

Regulation of Dendritic Cell Numbers and Maturation by Lipopolysaccharide In Vivo

By Thibaut De Smedt,* Bernard Pajak,† Eric Muraille,*
Laurence Lespagnard,* Ernst Heinen,§ Patrick De Baetselier,||
Jacques Urbain,* Oberdan Leo,* and Muriel Moser*

From the *Département de Biologie Moléculaire, Université Libre de Bruxelles, B-1640 Rhode-Saint-Genèse; †Département de Biologie Animale, Université Libre de Bruxelles, B-1050 Bruxelles; §Institut d'Histologie Humaine, Université de Liège, 4000 Liège; and ||Laboratorium of Cellular Immunology, Vrije Universiteit Brussel, B-1640 Rhode-Saint-Genèse, Belgium

Summary

Dendritic cells (DC) are described as "nature's adjuvant," since they have the capacity to sensitize T cells in vivo upon first encounter with the antigen. The potent accessory properties of DC appear to develop sequentially. In particular, the ability to process antigens and to sensitize naive T cells develops in sequence, a process termed "maturation" that is well described in vitro. Here, we obtain evidence for maturation in vivo in response to the bacterial product lipopolysaccharide (LPS). Before LPS treatment, many DC are found at the margin between the red and white pulp. These cells lack the M342 and DEC-205 markers, but process soluble proteins effectively. 6 h after LPS, DC with the M342 and DEC-205 markers are found in increased numbers in the T cell areas. These cells have a reduced capacity to process proteins, but show increases in the B7 costimulator and T cell stimulatory capacity. 48 h after LPS, the number of DC in the spleen is reduced markedly. We interpret these findings to mean that LPS can cause DC in the marginal zone to mature and to migrate into and then out of the T cell areas.

Optimal activation of T lymphocytes depends on TCR interactions with peptide/MHC complexes in conjunction with costimulatory signals that are typically delivered by the same APC. The costimulatory signal is antigen nonspecific, and is delivered by CD28 or CTLA4 on T cells that interact with a ligand, B7-1 (CD80) or B7-2 (CD86), that is present on the APC (1). The role of costimulatory molecules has been clearly demonstrated by studies showing that antigenic stimulation of T cells in vitro in the absence of costimulation leads to aborted T cell proliferation and the development of functional unresponsiveness or clonal anergy of T cells (2). Injection of CTLA4Ig, a soluble form of the extracellular domain of CTLA-4 with high avidity for B7-1 and B7-2, suppresses T cell-dependent antibody responses in vivo (3). CTLA4Ig is also capable of blocking in vitro proliferation in assays of alloreactivity (4, 5), as well as preventing xenograft rejection (6) and prolonging allograft rejection (7, 8). These data show that B7/CD28-CTLA4 activation pathways play an important role in regulating in vivo T cell responses.

Among the population of APC, which in the mouse comprises dendritic cells (DC)¹, B lymphocytes, and macrophages, DC appear to have the unique capacity to acti-

vate naive T cells (9). In vitro, they are essential to trigger a primary antibody response (10) and to generate antiviral CTL (11, 12), and they strongly stimulate CD4⁺ (13) and CD8⁺ (14) cells in the primary mixed leukocyte reaction. In vivo, DC appear to play a major role in initiating various T cell immune responses, such as contact sensitivity (15–17) and allograft rejection (18–21). DC pulsed extracorporeally can efficiently induce cellular (22) and T cell-dependent humoral (23, 24) responses in mice. The property of isolated DC to sensitize quiescent T cells upon the first encounter with the antigen correlates with their capacity to express very high levels of antigen/MHC peptides, as well as B7-1 and B7-2 costimulatory molecules (5, 25, 26).

The first step of the immune response leading to efficient activation of antigen-specific, naive T cells in situ is still poorly understood. DC are likely to be the adjuvant of the immune system in vivo. DC are motile and efficiently cluster T cells, are widely distributed in tissues, carry antigens that are administered intradermally or intravenously, and circulate through lymph or blood probably in route to lymphoid organs (for review see reference 9). The analysis of cryostat sections (26), however, revealed that B7-1 and B7-2 staining was weak or negative in most nonlymphoid organs, but strong in select sites in lymphoid organs. In particular, in the spleen, both MHC class II and B7-2 were expressed strongly on dendritic profiles in the T cell re-

¹Abbreviations used in this paper: BR, blocking reagent; DC, dendritic cells; SEB, staphylococcal enterotoxin B.

gions that occupy the center of the white pulp, but not in marginal zone where DC are abundant, and they carry high levels of the CD11c leukocyte integrin. Furthermore, the expression of both B7 molecules is weak on DC that are freshly isolated from the spleen, but is strongly upregulated in vitro, without intentional stimulation (26, 27). This process of maturation is characterized by the upregulation of the T cell-sensitizing function and the downregulation of the antigen-processing capacity during short-term culture (26–30). These observations indicate that DC do not display constitutive costimulatory function in situ, and suggest that they may mature into potent accessory cells during the early sensitization phase of immunity.

