Lipopolysaccharide or Whole Bacteria Block the Conversion of Inflammatory Monocytes into Dendritic Cells In Vivo

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Abstract

Monocytes can develop into dendritic cells (DCs) that migrate to lymph nodes (LNs) and present antigens to T cells. However, we find that this differentiation is blocked when monocytes accumulate subcutaneously in response to bacteria or lipopolysaccharide (LPS). The inhibition of DC differentiation is mediated by the bacteria and in conjunction with inflammatory cells recruited at the site of injection. Inhibition of migratory DC development was reversed in Toll-like receptor (TLR)4-mutated mice when LPS, but not whole bacteria, was injected, suggesting that TLR4 is one but not the only mediator of the inhibition. The block imposed by bacteria was partly relieved by the absence of interleukin (IL)-12 p40, but not by individual absence of several cytokines involved in DC differentiation or in inflammation, i.e., IL-6, IL-10, IL-12 p35, and interferon γ. Consistent with the inability of monocytes to yield migrating DCs, and the finding that other DCs had limited access to particulate or bacterial antigens, these antigens were weakly presented to T cells in the draining LN. These results illustrate that bacteria-associated signals can have a negative regulatory role on adaptive immunity and that local innate responses for containment of infectious bacteria can at least initially supersede development of adaptive responses.

Key words: *Salmonella typhimurium* • migration • inflammation • innate immunity • adaptive immunity

Introduction

DCs are professional APCs that are located in lymphoid and nonlymphoid tissues where they perform a sentinel function for incoming pathogens (1, 2). Although a steady-state migration of DCs to secondary lymphoid organs occurs, systemic injection of LPS or inflammatory cytokines has been shown to enhance the mobilization of tissue resident DCs from the periphery to secondary lymphoid organs (3, 4). Migrating DCs can also originate primarily from blood monocytes (5, 6). An estimated 25% of the circulating inflammatory monocytes will differentiate into migrating DCs, whereas the others will give rise to resident macrophages (5, 7). What drives the differentiation from monocytes into DCs or macrophages in vivo is still unknown, but it has been shown that cytokines or environmental factors can play a role in skewing either pathway of differentiation in vitro (8–15). It is conceivable that the differentiation of monocytes into DCs or macrophages in body tissues is shaped by the local environment present during their transit and that there can be situations that favor the development of one or the other cell type. As DCs have been shown to be required for cytotoxic T cell responses to intracellular bacteria (16) and priming of T cells takes place in the draining LN (DLN; 17), the capacity to generate migrating DCs after infection could be crucial to induce adaptive immunity

Abbreviations used in this paper: CFSE, carboxyl-fluorescein-succinimidyl ester; DLN, draining LN; GFP, green fluorescent protein; i.d., intradermal(ly); LB, lurian broth; LC, Langerhans cell; TLR, Toll-like receptor.

against the invading microbes. Thus, we have analyzed all the steps during the induction of an immune response to *Salmonella typhimurium* injected subcutaneously from DC differentiation and migration, to the activation of antigen-specific T lymphocytes.

We show that intradermal (i.d.) injection of dead or alive *S. typhimurium*, or simply LPS, induces a potent local innate inflammatory response that blocks monocyte-derived DC differentiation and migration to the DLN to present antigen to T cells. This is in agreement with our previous results showing that subcutaneous injection of *S. typhimurium*, recombinant for the melanoma antigen tyrosinase–related protein 2, does not protect from lethal challenge with B16 melanoma, whereas DCs loaded in vitro with the same bacteria can potently limit tumor growth (18). Although numerous studies show that inflammatory mediators can promote adaptive immune responses by promoting maturation of DCs, we show here that the result can be dramatically different when the precursors of the antigen-presenting DCs, in this case monocytes, encounter the inflammatory/pathogenic signal before committing to the DC pathway of differentiation in vivo. Our results reveal a capacity of the innate inflammatory reaction to inhibit adaptive immune responses to particulate antigens.

Materials and Methods

Cells and Reagents. Conditioning medium for DCs was IMDM (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (Life Technologies), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM ʟ-glutamine (all from Sigma-Aldrich), and 50 μM 2-mercaptoethanol, supplemented with 30% supernatant from GM-CSF–producing NIH/3T3 cells.

Inflammatory monocytes were collected from the peritoneal cavity of mice injected i.p. with 1 ml of a PBS solution containing 4% thioglycollate medium (Sigma-Aldrich) and then 18 h later with 0.5 ml FITC-conjugated 1-μm latex particles (0.02% wt/vol). Mice were killed 4 h later for peritoneal lavage. In some experiments this cell preparation was subjected to cell sorting, wherein latex^{+} cells (all F4/80 low) were gated and purified. These cells were then cultured in vitro in DC conditioning medium in the presence or absence of bacteria and in the presence or absence of the latex[−] fraction (>80% neutrophils) from the same sort of peritoneal cells.

Mice. 5–6-wk-old female C57/BL6J (Ly5.2), C57/BL6J (Ly5.1), C3H/HeJ, C3H/HeN, or BALB/c mice were purchased from Charles River Laboratories. DO11.10 OVA-TCR transgenic T cells were provided by D. Lo (Scripps Research Institute, La Jolla, CA). IFN-γ (BALB/c-Ifg[tm1 129]), IL-12p35 (C.129S1(B6)-Il12atm1Jm), and IL-12p40 (C.129S1-Il12btm1Jm) KO mice on a BALB/c background were purchased from The Jackson Laboratory. p35/IFN-γ double KO was obtained by interbreeding mice selected for carrying the double mutation from the F2 generation. BALB/c IL6 KO mice were provided by P. Sacerdote (University of Milan, Milan, Italy). MyD88–deficient mice were on a C57BL/6 background and were continuously fed antibiotics. The antibiotics had no effect on the migration of monocyte-derived DCs as assessed in control mice also fed antibiotics.

Antibodies, Cell Surface Phenotype, and Assessment of Cell Death. The following monoclonal antibodies were purchased from BD Biosciences: CD4 (L3T4), CD8a (Ly2), I/A-b, I/A-d, CD11b (Mac-1), CD11c (N418), CD45R (B220), Gr1 (Ly-6G), and CD45.2 (104). An IgG with specificity to neutralize mIL-10 was purchased from R&D Systems. F4/80 was purified from supernatant of the relevant hybridoma (HB198).

Staining was performed with 50 μg/ml propidium iodide to evaluate dead cells and flow cytometry analysis was performed with a FACScan™ (CELLQuest™ software; Becton Dickinson).

Bacterial Strains and Preparation for Injections. *S. typhimurium* SL7207 (*S. typhimurium* 233765 derivative hisG46, DEL407 [aroA::Tn10{Tc-s}]) was provided by B.A.D. Stocker (Stanford University School of Medicine, Stanford, CA). Two recombinant strains of SL7207 were generated expressing either the gene coding for GST-OVA fusion (SL7207-OVA), or with the GST alone (SL7207 pGEX; reference 18). 10^7 CFUs of SL7207-OVA express nearly 1 μg protein. *Bacillus subtilis* was provided by A. Galizzi (University of Pavia, Pavia, Italy). Single colonies were grown overnight at 37°C in lurian broth (LB; Difco) and restarted the next day at 1:10 of the original volume up to the following: for Salmonella an $0D_{600} = 0.35$, which corresponds to 7×10^8 CFU/ml and for *B. subtilis* to an $0D_{600} = 1.0$, which corresponds to 2×10^8 CFU/ml.

