vitro studies, we have now employed murine models to address whether monocytes become lymph-borne DCs in vivo after peripheral administration of phagocytic latex particles.

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Results

Phenotype of Murine Phagocytic Cells at the Site of Microsphere Injection

Intracutaneous injection of latex microspheres at various skin sites initiated the infiltration of monocytes (Figure 1A). By 18 hr following injection, essentially all particles had been phagocytosed by cells in the infiltrate. Consistent with the possibility that these cells were monocytes, the phagocytes expressed high levels of CD11b (Springer et al., 1979) (Figure 1B) but were negative for the macrophage marker F4/80 (Hume et al., 1983) at the time of infiltration. However, they upregulated expression of F4/80 by day 3 (Figure 1C). Monocytes express very low F4/80 antigen, but its expression increases during maturation to macrophages (Ajuebor et al., 1998).

F4/80 and CD11b are also expressed on some interstitial DCs (Larsen et al., 1990; Weinlich et al., 1998). Thus, to determine whether interstitial dermal DCs and/or epidermal DCs (Langerhans cells) had phagocytosed particles, skin sections were stained for expression of DEC-205, a marker that identifies epidermal and dermal skin DCs and many mature DCs in lymphoid organs (Kraal et al., 1986; Inaba et al., 1995) but that is not expressed on monocytes and macrophages. DEC-205$^+$ cells very rarely colocalized with microspheres, even though in some sections a few DEC-205$^+$ cells were observed in tissue surrounding the microspheres (Figure 1D). Occasional DEC-205$^+$ cells were seen bearing a very limited number of particles, suggesting that some resident DCs were modestly phagocytic in situ.

Fate of Phagocytes at the Site of Injection

In order to determine the fate of the injected microspheres, we performed acetone extraction of the tissue at the site of microsphere injection and then quantitated the units of fluorescence recovered. Approximately 25% of injected microspheres were cleared from the skin by day 4. The remaining 75% remained in situ. Histologic analysis up to day 18 showed that such particles were contained within a population of CD11b$^+$F4/80$^+$DEC-205$^-$ macrophages (data not shown).

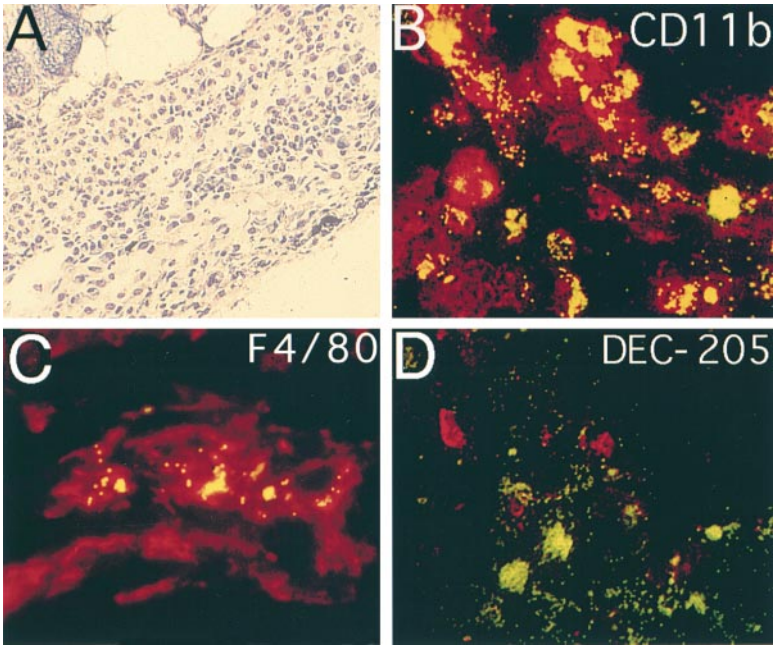

Figure 1. Monocytes Ingest Latex Microspheres In Situ

FITC-dyed microspheres were injected intracutaneously into the dorsal thoracic skin. Histologic and immunofluorescent analyses were conducted at different time points. By 18 hr after injection of microspheres, a predominantly mononuclear cell infiltrate was observed ([A], low power). Immunostaining for cell surface markers (red) CD11b (B), F4/80 (C), and DEC-205 (D) are shown from skin sections collected after 18 hr (B and D) or 3 days (C) following injection of microspheres ([B–D], high power). These data are representative of more than a dozen experiments using several mouse strains. (A), (B), and (D) show sections of skin from FVB/N mice, and (C) is from mice of mixed background (B6C3FEA/F1), which served as normal controls for the experiment using monocyte-deficient mutants shown in Figure 6C. Yellow staining indicates colocalization of FITC-labeled spheres (green) with Cy3-conjugated mAb–labeled cells (red).