In this paper, we show that systemic administration of endotoxin (LPS) induces the migration of most splenic DC from the marginal zone to the T cell areas within 4–6 h. This movement parallels a maturation process, as assessed by downregulation of processing capacity and upregulation of immunostimulatory properties. Unexpectedly, 48 h after LPS injection, the number of splenic DC decreased markedly, an observation that correlates with an impaired capacity to activate naive T lymphocytes in vitro and in vivo.

These data show that DC function is regulated by microbial products. LPS induces DC migration and maturation in vivo, resulting in colocalization of potent DC and T lymphocytes. The subsequent loss of DC after activation may be part of a negative feedback mechanism that controls in vivo inflammatory responses that may cause severe tissue damage.

Materials and Methods

Mice

Female BALB/c (H-2^d) and CBA/J (H-2^k), 6–8-wk old, were purchased from Charles River Wiga (Sulzfeld, Germany) and maintained in our own pathogen-free facility.

Reagents and Antibodies

LPS from *Escherichia coli* (serotype 055:B5) was purchased from Difco Laboratories (Detroit, MI). Purified exotoxin staphylococcal enterotoxin B (SEB) was purchased from Toxin Technology, Inc. (Sarasota, FL). Hamster mAb 37.51 to murine CD28 and control mAb F531 (kindly provided by J. Allison, University of California, Berkeley, CA; 31) were used at 1/10,000 dilution of ascitic fluids. Hamster mAb 145-2C11 to mouse CD3 ϵ (32) was purified in our laboratory. Myoglobin from sperm whale skeletal muscle was obtained from Fluka Chemie AG (Buchs, Switzerland). Pork insulin was purchased from Sigma Immunochemicals (St. Louis, MO).

In Vivo Treatment

Endotoxin Injection. BALB/c mice were injected intravenously into the lateral tail vein with 25 μ g LPS solubilized in

pyrogen-free NaCl 0.9%. Control animals were injected with the same volume of NaCl.

T Cell Activation In Vivo. LPS-treated and control BALB/c mice were injected with either 50 μ g SEB or 50 μ g anti-CD3 ϵ solubilized in pyrogen-free NaCl 0.9%. The mice were bled 2 h later, and the sera were assayed for IL-2 content using a standard bioassay using an IL-2-dependent, IL-4-insensitive subclone of the CTL.L line. IFN- γ was quantitated by two-site ELISA using mAbs F1 and Db-1, kindly provided by Dr. Billiau (Katholieke Universiteit Leuven, Leuven, Belgium) and P.H. Van Der Meide (TNO Health Research, Rijswijk, The Netherlands), respectively.

Cell Lines

The I-E^d-restricted, myoglobin-specific T cell hybridoma 13-26-8-HG.1 (33) was kindly provided by Dr. A. Livingstone (Basel Institute of Immunology, Basel, Switzerland). The I-E^d-restricted, pork insulin-specific T cell hybridoma B8P4.1C3 (34) was kindly provided by Dr T. Delovitch (John P. Roberts Research Institute, London, Ontario, Canada).