Leishmania mexicana (strain MNYC/BZ/62/M379) promastigotes, recently isolated from mice, were grown to stationary phase in culture. Parasites were washed thoroughly with PBS and injected at 3×10^6 parasites per site of injection.

Mice Treatments, Skin Inflammation Induction, and Latex Migration. Mice were anesthetized i.p. with Avertin 2.5% and shaved in four sites of the dorsal skin, which were drained either by the inguinal or brachial LNs. 10^7, 5×10^4, or 10^2 CFUs of bacteria were diluted to a final volume of 10 μl PBS and injected i.d. using a Hamilton syringe (Fisher Scientific). 10^7 FITC-conjugated latex particles of 1 μm in diameter (Polysciences) were injected as tracers in the presence or absence of bacteria. Migration of I-A[b+] or CD11c^{+} cells carrying latex to DLNs was assessed 1, 2, or 3 d after treatment. At indicated times, mice were killed and DLNs were collected, teased, and cells were released by treatment with 0.25% collagenase at 37°C for 25 min (Collagenase D; Roche). Cells were stained for I-A[b] or CD11c expression. For quantification, the entire population of DLN cells was acquired by cytofluorometry and absolute numbers of CD11c^{+} or I-Ab^{+} cells carrying latex beads were determined per LN. The region of the skin corresponding to the site of injection, as identified by faint visual appearance of green latex beads, was separated and cells were released after collagenase treatment and stained for FACS® analysis as described above.

Adoptive Transfer of Peritoneal Inflammatory Monocytes or of Cells from Infected Skins. Latex[+] inflammatory monocytes from C57BL/6 Ly5.2 mice were collected from peritoneal lavage as described above. $1–1.25 \times 10^6$ cells were injected i.d. in C57BL/6J Ly5.1 mice in the presence or absence of 10^7 CFUs of SL7207. 3 d later, mice were killed, DLNs were collected, and CD45.2[+]/latex^{+} cells were released and analyzed by FACS® analysis.

For adoptive transfer of cells from infected skins, mice were injected i.d. with 10^7 CFUs of SL7207. 2 d later, cells were collected and remaining extracellular bacteria were killed by incubation with 100 U/ml streptomycin, 10 μg/ml tetracycline, and 50 μg/ml gentamicin for 2 h at 37°C. 10^6 cells were injected i.d. in C57BL/6J Ly5.1 mice. 3 d later, mice were killed, DLNs were collected, and CD45.2[+]/latex^{+} cells were analyzed as described above.

Topical Application of Soluble FITC (FITC Paint). FITC (Sigma-Aldrich) was dissolved in acetone/dibutylphthalate at a concentration of 8 mg/ml and 25 μl were applied to each region of the shaved dorsal skin corresponding to the areas of bacterial injection as previously described (5).

Covalent Coupling of Latex Particles with OVA and Immunization Protocol. 1 μm carboxylated FITC-labeled latex beads were purchased from Polyscience. Covalent coupling with OVA was performed according to the manufacturer's instructions (Polyscience), except for 50 mM 2-(*N*-Morpholino)-ethanesulfonic acid, pH 6.5, which was used as coupling buffer. Spectrophotometric evaluation of sample showed that 10^7 microspheres were coated with 0.25 μg OVA. Mice were immunized with 10^7 microspheres or with an equivalent amount of soluble OVA (0.25 μg) in the presence or absence of increasing concentrations of bacteria (10^2–10^7). Four 10-μl injections were performed in the dorsal skin of mice.

TCR Transgenic T Cells Adoptive Transfer. DO11.10 TCR-OVA T cells, specific for the OVA$_{327–339}$ peptide in association with I-A^d, were collected from spleen, brachial, and inguinal LNs. CD4$^+$ T cells were purified by positive selection using α-CD4 (L3T4) magnetic microbeads (Mini-MACS; Miltenyi Biotec) and cells were labeled with 5 μM carboxyl-fluorescein-succinimidyl ester (CFSE) for 15 min at 37°C (Molecular Probes).

BALB/c recipient mice were injected i.v. with 3×10^6 DO11.10 CFSE-labeled T cells and then immunized with four different treatments. We injected 0.25 μg soluble or latex-bound OVA with increasing concentrations of bacteria (10^2–10^7 CFUs). Alternatively, mice were injected either with 3×10^5 BALB/c BM DCs loaded in vitro with 50 μg soluble OVA in the presence or absence of 10^7 CFUs of bacteria, or with 10^2, 5×10^4, or 10^7 CFUs of SLpOVA or SLpGEX (as a control). Mice injected with 10 μl of either 0.25 μg OVA, CFA, or 0.25 μg OVA emulsified in CFA were always included in each experiment as a control. 3 d later, DLNs were collected and DO11.10 T cell proliferation was assessed as reduction in CFSE labeling.

Intracellular Survival of Bacteria. 4×10^6 cells from spleen and DLNs of injected mice were lysed with 0.5% sodium deoxycholate. CFUs were counted after serial dilution of cellular lysates on LB agar to quantify the number of migrated live intracellular bacteria.

Online Supplemental Material. CD14$^+$ cells were positively selected from buffy coats using magnetic column separation (Miltenyi Biotech). Cells were cultured for 7 d at a concentration of 10^6/ml in RPMI (Cambrex) supplemented with 10% FBS (GIBCO BRL), 2 mM L-glutamine (Euroclone), 50 ng/ml rhGM-CSF, 20 ng/ml rhIL4 (PeproTech), and 100 mg/ml gentamicin (Roche). At days 2, 4, and 6, to generate "inflammatory monocytes," cells were preactivated with 20 ng/ml rhTNFα (Sigma-Aldrich) 4 h before bacterial encounter. SL7207 was then added at an MOI of 20:1 for 1 h in the absence of antibiotics. After incubation with bacteria, the medium was changed and antibiotics were added again. At d 7, cells were collected and analyzed by flow cytometry with the indicated antibodies. Fig. S1 is available at http://www.jem.org/cgi/content/full/jem.20030335/DC1.

Results

Gram-negative Bacteria Block DC Migration to the DLN. To evaluate the ability of DCs to take up, migrate, and transport skin-injected bacteria to DLNs, we used a metabolically defective strain of *S. typhimurium* (aroA strain SL7207) expressing the green fluorescent protein (GFP).
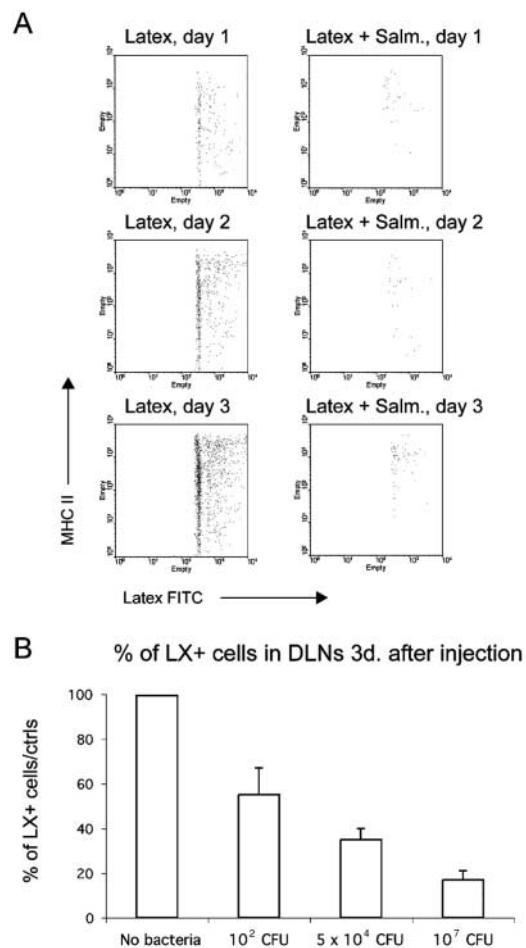