Previous work was consistent with the possibility that some monocytes may leave tissues by retraversing the vasculature (Randolph and Furie, 1996). Thus, we examined the blood and many body organs for the presence of FITC-latex-bearing cells. Such cells were very rarely observed in the spleen and were not detectable in the blood (data not shown). However, a large population of FITC-latex$^+$ cells accumulated in draining lymph nodes, reaching a maximum at 3–4 days (Figure 2A). Immunostaining for the B cell marker B220 to identify the follicles within lymph nodes showed that particle-bearing cells localized to the paracortical T cell area, just beneath the B cell follicles (Figure 2B). Microsphere$^+$ cells were rare in the macrophage-rich subcapsular sinus or medulla of the lymph nodes.

Control experiments were conducted, as previously described (Harmsen et al., 1985), to ensure that we were examining the trafficking of phagocytes from the periphery to draining lymph nodes rather than the possible escape of free microspheres into afferent lymph and subsequent uptake by cells already present in the lymph node. Red fluorescent microspheres were injected into a skin site adjacent to but not overlapping with the site at which FITC-dyed microspheres had been injected. The selected sites all drained to the same lymph nodes (brachial). Cells that accumulated by day 4 in draining lymph nodes contained either green or red microspheres, but not both (Figure 2C), demonstrating that the particles were engulfed by phagocytes in the tissues before they migrated to the lymph node. Had the particles entered lymph on their own, beads of both colors would have been found together within individual cells in the lymph node.

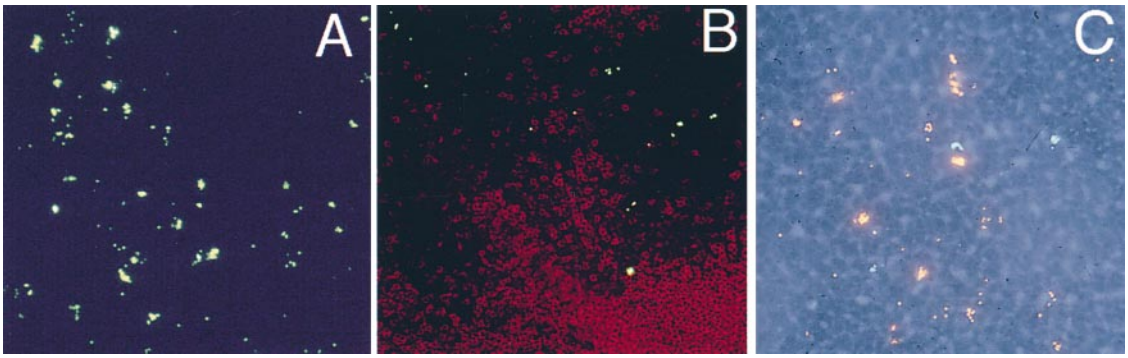

Figure 2. Migration of Microsphere$^+$ Cells to the T Cell Zone of Draining Lymph Nodes

Lymph node sections were examined daily up to 8 days. Peak accumulation of microspheres occurred at day 3–4 (A). Microsphere$^+$ cells localized to the T cell paracortical region that lies just beneath the B cell follicles, which were stained with B220 mAb followed by Cy3-conjugated secondary mAb (B). Injection of green and red microspheres, respectively, at adjacent but nonoverlapping sites in skin showed individual cells in the lymph node at day 4 that contained microspheres of one color or the other, but not both (C). Panel was photographed during UV light excitation, such that green microspheres appear turquoise and red beads appear orange.