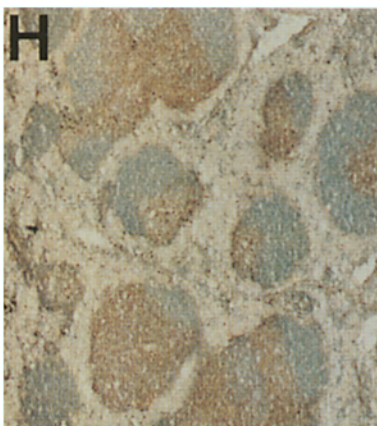
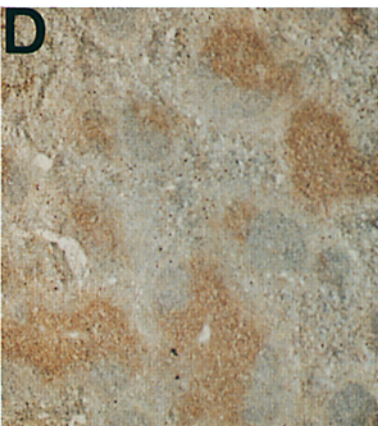
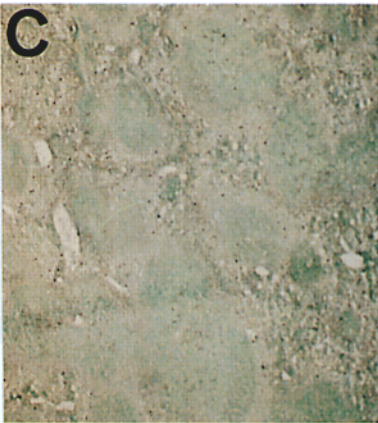
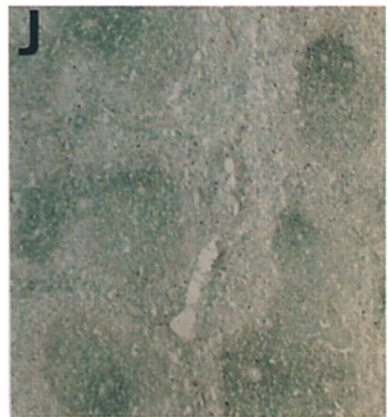
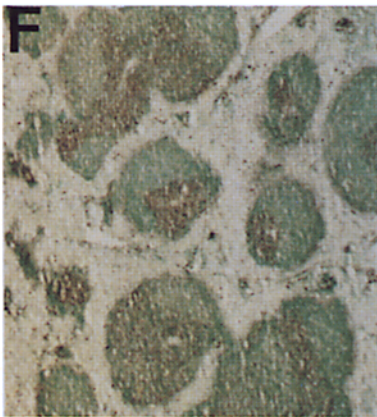
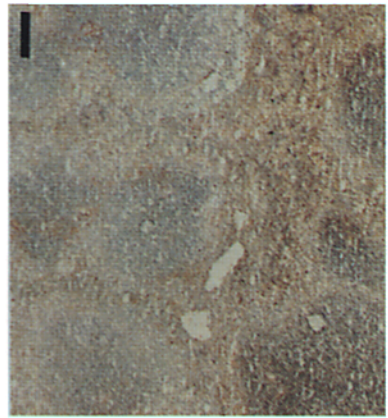
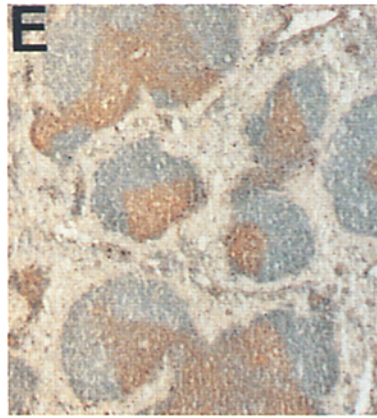
Flow Cytometry

Cells were analyzed by flow cytometry with a FACScan[®] cytometer (Becton Dickinson & Co., Mountain View, CA). The cells were preincubated or not (see figure legends) with 2.4G2 (a rat anti-mouse Fc receptor mAb) for 10 min before staining to prevent antibody binding to FcR, and were incubated with fluorescein-coupled mAb 14.4.4 (murine IgG2a anti-I-E^d, available through American Type Culture Collection, Rockville, MD), N418 (hamster antimurine CD11c; 35), 1610A1 (hamster anti-B7-1; 36), GL1 (rat IgG2a anti-B7-2; 37) with PE-coupled CD45R/B220 (rat IgG2a; PharMingen, San Diego, CA) or with biotinylated N418 followed by avidin coupled to PE or with biotinylated CD11b/Mac1 (PharMingen) revealed by avidin coupled to fluorescein. Cells were gated according to size and scatter to eliminate dead cells and debris from analysis.

Purification of Low Density Spleen Cells and Fresh DC

Spleens were digested with collagenase (CLSIII; Worthington Biochemical Corp., Freehold, NJ) and separated into low and high density fractions on a BSA gradient (Bovuminar Cohn fraction V powder; Armour Pharmaceutical Co., Tarrytown, NY), according to a procedure described by Crowley et al. (38). A further purification of fresh DC (without in vitro culture) by enrichment on a Mini Macs column was sometimes performed, according to the manufacturer's recommendations (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). In brief, low density spleen cells were incubated with biotinylated N418, washed, and incubated with streptavidin microbeads. Cells were then positively enriched on a Mini Macs column. More than 85% of these cells expressed N418 and MHC class II. For FACS[®] analysis, a gate was performed on N418-positive cells.