Figure 1. Bacteria block latex$^+$/I-A^{b+} cells migration from the injection site. (A) Bacteria induce a complete and immediate block of latex$^+$ DC migration. 10^7 fluorescent latex microspheres/site were injected i.d. in mice in four different sites in the presence or absence of 10^7 CFUs of Salmonella. Mice were killed at days 1, 2, and 3 after treatment and the total number of I-A^{b+} cells carrying FITC$^+$ latex particles per DLN was assessed after acquisition of the whole sample. Log fluorescence of latex microspheres is indicated on the x axis where at least two alignments of latex are clearly detectable, indicating cells containing one and two particles, respectively. Log fluorescence of I-A^{b+} is indicated on the y axis. These data are representative of several experiments, each performed in triplicate. (B) The block of DC migration is dependent on bacteria concentration. Again the total cellular content of the DLN for each condition was acquired and the efficiency of total latex$^+$ cell migration 3 d after treatment as compared with that in control mice (100%) is reported.

We performed i.d. injections in the dorsal skin of C57BL/6 mice using different concentrations of bacteria ranging from 10^2 to 10^7 CFUs. 1, 2, or 3 d later we collected the draining brachial and inguinal LNs and analyzed the total cellular content for GFP$^+$ cells. To our surprise, we could not detect any GFP$^+$ cells present in the DLNs (unpublished data). As this was possibly due to the capacity of DCs to efficiently kill salmonella and metabolize the GFP, we coinjected the bacteria with a nondegradable fluorescent tracer (FITC latex beads) to follow DC migration, using a previously described method (5). In Fig. 1 A (left), the total DLN cellular content of I-A^{b+}/bead$^+$ cells, which

paralleled the migration of CD11c$^+$/bead$^+$ cells (unpublished data), is shown. Cells carrying one, two, or more beads could be easily observed (especially on or after the second day) in the absence of bacteria. By contrast, coinjection of bacteria (10^7 CFUs) with 10^7 FITC latex completely blocked migration of I-A^{b+}/bead$^+$ cells from the infected site to the DLN at days 1, 2, or 3 after injection (Fig. 1 A, right). The block of bead$^+$ cell migration was dependent on the dose of bacteria injected, being maximal at 10^7 CFUs (Fig. 1 B), but substantial inhibition was observed at much lower doses (65% inhibition using 5 $\times$ 10^4 CFUs). We also tested the capacity of *Escherichia coli* (DH5α) to block the migration of bead$^+$ DCs and we observed similar results (unpublished data). Therefore, Gram-negative bac-

teria inhibit the migration of bead-bearing DCs from peripheral tissues to DLNs in a dose-dependent manner.

Inhibition of Latex-bearing DC Mobilization Is Due to Local Factors. Next, we investigated whether bacteria were inducing a systemic or a local block of latex-bearing DC migration. 10^7 FITC latex beads were injected i.d. either alone, as a positive control, or together with bacteria (10^7 CFUs) as a negative control, or contralaterally from bacterial injection to assess whether bacteria were controlling DC migration from a distant site. Injection of bacteria contralaterally from FITC latex beads did not affect DC migration, whereas coinjection of latex plus bacteria resulted again in block of DC migration (unpublished data). Therefore, local but not distal factors released by bacteria or by neighboring tissue cells mediate the block of DC migration. Similarly, also Gram-positive bacteria, such as Bacillus subtilis (10^7 CFUs), or parasites, such as *L. mexicana* (3 $\times$ 10^6 stationary phase promastigotes), impaired DC migration, although the block was not as complete as with Gram-negative bacteria (Fig. 2 A).

Inactivated Bacteria or Simply LPS Are Sufficient to Inhibit DC Migration. It has been shown that *Schistosoma mansoni* can release soluble lipophilic factors able to block Langerhans cells (LCs) migration induced by TNF-α via binding to the adenylate cyclase-coupled PGD$_2$ receptor (19). Similarly, live bacteria could release metabolites or factors in situ which could interfere with DC function and migration. Thus, we analyzed whether inactivated bacteria could still influence DC mobilization. *S. typhimurium* was inactivated as described in Materials and Methods. In all cases, a block in DC migration, similar to that induced by live bacteria, was observed (Fig. 2 B). Finally, we asked if a component of Gram-negative bacteria cell wall, such as LPS, was also able to affect DC migration. As shown in Fig. 2 C, increasing the concentration of i.d. injected LPS ($\geq$1 μg) inhibited DC migration. Thus, neither viable nor intact Gram-negative bacteria are required to block DC mobilization, excluding the possibility that abrogation of DC migration is due to metabolic factors released by the microorganisms. Moreover, the fact that LPS was able to reduce DC migration favors the possibility that engagement of Toll-like receptor (TLR)4 regulates DC differentiation and/or migration.
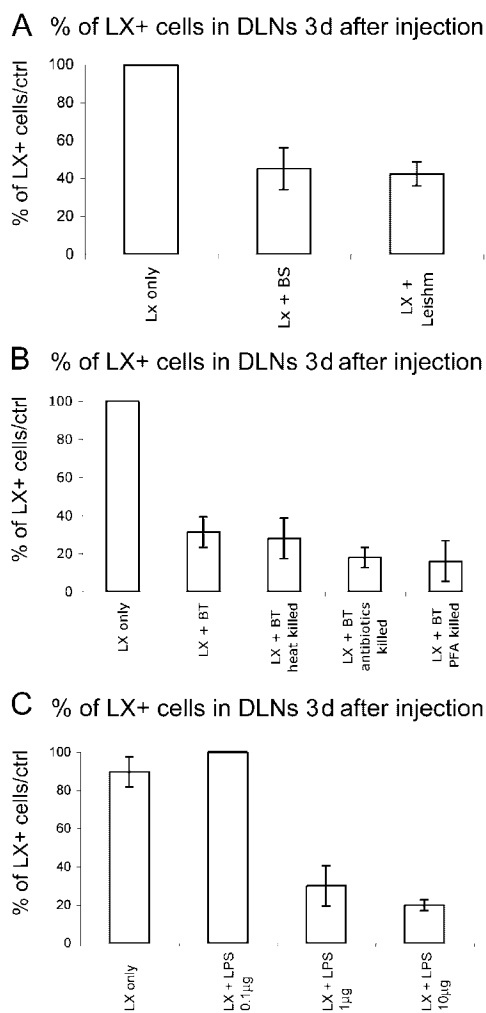
Figure 2. Inhibition of DC mobilization can be induced also by Gram-positive bacteria, parasites, inactivated bacteria, and bacterial LPS. (A) Not only Gram-negative, but also Gram-positive bacteria like *Bacillus subtilis* (BS) or parasites like *L. mexicana* are able to reduce latex$^+$ cell migration. Efficiency of latex$^+$ cell migration as compared with migration of latex$^+$ cells in control mice (100%) 3 d after injection, is reported. (B) Viability of bacteria is not necessary to impede DC migration. Bacteria were killed in different ways to ensure that the method of inactivation was not affecting latex$^+$ cell migration. (C) LPS from *E. coli* blocks latex$^+$ cell migration to DLNs at concentrations $\geq$1 μg.
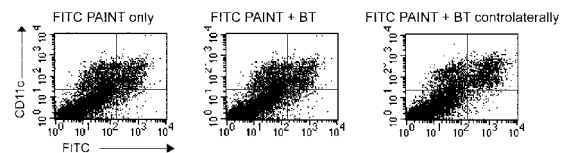
Figure 3. Bacteria do not substantially affect LCs migratory properties. (A) LCs are able to migrate to DLNs even in the presence of subcutaneously injected bacteria. LCs residing in the epidermis were sensitized with soluble FITC directly painted on the skin of shaved mice. Cytofluorometry analysis of CD11c$^+$ FITC$^+$ cells migrated from skins of mice injected in the same site (FITC paint + BT), contralaterally (FITC PAINT + BT contralaterally), or noninjected (FITC paint only) with bacteria is reported. All these data are representative of two or more experiments each performed in triplicate.