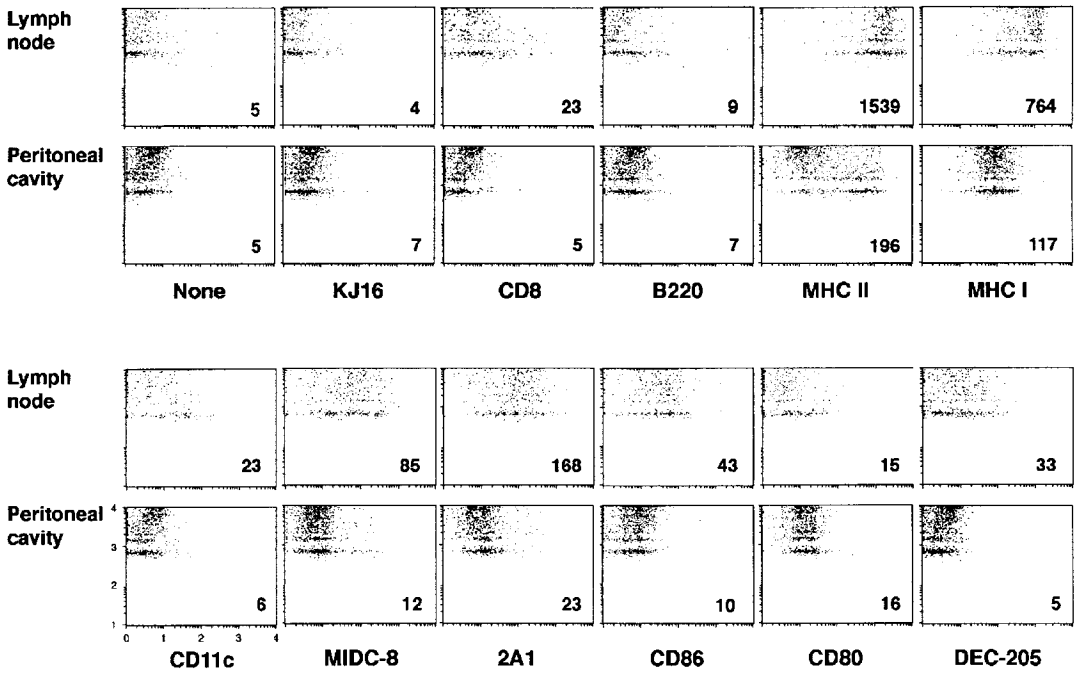

Figure 3. Cell Surface Phenotype of Microsphere$^+$ Cells in the Lymph Node

Single-cell suspensions of the lymph nodes (day 3) were prepared and stained with mAbs to a number of cell surface markers, control mAb KJ16, or no primary mAb ("None"). In other animals, latex was injected into the peritoneal cavities to compare the phenotype of lymph node microsphere$^+$ cells to peritoneal macrophages that engulfed beads during the 3 days following bead injection. Profiles have been gated on microsphere$^+$ cells. Log fluorescence of surface markers is indicated on the x axis; log fluorescence of ingested microspheres is indicated on the y axis. Note the banding pattern of the microspheres, in which the first band of lowest fluorescence intensity represents cells containing one bead per cell, the second band two beads per cell, and so on. The mean fluorescence intensity with respect to cell surface markers is shown in the lower right of each profile.

Microsphere$^+$ Cells in Lymph Node Exhibit a Cell Surface Phenotype Resembling Dendritic Cells

DCs and T cells are abundant in the lymph node paracortex, whereas macrophages accumulate predominantly in the subcapsular space and in the medulla. In agreement with the localization of bead$^+$ cells in the paracortex, flow cytometric evaluation of lymph node cells revealed a population of latex-bearing cells that displayed a cell surface phenotype that was consistent with DCs (Inaba et al., 1997) and was clearly distinct from the phenotype of mononuclear phagocytes recovered from the peritoneal cavity of additional mice injected intraperitoneally three days earlier with microspheres (Figure 3). Latex-containing lymph node cells expressed very high levels of MHC class I and II and the T cell costimulatory molecule CD86, but they were not B cells, as illustrated by a lack of B220. There was little to no expression of the T cell marker CD8α, which in mice identifies lymphoid DCs (Shortman and Caux, 1997). Microsphere$^+$ cells expressed a number of molecules restricted to DCs, including the MIDC-8 (Breel et al., 1987) and 2A1 (Inaba et al., 1992) antigens. A fraction of these cells also expressed DEC-205.

The flow cytometric analysis permitted an evaluation of phagocytic activity. Because the polystyrene particles were uniformly fluorescent, a banding pattern with respect to cell-associated fluorescence was observed. The band of least fluorescence intensity corresponded to cells that ingested a single microsphere, and the next band of cells with double the fluorescence intensity of the first band contained two microspheres, etc. Cells that had ingested a single microsphere showed expression of the DC marker CD11c, whereas cells with three or more particles had little or no CD11c. All together, these data suggest that particle-bearing cells in the lymph node are CD11c^{dim} DCs, not macrophages.

Microsphere$^+$ Cells Do Not Originate from Langerhans Cells

Migration of Langerhans cells is stimulated by epicutaneous application of contact sensitizers (Kripke et al., 1990). Soluble FITC serves as contact sensitizer, and FITC-labeled cells can be recovered in the draining lymph node (Kripke et al., 1990). FITC$^+$ lymph node cells appear to be derived entirely from the Langerhans cell population (Sato et al., 1998). When we utilized this model, we observed that all FITC$^+$ cells in the lymph node were CD11c$^+$ (Figure 4, upper left). To test whether microsphere-bearing DCs in the lymph node originated from Langerhans cells or other cells such as monocytes, we injected red fluorescent microspheres intracutaneously either 8 hr prior to or coincident with topical application of solubilized FITC. As analyzed by two-color flow cytometry, the microsphere-containing cells in the lymph node were distinct from the FITC$^+$ cells (Figure 4), indicating that the vast majority of microsphere$^+$ lymph

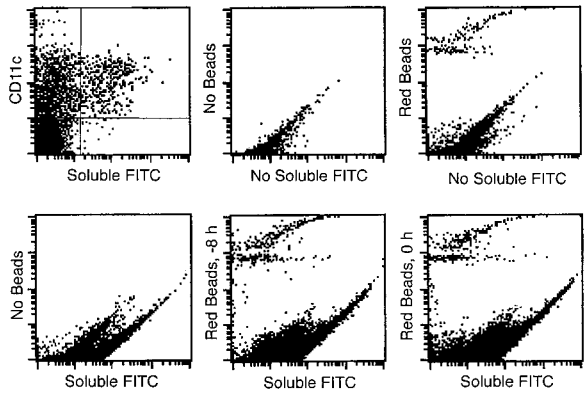

Figure 4. Bead$^+$ Lymph Node Cells Do Not Coincide with Langerhans Cells that Migrate to Lymph Nodes in Response to a Contact Sensitizer