Figure 1. LPS induces migration of N418-positive cells from the marginal zone of the spleen to the T cell area. Immunoperoxidase labeling of cryostat sections from spleens of control mice (A–D) and mice treated 6 h (E–H) or 48 h (I–L) previously with 25 μ g LPS intravenously. Sections were stained with anti-CD11c mAb (A, E, and I), with anti-DEC-205 mAb (B, F, and J), with M342 mAb (C, G, and K), or with anti-CD4 mAb (D, H, and L). The sections stained with anti-CD11c and anti-CD4 mAb were counterstained with hematoxylin; the sections stained with NLDC-145 and M342 were counterstained with methyl green. The original magnification was 10. \blacktriangledown , white pulp; *, red pulp; \rightarrow , marginal zone.



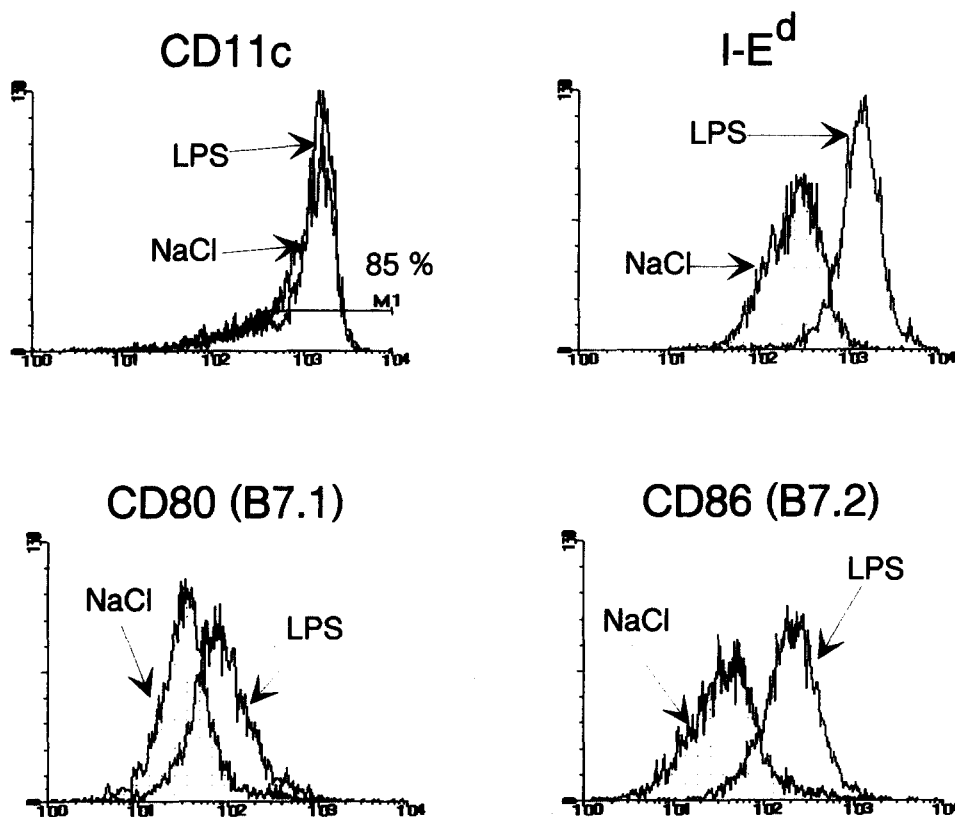


Figure 2. Splenic DC upregulate MHC class II and B7 expression early after LPS injection. N418-positive cells enriched from low density spleen cells from BALB/c mice injected with NaCl or LPS 6 h earlier were double stained for red fluorescence with biotinylated anti-CD11c mAb followed by streptavidin-PE and for green fluorescence with anti-I-E^d, anti-B7-1, or anti-B7-2 mAbs coupled to fluorescein. The DC-enriched population (see Materials and Methods) contained at least 85% CD11c-positive cells. The data represent the expression of MHC class II, B7-1, and B7-2 on cells gated for CD11c expression.