Block of DC Migration Affects Only DCs in the Site of Injection. If the inflammatory response generated in the immediate vicinity of the injection physically impeded DC mobilization, we would expect that DC migration from proximal regions, which are not directly localized within the inflammatory response, to be unaffected by the presence of bacteria. Thus, we investigated, by means of epicutaneous application of contact sensitizers, the capacity of epidermal LCs overlying the site of injection to migrate to DLNs in the presence or absence of bacteria injected i.d. Using the contact sensitizer FITC, we painted the skin of mice that received bacteria intracutaneously and followed FITC^{+} LCs migration to the draining node. As shown in Fig. 3, FITC^{+} CD11c^{+} DC migration to DLNs was not affected by the presence of bacteria. Rather, a slight increase of FITC^{+} cells was observed, confirming that the effect is confined strictly to the site injection and inflammatory focus, and/or is restricted in its effect to certain DCs or DC precursors, such as the monocyte-derived cells that most efficiently acquire phagocytic particulates in the dermis (5).

Absence of DC Migration Correlates with Defect in Immune Response. As already described, FITC microspheres as a particulate antigen are unable to gain access to the DLNs in the absence of cellular transport (5). Thus, we can assume that the majority of the beads recovered from the DLNs are actively transported by phagocytes migrating from the periphery. As injection of increasing concentrations of bacteria progressively blocks DC migration from peripheral tissues, we would expect to gradually dampen T cell proliferation to a model antigen covalently coupled to latex beads when coinjecting the beads with progressively higher doses of bacteria. Thus, latex beads were coupled with the model antigen OVA and OVA-specific transgenic T cells (from DO11.10 mice) were used to follow T cell expansion in vivo according to the protocol established by Kearney et al. (20). The population of transferred T cells was tracked by flow cytometry as CD4^{+} CFSE^{+} cells, and cell division was evaluated as subsequent reduction in the associated CFSE fluorescence. As shown in Fig. 4 A, proliferation of OVA-specific T cells to latex OVA is inhibited in a dose-dependent manner by bacteria. Keeping constant the number of OVA latex beads, the number of T cell divisions is progressively reduced to no detectable divisions at 10^7 bacteria. T cell proliferation induced by soluble OVA that could escape the inflammatory region and be taken up by APCs distally was not affected by increasing concentrations of bacteria (Fig. 4 B). We then injected *S. typhimurium* expressing the GST-OVA fusion protein (SL-pOVA), or the same strain expressing only the GST (SL-pGEX), as a control. As shown in Fig. 4 C, only at a very high concentration (10^7 CFUs) of SL-pOVA could some response be detected, which was much lower than that induced by latex OVA even though the amount of OVA delivered by the bacteria was four times higher than that released by the beads (refer to Materials and Methods). This correlated with the recovery of very few bacterial colonies in DLNs, probably due to free bacteria escaping from the inflammatory site (Fig. 4 D). These results were not due to a gener-
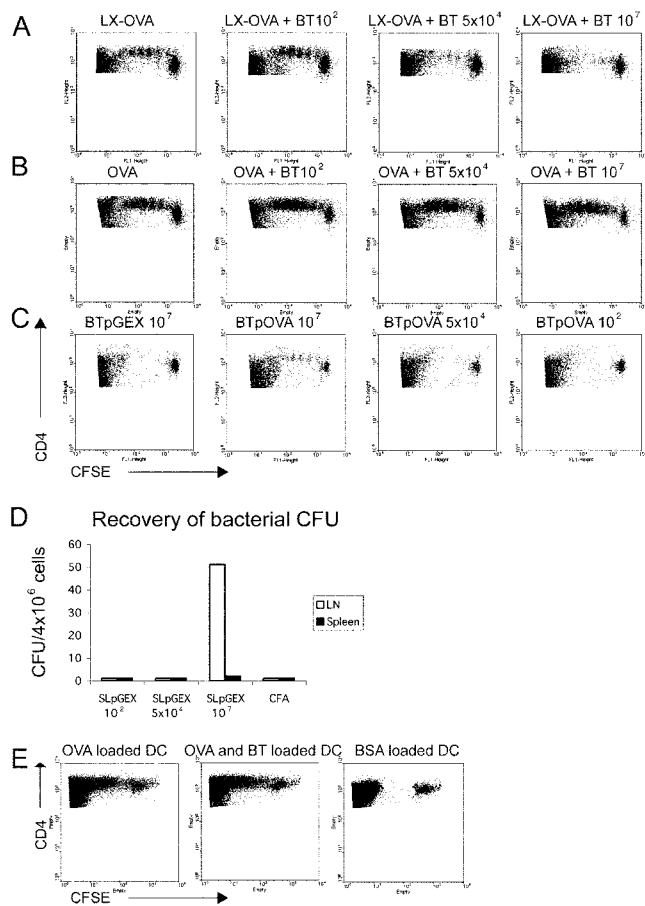

Figure 4. Bacteria block T cell proliferation after administration of particulate or "bacterial," but not soluble, antigens. (A) A dose-dependent inhibition of T cell proliferation to particulate antigen is observed in the presence of bacteria. BALB/c recipient mice were transferred with CFSE-labeled DO11.10 CD4^{+} T cells. 10^7 carboxylated microspheres covalently coupled with 0.25 μg OVA protein were injected i.d. together with increasing concentrations of bacteria (range: 10^2–10^7 CFUs). Dot plots of CD4^{+} CFSE^{+} T cells collected from DLNs 3 d after subcutaneous injections are reported. (B) 0.25 μg soluble OVA reaches the DLNs in the absence of cellular transport and is presented by resident APCs to activate T cells also when coinjected with increasing concentrations of bacteria. (C) "Bacterial" recombinant OVA induces the proliferation of DO11.10 T cells only after injection of high amounts (10^7 CFUs) of bacteria. Lower doses of bacteria or control nonrecombinant BTpGEX (first panel) did not induce DO11.10 T cell proliferation. (D) Only after injection of 10^7 CFUs of bacteria could some CFUs be recovered in DLNs, but not in the spleen. Bacterial escape from the site of injection was evaluated by collecting cells from spleen and DLNs of treated mice and by plating cellular lysates on LB agar. (E) DCs loaded with soluble OVA induce good T cell proliferative responses when either injected alone or after washing out coincubated bacteria. BSA-loaded BM DCs were used as a negative control in these experiments.