Red fluorescent microspheres were injected intracutaneously 8 hr prior to (y axis "Red Beads", −8 hr) or coincident (y axis "Red Beads", 0 hr) with the topical application of soluble FITC, which acts as a contact sensitizer. In some mice, only red beads were injected or only FITC was applied without injection of beads. Total lymph node cells were analyzed by flow cytometry 2 days later. In a sample in which only FITC was applied, lymph node cells were double stained for CD11c expression (upper left panel). For two-color analysis with beads and soluble FITC, the extreme brightness of the red beads in the FL2 channel made establishing voltage and compensation parameters difficult. Nevertheless, the two colors could be distinguished. Data shown are derived from one of three independent experiments conducted that show similar results.

node DCs do not originate from Langerhans cells. Only a few single bead-containing cells may also have acquired soluble FITC (Figure 4).

Characterization of Bead$^+$ Cells that Emigrate from Skin Explants in the Presence and Absence of Inflammatory Monocytes

Using an ex vivo model of cellular migration via lymphatics, we tested further whether a subset of inflammatory monocytes, rather than resident skin DCs, served as precursors for particle-laden lymph node DCs. Previous research has shown that DCs and T lymphocytes migrate out of cultured mouse ear explants (Larsen et al., 1990; Ortner et al., 1996) by entering the lymphatic vessels (Weinlich et al., 1998). To investigate the phagocytic capacity and phenotype of cells that emigrated from inflamed skin, microspheres were injected into the intracutaneous tissue of mouse ears 18 hr prior to sacrifice and preparation of ear explants. Thus, ear tissue from the mice would contain inflammatory monocytes. Alternatively, phagocytic activity and migration of resident dermal and epidermal DCs in the absence of inflammatory monocytes were studied by injecting the microspheres into the skin just following sacrifice. A population of microsphere$^+$ cells was observed under both conditions. However, the microsphere$^+$ MHC II^{hi} cells that migrated from noninflamed skin had phagocytosed fewer particles per cell than emigrés collected from cultures containing inflammatory cells (Figure 5A). Interestingly, nonphagocytic DCs and those ingesting only 1–2 beads per cell expressed CD11c (Figure 5A) typical of dermal DCs and Langerhans cells. In contrast, the more phagocytic MHC II^{hi} cells that appeared only in explants containing inflammatory cells did not express CD11c or were CD11c^{dim} (Figure 5A, bracket). Likewise, only some of the bead$^+$ cells in the lymph node expressed CD11c, and those expressing the highest levels tended to be the least phagocytic (Figure 3). These bead$^+$ MHC II^{hi} cells were derived from cells that did not express MHC II when they first acquired the microparticles, because bead$^+$ cells within the skin did not express MHC II (Figure 5B) except for an occasional dermal DC that had ingested a single particle (arrowhead, Figure 5B). The number of microsphere$^+$ DCs was approximately four times higher from explants containing inflammatory cells, with the majority of the increased emigration occurring in the first 2 days of culture. These data suggest that monocytes recruited during inflammatory reactions give rise to a population of microsphere-bearing CD11c^{dim}MHC II^{hi} DCs.

To study the immunostimulatory potential of microsphere-bearing cells, we first attempted to isolate bead$^+$ cells from the lymph node, mainly by using paramagnetic microspheres and corresponding isolation protocols. However, we were unable to isolate them to sufficient purity and yield, probably because these cells represented only about 2% of lymph node DCs, or approximately 0.04% of total lymph node cells. Because bead$^+$ cells that migrated from the skin explants, apparently via lymphatics, are naturally enriched for DCs, we could successfully sort these cells by flow cytometry. Sorting for cells that expressed high levels of MHC II, microsphere$^+$ cells derived from inflamed ear explants were isolated and cultured with allogeneic T cells. We sorted microsphere$^+$ cells that had phagocytosed more than one particle per cell. Bead$^+$ emigrés from inflamed mouse ears induced robust proliferation (counts per minute at 1 bead$^+$ cell per 300 T cells) of allogeneic T cells to a somewhat higher but generally similar degree as the nonphagocytic population of DCs that also migrated out of the explants (Figure 5C). We cannot eliminate the possibility that a few phagocytic resident DCs (such as dermal DCs) contaminated our sorted preparation. The number of CD11c$^+$bead$^+$ cells was less than 5% of the total MHC II$^+$bead$^+$ cells in the inflammatory cell population, suggesting that such contamination was minimal.

Characterization of Microsphere Transport to Lymph Nodes in Monocyte-Deficient Mice

To extend our investigation into the possibility that the more phagocytic CD11c^{dim} MHC II^{hi} cells that emigrated from skin to the draining lymph nodes were DCs derived from monocytes, we studied DC-mediated lymphatic transport of microspheres in monocyte-deficient mice. Osteopetrotic mice (op/op) harbor a null mutation in the M-CSF (CSF-1) gene and therefore are severely deficient in monocytes and many populations of tissue macrophages (Wiktor-Jedrzejczak et al., 1982). Previously characterized, monocyte-independent populations of DCs are intact in these mice (Witmer-Pack et al., 1993), and we observed normal migration of DCs from noninflamed ear explants (Langerhans cells and dermal DCs) established from op/op and normal littermates (data not shown). Thus, these mice permitted the analysis of monocyte-derived DCs without apparent absence or dysfunction among other DC subsets.