In Vitro Responses

The complete medium used in all experiments was RPMI 1640 (Seromed Biochem KG, Berlin, Germany) supplemented with 2% HY ultrosor (a serum-free medium supplement purchased from GIBCO BRL, Merelbeke, Belgium) or 10% FCS, penicillin, streptomycin, nonessential aminoacids, sodium pyruvate, 2-ME, and L-glutamine (Flow ICN Biomedicals, Bucks, UK). Splenic T cells were purified by depletion of adherent cells by passage over Sephadex G10 (Pharmacia Bioprocess, Uppsala, Sweden) and complement mediated lysis with a cocktail of anti-APC antibodies. T cells were stimulated with graded doses of SEB in the presence of γ -irradiated (3,000 rads) spleen cells or fresh DC in round-bottom 96-well plates, or were cultured in the presence of various numbers of γ -irradiated (3,000 rads) allogeneic spleen cells. Cultures were maintained at 37°C in a humidified incubator (7% CO₂). The supernatants were collected after 12–48 h of culture, frozen, and assayed for IL-2 content (see above).

Immunohistochemistry

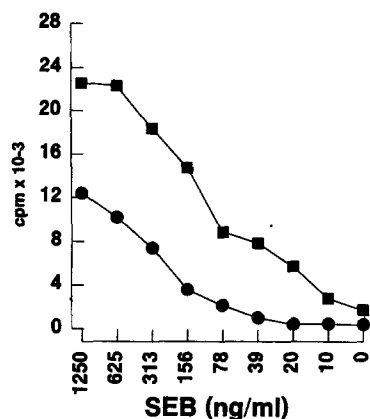
Tissues samples were frozen in isopentane and 12- μ m cryostat sections were prepared. Samples were fixed in neat acetone for 10 min, air-dried, and incubated in PBS containing 0.5% of blocking reagent (BR) from Boehringer for 30 min. Sections were incubated for 20 min with culture supernatant of the monoclonal rat anti-mouse antibody 2.4G2 to Fc receptors to prevent nonspecific staining, except when culture supernatants were used for primary antibodies. Slides were washed in PBS, incubated for 1 h at room temperature with 10 μ g/ml in PBS-BR 0.5% biotinylated mAbs (N418, anti-CD11c, and anti-mouse-CD4 [PharMingen]) or with culture supernatants from rat NLDC-145, (also termed

anti-DEC-205 [39; kindly provided by Dr. J. Austyn, University of Oxford, UK] or hamster M342 [40; kindly provided by Dr. R. Steinman, The Rockefeller University, New York] and washed in PBS. Slides were then incubated in a 1:100 dilution of avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA) in PBS-BR 0.5% or goat F(ab')₂ anti-rat Ig-peroxidase (POD) (BioSource International, Camarillo, CA) or rabbit F(ab')₂ anti-hamster Ig-POD (Rockland Inc., Gilbertsville, PA) for 30 min and washed in PBS. The peroxidase activity was revealed with a solution of diaminobenzidine tetrahydrochloride with or without metal enhancer (DAB tablets, SigmaFAST; Sigma). The sections were stained for 10 min at room temperature, counterstained with hematoxylin or methyl green, and mounted with Poly-mount (Polysciences Inc., Warrington, PA).

Results

LPS Induces Migration of N418-positive Cells from the Marginal Zone of the Spleen to the T Cell Area. LPS was administered to mice, and DC and T cells were visualized in serial cryosections from spleens 6 and 48 h later using specific mAbs (Fig. 1). Two populations of DC were detected in the spleens of untreated mice (*left panel*), as described previously (35, 39, 40). The majority of N418-positive cells (*A*) were detected in the marginal zone between red and white pulp, whereas a few cells were labeled in the T cell area (*D*) in the white pulp. Staining with NLDC-145 (*B*) and M342 (*C*) antibodies outlined some positive cells only in the T cell area.

A SEB + naive T cells



B insulin + hybridoma

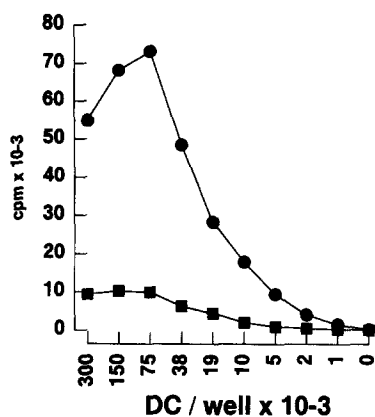


Figure 3. LPS induces functional maturation of splenic DC within a few hours. N418-positive cells enriched from low density spleen cells from BALB/c mice injected with NaCl (circles) or LPS 6 h earlier (squares) were cultured with (A) 2×10^5 syngeneic T cells in the presence of serial dilutions of SEB or (B) with 5×10^4 T cell hybridoma B8P4.1C3 and 200 $\mu\text{g/ml}$ pork insulin. IL-2 production was quantitated from the 12-h (A) or 24-h (B) culture supernatants.