alized and direct inhibitory effect of the bacteria on DC migration because when OVA-loaded, bone marrow–derived DCs were coincubated with bacteria (10^7 CFUs) in vitro, and then extensively washed before injection in vivo, a good T cell proliferative response was detected (Fig. 4 E). Altogether, these experiments indicate that not only is presentation of a model antigen coupled to latex impaired, but also the same antigen expressed within bacteria is not pre-
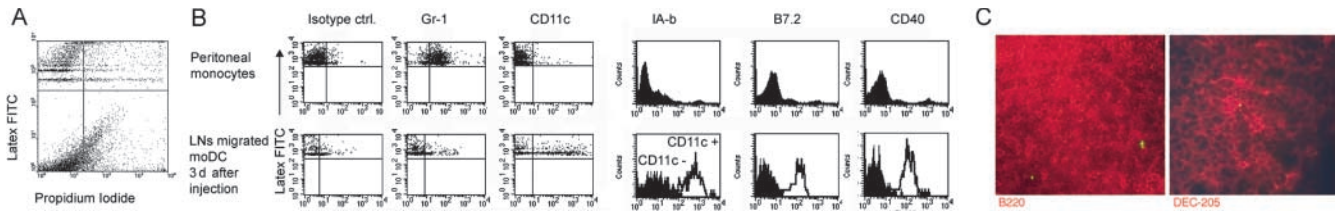
Figure 5. Inflamed skin cells are not undergoing massive cell death and LN latex^{+} cells derived from peritoneal monocytes display features of mature DCs. (A) Only 10% of latex^{+} cells from the inflamed skin cell suspension are also propidium iodide[+] (dead). Two-color FACS® analysis of FITC latex^{+} cells and propidium iodide was performed on cells collected from injected site 36 h after injection of 10[7] bacterial CFUs. (B) Peritoneal monocytes loaded with FITC[+] latex beads display features of mature DCs when they reach the DLN. Isolated peritoneal monocytes express intermediate levels of Gr1, are negative for CD11c, B7.2, and CD40, and are all positive for FITC latex beads (top). LN latex[+] monocyte-derived DCs down-regulate the expression of Gr1 and up-regulate the expression of CD11c, MHC II, B7.2, and CD40. Open histogram profiles show phenotype of gated CD11c^{+} cells and filled profiles show gated CD11c^{−} cells. (C) Frozen sections of LNs examined 3 d after adoptive transfer were stained with B220 or DEC-205, detected with Cy3-conjugated secondary mAb (red). Latex-bearing cells are visualized by green fluorescence.

sented due to failure of the antigen to reach the DLN within DCs and thereby promote T cell proliferation.

DCs Are Not Trapped at the Site of Bacterial Injection. A common feature that accompanied the defect in DC migration was the formation of a strong inflammatory reaction at the site of bacterial injection. We pursued three hypotheses for the block of DC migration: (a) the possibility that the massive presence of Salmonella might be toxic and induce the death of latex^{+} cells; (b) that latex[+] DCs might be trapped in the inflammatory area; and (c) alternatively, as latex[+] DCs migrating to DLNs from the skin derive primarily from monocytes during their transit into the tissue (5), it seemed possible that the presence of bacteria at the site of injection inhibits DC differentiation from monocytes, thereby blocking their migration into DLNs. Thus, we addressed each one of these possibilities. At days 1, 2, or 3, the tissue corresponding to the injection site was analyzed either by cytofluorometry or by immunohistochem-
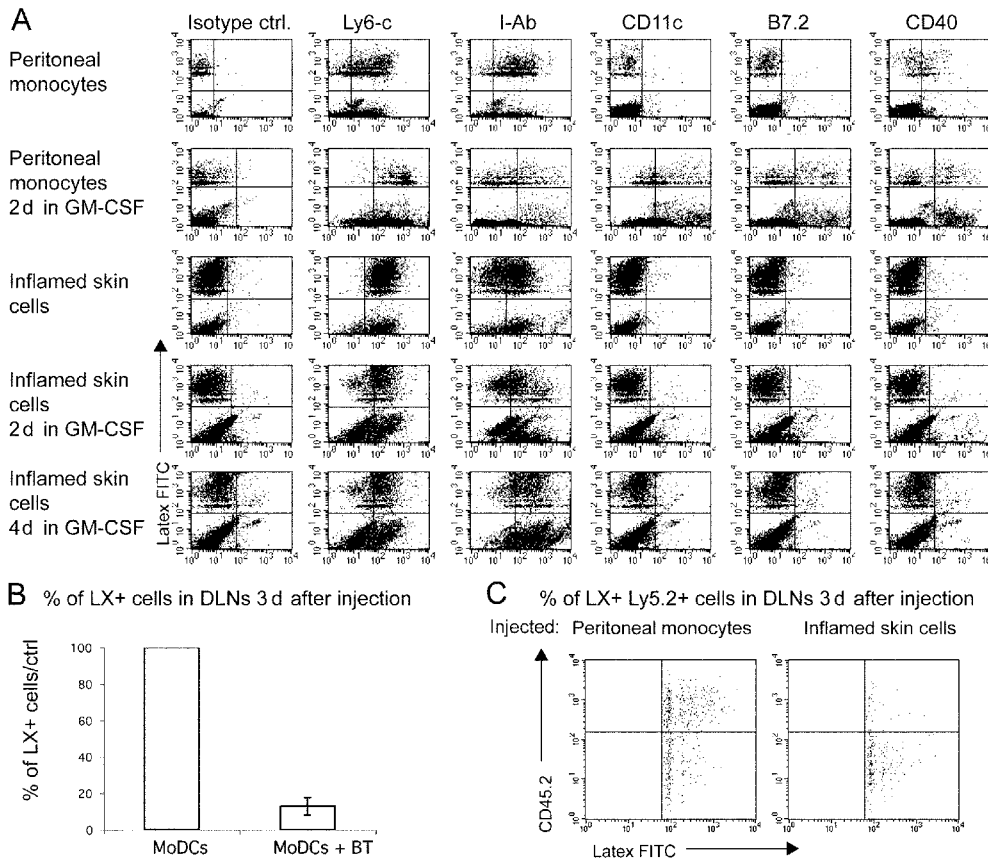

Figure 6. Bacteria impede DC differentiation from monocytes. (A) Latex^{+} cells from peritoneal lavage displayed typical features of inflammatory monocytes: intermediate levels of Ly6-c and I-A[b], no CD11c, no B7.2, and low CD40. Peritoneal monocytes differentiated in DCs after 2 d of culture in GM-CSF–containing medium. By contrast cells obtained at day 2 from the site of bacteria/latex injection in skin did not show signs of DC development after recovery from the skin, and they retained a "monocyte" phenotype also after 2 or 4 d of culture in GM-CSF–containing medium. (B) Bacteria impede the migration of coinjected latex-bearing inflammatory monocytes. Latex[+] monocytes were collected from peritoneal lavage of mice treated with thioglycollate for 18 h and with FITC latex solution for an additional 4 h. Inflammatory monocytes were injected i.d. in the presence or absence of bacteria. DLNs were analyzed 3 d after injection for the presence of latex^{+} cells. (C) Inflammatory monocytes but not inflammatory cells from bacteria-injected skins are able to produce migrating monocyte-derived DCs. CD45.2[+] peritoneal inflammatory monocytes (left) or 2 d–infected skin cells (right) were cultured for 2 h in medium containing antibiotics to kill extracellular bacteria, washed, and injected i.d. in Ly5.1 mice in the absence of bacteria. LN Ly5.2-latex^{+} migrated cells are shown 3 d after injection. These results are representative of three independent experiments.