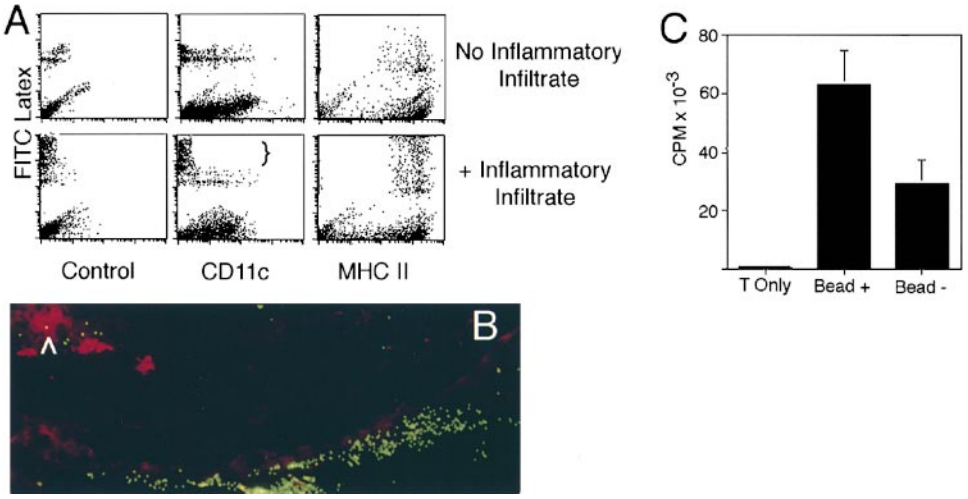

Figure 5. Phagocytic Load, Phenotype, and Allostimulatory Capacity of Cells that Emigrated from Dermal Lymphatics of Inflamed and Noninflamed Skin

(A) Microspheres were injected into the intracutaneous tissue of mouse ears 18 hr prior to ("+ Inflammatory Infiltrate") or at the time of sacrifice ("No Inflammatory Infiltrate"). The former condition permits the influx of inflammatory cells, but the latter does not and therefore would allow microsphere uptake only by resident Langerhans cells or interstitial DCs and macrophages. Explants of the dorsal leaflet of the ears were cultured for five days, with transfer to fresh medium daily. Data shown are cells that emigrated between the first and second day of culture, when DCs derived from inflammatory cells were particularly abundant. Flow cytometric evaluations show isotype-matched control mAb, anti-CD11c mAb, and anti-MHC II mAb. Data are representative of five independent experiments.

(B) Skin sections were examined for expression of MHC II. Most inflammatory bead$^+$ cells (green) did not express MHC II (red), and resident MHC II$^+$ cells contained only a single or no microspheres (arrowhead).

(C) Microspheres were injected into the intracutaneous tissue of mouse ears 18 hr prior to sacrifice and establishment of ear explant cultures. Emigrated cells from the second day of these cultures were stained with PE-conjugated anti-MHC II mAb and sorted by flow cytometry for MHC II^{hi} expressing cells lacking microspheres ("No Beads") and those containing more than a single microsphere ("+ Beads"). For analysis of allogeneic T cell proliferation, the sorted cells were mixed with allogeneic CD4$^+$/CD8$^+$ splenic and lymph node T cells at a ratio of 5×10^2 sorted cells/1.5×10^5 T cells. DNA synthesis was measured at 66–72 hr.

A very limited to absent inflammatory reaction was observed in op/op mice at the site of microsphere injection, as observed by histologic evaluation (Figures 6A and 6B) and the relative paucity of F4/80$^+$ cells that were present (Figure 6C, compare to Figure 1C). These scattered F4/80$^+$ resident dermal macrophages, which represent one of the few macrophage populations found in op/op mice (24), phagocytosed numerous microspheres (Figure 6C, yellow). Dermal DCs were present in these mice, but just as observed in wild-type and op/+ littermates, DEC-205$^+$ DCs (red) did not colocalize with FITC microspheres (Figure 6D).

Particle transport to lymph nodes by DCs in op/op mice and normal littermates (op/+ or +/+) was compared. Normal (op/+) mice possessed a population of lymph node MHC II^{hi}2A1$^+$ paracortical DCs that contained three or more microspheres per cell (Figure 6E, arrows), but op/op mice essentially lacked this population (Figures 6F and 6G). However, like their normal counterparts, op/op lymph nodes contained a population of DCs associated with a single microsphere per cell. As in previous experiments (Figures 3 and 5), only these modestly phagocytic cells (1 bead/cell) were CD11c$^+$ (data not shown).

Discussion

Questions regarding the ontogenic relationship of DCs with monocytes and macrophages have been longstanding. When tissues are broadly surveyed by immunohistochemistry for DC number and organization, only slight differences are observed between normal mice and monocyte-deficient op/op mice (Witmer-Pack et al., 1993), leading to the conclusion that most DCs do not derive from monocytes. However, using these mutant mice, we have conducted assays that highlight the existence of a subpopulation of monocyte-derived DCs. These cells express high levels of MHC II, CD86, and DC-restricted markers 2A1 and MIDC-8 and localize to the T cell area of lymph nodes. In contrast to other murine DCs, there is little to no expression of CD11c, and only some express DEC-205. These monocyte-derived DCs exhibit the highest phagocytic capacity among peripheral DC subsets. The phagocytic activity of these cells is reminiscent of the phagocytic rat lymph-borne DCs previously reported (Matsuno et al., 1996), suggesting that such DCs may also be derived from monocytes.

In addition, a population of DCs that had ingested only 1–2 microspheres per cell migrated out of skin explants and was observed in lymph nodes. The appearance of these cells in draining lymph nodes was not affected by the presence or absence of monocytes. Very few of these cells appear to be derived from Langerhans cells, but occasional single bead$^+$ cells were observed in the dermis. Thus, it is likely that these modestly phagocytic cells are dermal DCs.

Several past studies have traced the trafficking of particles from the periphery to draining lymph nodes, and it has been shown that such trafficking is mediated by mononuclear phagocytes (Harmsen et al., 1985). Indeed, data obtained in these studies (Smith et al., 1970;

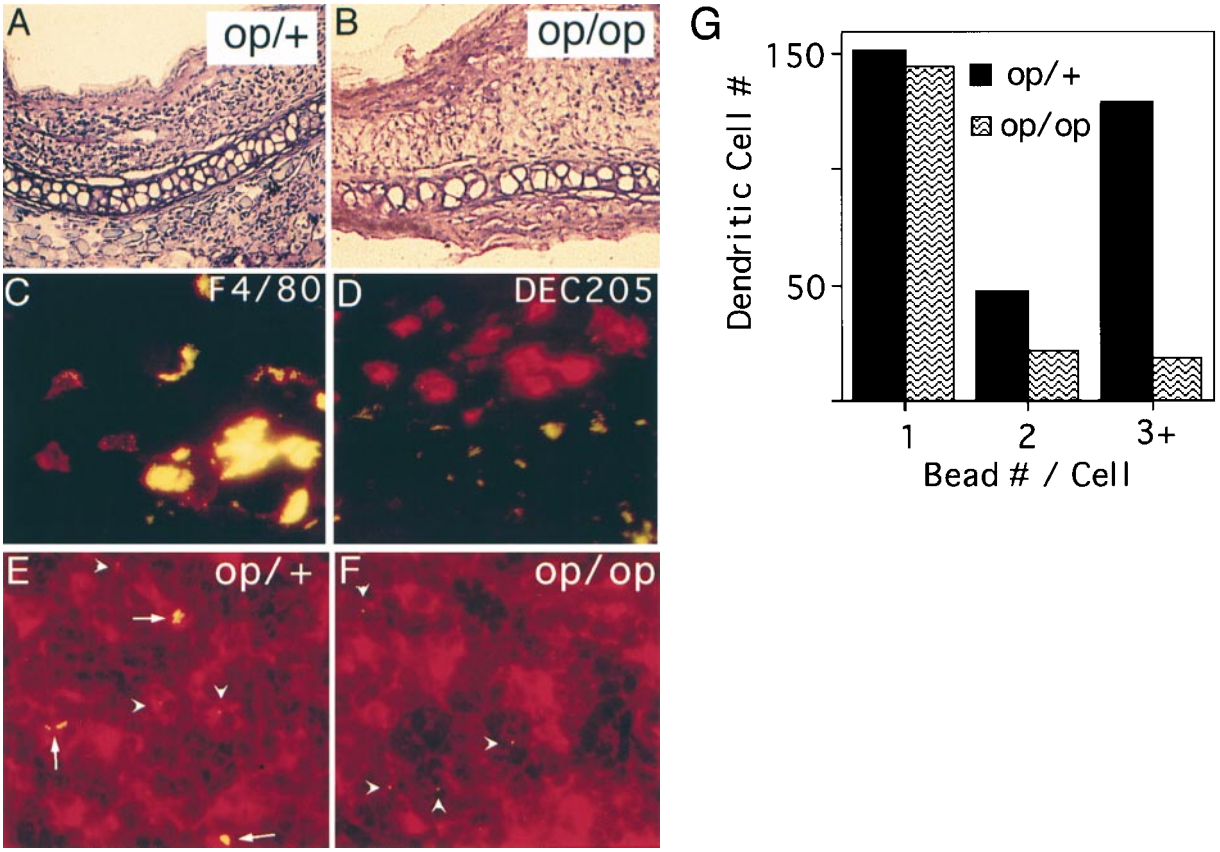

Figure 6. Phagocytic Load and Phenotype of Lymph Node DCs in Monocyte-Deficient Mice or Normal Littermates