istry for the expression of the following markers: Gr-1, CD11c, CD11b, F4/80, B220, I-A^b, CD4, and CD8. The cell suspension was also stained for propidium iodide to evaluate the amount of cell death. It is unlikely that apoptosis or necrosis accounts for diminished DC migration because a maximum of 10% latex$^+$ cells were also positive for propidium iodide (Fig. 5 A). Only a few macrophages (F4/80^{hi} cells) were found the first day but their number was increased at later time points, whereas very few CD11c$^+$ were found at any time points. Thus, we conclude that latex$^+$ cells were confined to the infection site. This conclusion was supported by quantitation of the units of fluorescence recovered after acetone extraction of the tissue at the site of microsphere injection. We found that 100% of initial injected fluorescence was recovered 3 d after coinjection of latex and bacteria (unpublished data), whereas ~25% of the fluorescence units leave the injection site within migratory cells in the absence of bacteria (5). The immunohistochemistry of the Salmonella-infected skin was in complete agreement with the FACS® analysis (unpublished data). Hence, we can exclude that abrogation of DC migration is due either to cell death of latex$^+$ cells or to trapping of CD11c$^+$/latex$^+$ cells in the skin.

Bacteria Irreversibly Block DC Differentiation from Monocytes. Next, we addressed the issue of whether DC differentiation from monocytes was taking place normally in the inflamed tissue. We derived monocytes from peritoneal lavage (21) of Ly5.2 mice as described in Materials and Methods to yield latex$^+$ monocytes. The majority of collected latex$^+$ cells displayed typical features of inflammatory monocytes (Fig. 6 A: intermediate levels of Ly6-c, intermediate F4/80 [not depicted], and I-A^b, no CD11c, no B7.2, no CD40, and low CD115 [M-CSF receptor]). The absence of CD11c$^+$ cells suggests that classical DCs are not present. F4/80^{hi} macrophages, likely derived from resident macrophages, were present but represent <10% of monocyte-derived cells (unpublished data) and were surprisingly less efficient at engulfing microspheres than their newly recruited counterparts. Thus, although a few of the recovered cells could have originated from resident macrophages, most must derive from newly recruited monocytes. FITC latex$^+$ monocytes were injected i.d. in C57/BL6J Ly5.1 mice either alone or together with bacteria at a ratio of 25 bacteria to 1 monocyte. Adoptively transferred monocytes could be tracked in recipient mice as CD45.2$^+$/latex$^+$ cells. As shown in Fig. 6 B, latex$^+$ adoptively transferred cells could be detected in DLNs only when injected in the absence of bacteria. LN latex$^+$ cells displayed a DC phenotype (Fig. 5 B) similarly to endogenously migrated monocytes (5). Many migrated monocytes gave rise to mature latex$^+$ DCs having high expression of MHC-II, B7.2, and CD40 (Fig. 5 B). In situ these cells were found subjacent to the B cell follicles within the T cell zone and in the interfollicular spaces, where they stained positively for DEC–205 (Fig. 5 C).

As per our earlier conclusion, nonmigrated latex$^+$ skin cells of mice coinjected with latex and bacteria did not display any marker of DCs (Fig. 6 A). Moreover, when latex$^+$

cells were collected from the skin inflammatory reaction and reinjected i.d. in the absence of bacteria, they did not produce migrating DCs, indicating that the block of DC differentiation from monocytes was irreversible (Fig. 6 C). The only latex$^+$ cells that could be found in the LN were of recipient origin. Consistently, culture of latex$^+$ cells from infected skin in conditioned medium containing GM-CSF for up to 4 d did not yield to DCs (Fig. 6 A). By contrast, peritoneal monocytes rapidly up-regulated the expression of CD11c, costimulatory molecules (CD40 and B7.2), and MHC class II (I-A^b) after 2 d of culture in DC-conditioned medium (Fig. 6 A). These results support the hypothesis that i.d. bacterial injection irreversibly inhibits DC differentiation from inflammatory monocytes recruited at the site of infection, thus explaining their inability to migrate to DLNs.
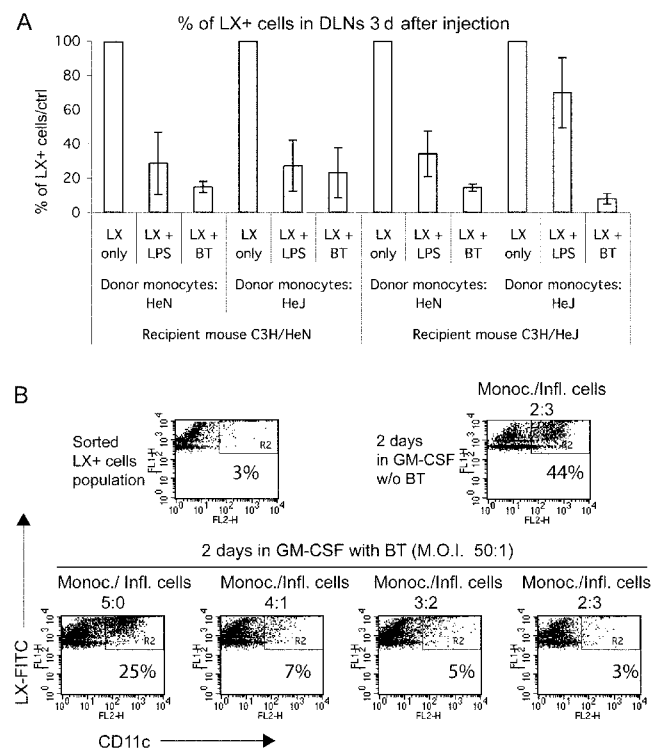

Figure 7. LPS is not fully responsible for bacteria-dependent inhibition of DC differentiation and also exerts its effect on recruited inflammatory cells. (A) C3H/HeN and C3H/HeJ mice were i.d. injected with latex$^+$ inflammatory monocytes. Monocytes were derived from either HeN or HeJ mice, and then i.d. injected alone or together with 10^7 CFUs of bacteria or 10 μg LPS in the syngeneic and congenic strain. Latex$^+$ cells were recovered from DLNs 3 d after injection. Bacteria always impede migration of latex$^+$ monocytes. The block of DC migration induced by LPS is completely reverted when injecting nonresponsive monocytes (HeJ monocytes) but not when injecting LPS-responsive monocytes (HeN monocytes) into nonresponsive mice (HeJ mice). (B) The block of DC differentiation from monocytes is mediated by a concerted action between bacteria and inflammatory cells. Thioglycollate-elicited inflammatory monocytes were enriched after sorting of latex-bearing cells and were incubated either alone or with Salmonella (50:1 bacteria/monocytes) in GM-CSF–containing medium. Maximum 50% inhibition of DC differentiation was observed after incubation of inflammatory cells with bacteria. 100% inhibition was observed when increasing ratios of inflammatory cells were added in addition to bacteria.