FITC-latex microspheres were injected into the ear tissue and/or footpads of 5-week-old op/+ mice or op/op mutants. The former exhibited a vigorous inflammatory response to the injection (A), but the latter did not (B–D). After 3 days, the local cellular response was evaluated at the site of injection by examining frozen sections histologically and immunostaining for macrophages using F4/80 (C) and DCs using anti-DEC-205 mAbs (D), respectively. The micrograph of F4/80 staining (red, [C]) shows a few large resident macrophages in op/op mice that stain yellow (green latex + red F4/80; compare with op/+ mice in Figure 1C), whereas the area shown for DEC-205$^+$ cells (red) represents an area with a somewhat higher abundance of DCs than is typical for either mutant or control littermates. Sections of draining lymph nodes collected at day 3 from op/+ (E) and op/op (F) mice were stained for the DC marker 2A1. 2A1$^+$ cells containing only one microsphere are highlighted by arrowheads. 2A1$^+$ cells that had ingested numerous microspheres are evident in sections of op/+ lymph nodes (arrows) but not op/op lymph nodes. (G) Single-cell suspensions from op/+ and op/op lymph nodes were prepared, and MHC II^{hi} labeled cells were evaluated by flow cytometry to determine the number of microsphere$^+$ DCs per lymph node and the extent of their phagocytic load. Data show the averaged results from two independent experiments that were conducted using two 5-week-old mice per experiment. The graph illustrates the mean number of microsphere$^+$ DCs recovered from the pooled brachial lymph nodes of individual mice, and the particle content of these cells is plotted as one bead/cell ("1"), two beads/cell ("2"), and three or more beads/cell ("3+").

Yamashita et al., 1978; Corry et al., 1984; Harmsen et al., 1985; Hardonk et al., 1986; Bellingan et al., 1996) are consistent with our findings. However, previous work assumed that particle-bearing cells in the lymph node were macrophages without a comprehensive analysis of cell surface phenotype, localization within the lymph node, or function. Thus, we were surprised to discover that few particle-bearing cells in the lymph node were macrophages, but that instead these cells were DCs. Importantly, our experiments were designed to exclude conditions in which particles may enter lymph without involving active cellular transport. Some previous studies examined cellular transport from the peritoneal cavity (Rosen and Gordon, 1990; Bellingan et al., 1996). When we performed experiments by injecting microspheres into the peritoneal cavity, we observed that in contrast to skin injections, both DCs and subcortical macrophages became heavily laden with microspheres (data not shown). Thus, the pattern of particle drainage from the peritoneal cavity appears to be different from other body tissues. Possibly, the lymphatics of the peritoneal cavity are relatively leaky, allowing a higher level of passive translocation of microspheres not seen in other anatomic sites. Passive particle translocation may also have occurred in previous studies that used very small carbon particles instead of the larger particles used herein, thereby explaining the strong macrophage labeling in those studies (Cohen et al., 1966; Fossum, 1980).

Particulate antigens are thought to be among the most potent inducers of immunity (Raychaudhuri and Rock, 1998). When soluble antigen is administered with Freund's adjuvant to op/op mice, the resulting immune response appears similar to that mounted in their normal counterparts (Wiktor-Jedrzejczak et al., 1992; Chang et al., 1995). However, immunity to the particulate micro-

bial antigen, Mycobacterium tuberculosis, is dramatically diminished in op/op mice, such that the mice succumb rapidly (Teitelbaum et al., 1999). Importantly, colony forming units corresponding to the spread of tubercle bacilli fail to accumulate in draining lymph nodes (Teitelbaum et al., 1999). Combining such observations with our studies, it seems that the acquisition and presentation of *particulate* antigens may define a major role for DCs derived from monocytes. Thus, immune responses to antigens acquired by phagocytosis would likely be enhanced by the recruitment of monocytes and rapid clearance from peripheral tissues as they differentiate into DCs.

Approaches to promote regression of atherosclerotic lesions, which are replete with lipid-laden macrophages, are still a major clinical challenge. The present findings suggest that monocyte-derived macrophages remain predominantly in the periphery and that monocytes that become DCs readily migrate to the lymph node. This observation is in agreement with our in vitro transendothelial trafficking model, in which human monocyte-derived DCs reverse transmigrate but macrophages remain in the subendothelial tissue (Randolph et al., 1998). Our data underscore the possibility that an increase in DC development from monocytes could lead to the clearance of monocyte-derived cells from tissues in chronic disorders in which macrophage accumulation is excessive and pathologic, as in atherosclerosis. Additional studies are needed to investigate the mechanism(s) that regulate the commitment and differentiation of monocytes to macrophages versus DCs in situ.

Experimental Procedures

Mice

A variety of strains were employed in these experiments, with similar results. Most experiments used female mice at 8 weeks of age. Strains CD1 and CD2/F1 were purchased from Charles River Labs, and strains FVB/N and C57BL/6 were purchased from Jackson Labs or were bred in our own facilities. Heterozygous breeders (B6C3FEA/F1 background) for the osteopetrotic mutation (op) were purchased from Jackson Labs. Op/op mutants were recognized by their smaller size and lack of teeth and were maintained on a diet of powdered chow supplemented with daily feedings of Enfamil (Chang et al., 1995). Mutants and normal littermates were used in experiments at 5 weeks of age, before the mutant phenotype begins to be corrected by the M-CSF-like activity of vascular endothelial cell growth factor (Niida et al., 1999). At this time, the average weight of the mutants was 9 g, compared with about 20 g for the op/+ and +/+ controls.