Inhibition of DC Differentiation Is Not Due Only to TLR4 Engagement. We have shown that i.d. injection of amounts of LPS ≥1 μg induces a block of DC migration similar to that induced by the bacteria. Thus, we have investigated whether the effect exerted by the bacteria was primarily mediated by LPS. We have analyzed the migratory capacity of adoptively transferred inflammatory monocytes coinjected with LPS or bacteria in mice nonresponsive to LPS (C3H/HeJ) or in mice responsive to LPS (C3H/HeN). As expected, HeN monocytes were unable to migrate in LPS-responsive mice (HeN) when coinjected both with LPS or bacteria (Fig. 7 A). By contrast, HeJ monocytes were perfectly capable of migrating in LPS-nonresponsive mice only when LPS but not bacteria was coinjected (Fig. 7 A). As C3H/HeJ mice have a point mutation which affects only the function of TLR4, we can conclude that when we injected bacteria, other bacterial components that can transduce via TLRs other than TLR4 are also involved in the block of DC differentiation. We investigated whether the inhibitory effect of bacteria might be reversed in MyD88$^{-/-}$ mice. In this strain, migration of monocyte-derived DCs was normal in the absence of bacteria, and the block induced by bacteria was not reversed. If the effects of bacteria are dependent on TLRs, the response must involve MyD88-independent signaling pathways (22) that may dominate over or be redundant with MyD88 signaling.

The Inhibitory Effect of Bacteria Is Mediated in Conjunction with Inflammatory Cells. Interestingly, when HeN monocytes were coinjected with LPS into the LPS nonresponsive strain their migratory capacity was not reverted. There could be two explanations, not mutually exclusive, that either LPS itself blocks to some extent DC differentiation from monocytes or that some contaminating inflammatory cells might release factors that play a role in inhibiting monocyte differentiation to DCs. The latter possibility is supported by the fact that LPS nonresponsive monocytes are unable to differentiate to DCs into LPS-responsive recipients, suggesting that the inflammatory environment, rather than a cell autonomous effect, induced by LPS plays a decisive role in the development or function of migratory DCs (Fig. 7 A). However, we could not exclude that bacteria or LPS might have a direct effect on DC differentiation. Thus, we derived inflammatory monocytes from peritoneal wash as described above, and we purified the population of inflammatory monocytes by sorting latex$^+$ cells. We then incubated the cells with or without external bacteria in GM-CSF–containing medium and we analyzed the percentage of cells giving rise to CD11c$^+$ cells. As shown in Fig. 7 B, the presence of bacteria reduced 50% of the potential of inflammatory monocytes to differentiate into DCs. A much more robust inhibition of differentiation, more closely resembling the massive block observed in vivo, was observed when we added back even a relatively small number of the latex$^-$ inflammatory cells from the cell sort. From Fig. 6 (where unsorted peritoneal cells were used), we know that leaving the latex$^-$ inflammatory cells in the 2-d culture containing GM-CSF does not adversely affect the differentiation of monocytes into DCs in

the absence of bacteria. When inflammatory cells (predominantly neutrophils) are present with bacteria and labeled monocytes, the differentiation of the monocyte-derived cells to DCs nearly ablated. These data suggest that complete inhibition of DC differentiation is achieved by a combination of events mediated by the bacteria and by the inflammatory cells recruited at the infection site.

Single Action of IL-10, IL-6, IL-12, or IFN-γ Is Not Sufficient to Block DC Differentiation. To identify soluble factors released by inflammatory cells and responsible for the block of DC differentiation, we evaluated the role of cytokines recognized as having a negative effect on DC differentiation. We found that during the first and second day after infection, high amounts of IL-10 (corresponding to 300 and 500 pg, respectively) were detected by ELISA in the dissected tissue corresponding to the site of injection. IL-10 plays a regulatory role on DC function by blocking either DC differentiation from monocytes (10, 11) or DC and LCs maturation and function induced by several stimuli (23–26). Moreover, IL-10 converts monocytes into macrophages with increased antibacterial activity (15) and reduces the migratory capacity of DCs infected with *Mycobacterium bovis bacillus calmette guerin* (27). Thus, we investigated whether IL-10 released during inflammation played a role
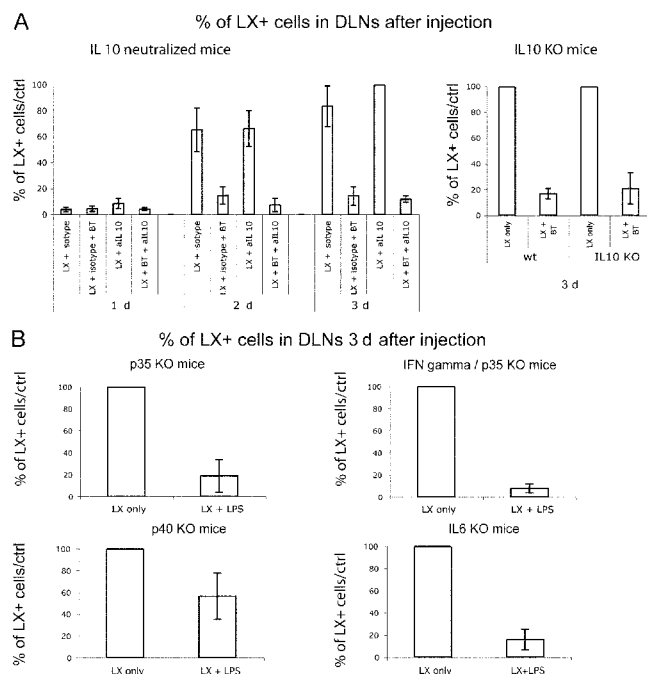

Figure 8. Absence of IL-12-p40, but not p35, IL-10, IL-6, or IFN-γ partly relieves the block imposed by LPS. (A) IL-10 neutralization with anti–IL-10 antibodies does not restore DC mobilization from the periphery to DLNs. Similar results were obtained in IL-10 KO mice. Latex$^+$ cells recovered from the DLNs are shown 1, 2, or 3 d after injection in IL-10–neutralized mice, and 3 d after injection in IL-10 KO mice. Results are representative of three independent experiments. (B) Only IL-12-p40 KO mice can partly revert the block of latex$^+$ cell migration induced by LPS injection. Migration of latex$^+$ cells was evaluated in different mice strains lacking, respectively, the expression of IL-12-p35, IFN-γ and IL-12-p35, IL-12-p40, and IL-6. Latex microspheres were i.d. injected alone or together with 10 μg LPS.

in blocking DC differentiation and we coinjected neutralizing anti–IL-10 antibodies together with FITC latex only, or with FITC latex and bacteria. As a control we injected an isotype-matched irrelevant antibody. As shown in Fig. 8 A, IL-10 neutralization was not able to restore even partially DC migration. Injection of latex beads in the presence of 10^7 CFUs of bacteria blocked DC migration also in mice lacking IL-10 (Fig. 8 A), indicating that if IL-10 was involved in modulating DC function, this was in combination with other factors. Another possible candidate responsible for skewing monocyte differentiation to macrophages is IL-6, which could be released by many inflammatory cells (14). Thus, we investigated the role of IL-6 in the block of DC differentiation and we injected FITC latex beads in the absence or presence of bacteria (unpublished data) or LPS in IL-6 KO mice. As shown in Fig. 8 B, the absence of IL-6 does not restore the migration of latex$^+$ beads, suggesting that IL-6 does not play a primary role in the block of DC differentiation from monocytes. Finally, it has been recently shown that IL-12 and IFN-γ, which are important early in the primary response to Salmonella infection (28), are released principally by neutrophils (29, 30). As the most populous cell type recruited early in the inflammatory reaction are neutrophils, we asked whether IL-12 or IFN-γ could play a role in the block of DC migration either by influencing DC differentiation or the generation of the inflammatory reaction. As shown in Fig. 8 B, in mice lacking either p35 or both IFN-γ and p35, the block of DC migration was not reverted.

IL-12-p40 Is Partly Involved in the Block of DC Migration. A prominent role for p40 in the clearance of *Mycobacterium tuberculosis* as well as of the bacterium *Francisella tularensis* has been recently described (31, 32). Thus, we investigated the capacity of latex-bearing cells to migrate to DLNs in p40 KO mice after coinjection of latex beads and LPS. As shown in Fig. 8 B, DC migration was partially restored in p40 KO mice, suggesting that p40 alone or its combination with other proteins, such as p19 (33) rather than p35, are important either in the block of DC differentiation or migration and presumably in the containment of bacteria at the site of infection.

Discussion

It is clear for various infections that the establishment of containment reactions that limit expansion and spread of infectious agents is protective for the host, a typical example being the induction of granulomas (for review see reference 34). However, the requirement for microbial containment might have a parallel effect of inhibiting total cell emigration from the inflamed area, including APCs, with detrimental effects on the induction of adaptive immune responses. We came across this possibility when we found that recombinant Salmonellae injected i.d. are unable to induce efficient adaptive immune responses to bacteria-associated antigens (18). Here we have shown that indeed, i.d. injection of *S. typhimurium* or LPS (at concentrations ≥ 1 μg) induce the initiation of local inflammatory reactions, which impede the differentiation of DCs from monocytes and their subsequent migration to DLNs.

The block of DC differentiation is due to a combination of effects mediated both by the bacteria and by inflammatory cells recruited at the site of injection. In fact, in vitro incubation of inflammatory monocytes with bacteria blocked nearly 50% of their differentiation into DCs, consistent with earlier analysis of the effects of LPS on the differentiation of human monocytes to DCs in vitro (35). More substantial inhibition, however, was observed when monocytes were coincubated with bacteria and inflammatory cells in vitro. This condition blocked completely DC differentiation. A direct involvement in vivo of recipient inflammatory cells after LPS injection was attested by the migratory defect of adoptively transferred LPS-nonresponsive inflammatory monocytes into LPS-responsive, but not LPS-nonresponsive, mice. This highlights an unexpected negative regulatory role of TLR4 engagement on DC differentiation from precursor cells and poses a new perspective on TLRs. That is, TLR engagement may not unequivocally link innate and adaptive immune responses. Although numerous studies show that inflammatory mediators and engagement of TLRs can promote adaptive immune responses by promoting maturation of DCs, the outcome is quite different when monocytes, which are not a priori committed to become DCs but can also become macrophages, encounter the inflammatory/pathogenic signal before committing to the DC pathway of differentiation in vivo.

The block of DC differentiation from monocytes correlated with the inability of particulate or bacteria-associated antigen to reach the DLN. We observed a progressive decline of T cell activation toward particulate antigen (OVA latex beads), which can gain access to DLNs primarily via cellular transport (5) in the presence of increasing concentrations of coinjected Salmonellae. Accumulation of T cells was also not found within the skin inflammatory infiltrate (unpublished data), suggesting that our failure to find them in DLN was not due to their preferential recruitment in the inflammatory focus at the site of injection. We can also exclude that bacteria might be toxic or affect the intrinsic migratory properties of fully differentiated DCs as reported for *Leishmania major* (36, 37) because DCs preloaded in vitro with bacteria and soluble OVA before injection in vivo were not impaired in their capacity to stimulate T cells. Consistent with the inability of Salmonella vaccination to protect from tumor challenge, we observed very little T cell proliferation to bacteria expressing OVA and only at a high concentration of injected bacteria (10^7 CFUs), probably due to few bacteria freely migrating to the DLN. By contrast, we did not detect any reduction in T cell proliferation after injection of soluble OVA with increasing concentrations of bacteria, indicating that soluble antigens can freely enter afferent lymph to reach DLNs and be presented by resident APCs.

Indeed, dissemination of free antigens might be the main way in which adaptive responses develop against pathogens that elicit strong local inflammatory responses.

As mentioned above, a prompt recruitment of inflammatory cells was observed at the site of bacterial injection. When latex alone is administered intracutaneously, the predominant cell types attracted to the site of injection are mononuclear cells, including many monocytes. Very few polymorphonuclear cells are observed (5). The inclusion of bacteria in the injections may substantially modify the profile of the recruited cells, as neutrophils are well known to be recruited by bacterial products. Our analysis of the skin inflammatory site supports this conclusion. We found by microscopic analysis of the inflamed skin, the presence of many macrophages and granulocytes. By contrast we could hardly detect any cells having the typical phenotype of DCs. It is likely that some, if not all, of the monocytes that are recruited at the site of inflammation differentiate into highly phagocytic cells as macrophages and are not available to develop into migratory DCs. This is supported by the observation that DC differentiation is irreversibly inhibited by bacteria because cells from infected skins were still unable to differentiate into DCs after 4 d of culture in medium containing GM-CSF, whereas inflammatory monocytes differentiated into DCs already after 2 d of culture. We postulated that cytokines released by the recruited inflammatory cells might have a regulatory activity on DC differentiation. However, we could not revert the block of DC migration by the individual absence of several cytokines involved in DC differentiation or in inflammation, i.e., IL-6, IL-10, IL-12-p35, and IFN-γ, suggesting that the inhibition of DC differentiation is probably due to the coexistence of multiple events. The only cytokine KO mouse that partially reverted the observed phenotype in the presence of LPS was IL-12-p40 KO. p40 could act as a homodimer (p40$_2$) or together with other proteins rather than p35 (i.e., p19 to yield IL-23), and this is in agreement with data in the literature showing a crucial role of p40, but not of p35, in bacterial clearance (31, 32). Further, p40$_2$ has been reported to have chemotactic (38) and activating properties on macrophages and microglia (39). Thus, p40 could have similar properties also on monocytes and somehow influence their differentiation, migratory clearance, and function. The role of p40 in DC differentiation from monocytes and whether its origin from neutrophils explains the inhibitory effect of the inflammatory cells is under investigation.

In conclusion, these results tell us that when the skin is accidentally injured, by a cut for instance, and the dermis is exposed to environmental microorganisms, the immune system responds with the induction of a vigorous innate response aimed at microbial containment. We could envisage the existence of a hierarchical mechanism, whereby an inflammatory focus delays the onset of the adaptive response until the cellular (phagocyte) response has a chance to develop in the periphery and clear the infecting agent. This is achieved by (a) inhibiting monocyte differentiation into DCs, which may favor the development of cells with higher microbicidal potential, and (b) by blocking DC mobilization to DLNs, which may reduce the possibility for the pathogen to use DCs as carriers to reach DLNs. Thus, we have identified a checkpoint in the induction of adaptive responses to bacteria that is the regulation of DC differentiation from monocytes. Although this mechanism would decrease the chances of microbial spreading, it could be harnessed by intracellular bacteria particularly resistant to phagocyte killing to hide from the immune system and create a niche to their own interest.

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