Injection of Microspheres

All protocols were approved by institutional animal review committees at The Rockefeller University and Weill Medical College of Cornell University. For injection, microspheres of 0.5 or 1.0 μm in diameter were resuspended to 0.0025% or 0.025% (w/v) in PBS. Preparations of these microspheres lacked detectable levels of LPS, using the Limulus detection system from Cape Cod Associates. Mice were anesthetized by inhalation of Metofane (Schering-Plough) and injections were administered into the intracutaneous tissue of the front or rear footpads (10 and 25 μl, respectively). Alternatively, intracutaneous injections were made at shaved areas of the dorsal thoracic skin or into the intracutaneous tissue of the ear (10 μl/injection). All injections were made with Hamilton syringes (Fisher). Similar results were obtained using FITC-dyed polystyrene (FITC-latex) or carboxy-modified microspheres that fluoresce in the red range (488 nm/605 nm; Molecular Probes). In other experiments, microspheres were injected into the peritoneal cavity. At times indicated (typically 3 days) following microsphere injection, mice were sacrificed by CO_2 inhalation and lymph nodes were collected. The front footpad and dorsal thoracic skin drain to the brachial lymph nodes, the rear footpad to the popliteal nodes, and the peritoneal cavity to the parathymic lymph nodes (Tilney, 1971).

Acetone Extractions

Skin surrounding the site of injection was excised and frozen at −20°C until all time points were collected. Thawed skin samples were immersed in 500 μl of acetone to dissolve the polystyrene microspheres and allowed to soak for at least 1 hr. Then the tissue was crushed in a Dounce homogenizer. The supernatant was mixed with H_20 at a 1:1 ratio, and the fluorescence in a 100 μl aliquot was read using a Cytofluor 2350 fluorescence measurement system from PerSeptive Biosystems.

Topical Application of Soluble FITC

FITC was dissolved in acetone/dibutylphthalate and applied to the shaved dorsal thoracic skin of mice according to the "low dose" procedure previously described (Gunn et al., 1999).

Ear Explant Cultures

Microspheres were injected at three locations per ear (10 μl each) either 18 hr before or just after sacrifice of the mice. Ears were then resected and cultured as previously described, with transfer of the explants to fresh medium daily (Ortner et al., 1996).

Immunostaining

Skin or lymph nodes were snap-frozen in OCT compound (Miles), and 10 μm sections were prepared and affixed to polylysine-coated slides. Sections were fixed in freshly prepared 3% neutral-buffered paraformaldehyde for 5 min, rinsed in PBS, and blocked with 0.2% chicken ovalbumin plus 5% normal serum for 10 min. Sequential mAb incubations were for 45 min each, with mAb incubations separated by three washes in PBS. Primary antibodies used included anti-DEC-205 mAb NLDC 145 (Cedarlane), biotinylated anti-F4/80 mAb (Serotec), F4/80 supernatant (Biosource International), 2A1 supernatant, anti-CD11b mAb 5C6, anti-CD11c supernatant N418, anti-B220 mAb (Cedarlane), anti-CD4 mAb (PharMingen), biotinylated anti-MHC II mAb, and purified rat IgG2a (PharMingen). Cy3-conjugated donkey anti-rat mAb (Jackson Immunoresearch) or Cy3-conjugated strepavidin (Sigma) was used for detection.

Flow Cytometry

Flow cytometric staining and analysis was conducted as previously described (Inaba et al., 1992). Anti-MHC II mAb M5/114.15.2 and KJ16 were from the American Type Culture Collection; mAbs to CD8, CD11b, CD11c, CD86, and B220 were purchased from PharMingen, and mAb 2A1 was generated in this laboratory. Anti-DEC-205 (NLDC 145) and MIDC-8 mAbs were kindly provided by Dr. George Kraal (Amsterdam). To detect the intracellular epitopes recognized by 2A1 and MIDC-8, cells were fixed and permeabilized as described (Inaba et al., 1992).

Mixed Leukocyte Reaction

Following injection of microspheres 18 hr prior to sacrifice and establishment of ear explant cultures, DCs that emigrated between 24 and 48 hr of culture from 40 ear explants were pooled, stained with anti-MHC II mAb, and sorted. Gates were set to collect MHC II^+ cells with 0–1 beads/cell or ≥2 beads/cell. The cell fractions recovered were incubated with 3×10^5 allogeneic T cells/well. T cells were purified from pooled splenic and lymph node cells that had been depleted of MHC II–expressing cells by treatment with rat anti-MHC II mAb followed by anti-rat magnetic beads (Dynal). Candidate antigen-presenting cells were mixed with T cells at indicated ratios, cultured for 3–4 days, and finally cultured in [^{3}H]thymidine (4 μCi/ml) for 13 hr.

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