The injection of LPS leads to a redistribution of DC markers in the spleen, suggesting that DC have migrated from the marginal zone to the T cell area. Fig. 1 (*middle panel*) shows serial sections stained with various antibodies. Most N418-positive (E) cells were detected in association with T cells (H) 6 h after injection of LPS. Adjacent sections of the T cell areas were strongly stained with NLDC-145 (F) and M342 (G) antibodies. 48 h after endotoxin administration (Fig. 1, *right panel*), the overall number of splenic DC was strongly reduced, since only few cells in the T cell areas (L) stained with N418 (I), NLDC-145 (J), or M342 (K).

Splenic DC Upregulate MHC Class II and B7 Expression Early after LPS Injection. Since the expression of M342 and NLDC-145 is weak or absent on freshly isolated DC and increased after culture and maturation in vitro (40, 41), we tested whether LPS could induce DC maturation in vivo. LPS was injected intravenously, and 6 h later, low density spleen cells were enriched for N418⁺ cells (see Materials and Methods), and the expression of MHC class II and costimulatory molecules (B7.1 and B7.2) was analyzed by FACS[®]. As shown in Fig. 2, DC isolated from LPS-treated mice displayed increased levels of I-E, B7-1, and B7-2 molecules, as compared to control animals, whereas the expression of CD11c remained unchanged.

LPS Induces Functional Maturation of Splenic DC within a Few Hours. One of the hallmarks of maturation of DC upon culture in vitro is an increased capacity to sensitize naive T cells and a reciprocal downregulation of the capacity to process protein antigens (26–30). To test whether the injection of LPS and the subsequent upregulation of MHC class II and costimulatory molecules on DC were associated with a functional maturation in vivo, splenic DC were purified from naive or LPS-injected animals and immediately tested as accessory cells for the presentation of SEB to syngeneic naive T cells. This superantigen does not require processing and is best presented by DC (42, 43). As shown in Fig. 3 A, DC enriched from animals that had been injected with LPS 6 h earlier were better activators of T cells than DC from untreated animals. The increased immuno-

stimulatory properties of DC from LPS-treated mice were inversely correlated with their capacity to process and present a protein antigen, as assessed by a decreased ability to present insulin to T cell hybridoma (Fig. 3 B). The capacity of DC to present antigens that do not need processing (SEB or peptides) to T cell hybridoma remained unchanged 6 h after endotoxin administration (data not shown).

LPS Injection Results in the Loss of N418-positive Cells from Mouse Spleen after 2 d. The staining of spleen cryosections (Fig. 1) revealed that the movement of DC induced early by LPS was followed by a strong reduction of N418⁺ cells 48 h later. To further document this observation and to analyze whether the loss of N418 staining in cryosections corresponded to the absence of DC or to a selective loss of N418 marker, we performed immunofluorescence studies on DC-enriched, low-density spleen cells. Fig. 4 represents the contour profiles of cells double-stained with B220 in red, and either N418 or 14-4-4 (anti-I-E^d) in green. The data show that low density spleen cells from LPS-injected mice lack the DC population identified as B220⁻, 14-4-4^{high}, or B220⁻, N418⁺. The numbers of DC returned to control levels 15 d after endotoxin administration. The APC populations were further stained in unseparated spleens. As shown in Table 1, LPS-injected mice had reduced numbers of DC, as compared to control animals. By contrast, the numbers of B lymphocytes and macrophages were increased in treated animals. Taken together, these data strongly suggest that a single injection of LPS results in a selective loss of a major population of splenic DC.

LPS Administration Results in Decreased Capacity to Sensitize Naive T Lymphocytes In Vitro 2 d after Injection. Since DC appear to have the unique property to activate naive T cells in vitro, we tested the capacity of splenic cells from control and LPS-injected (2 d earlier) mice to induce primary immune responses in vitro. Irradiated spleen cells from LPS-injected animals induced lower IL-2 secretion (Fig. 5 A) by naive, allogeneic T cells than spleen cells from control mice. Equal numbers of spleen cells from control and LPS-treated mice stimulated T cells at intermediate levels, suggesting that no mechanism of suppression was induced after

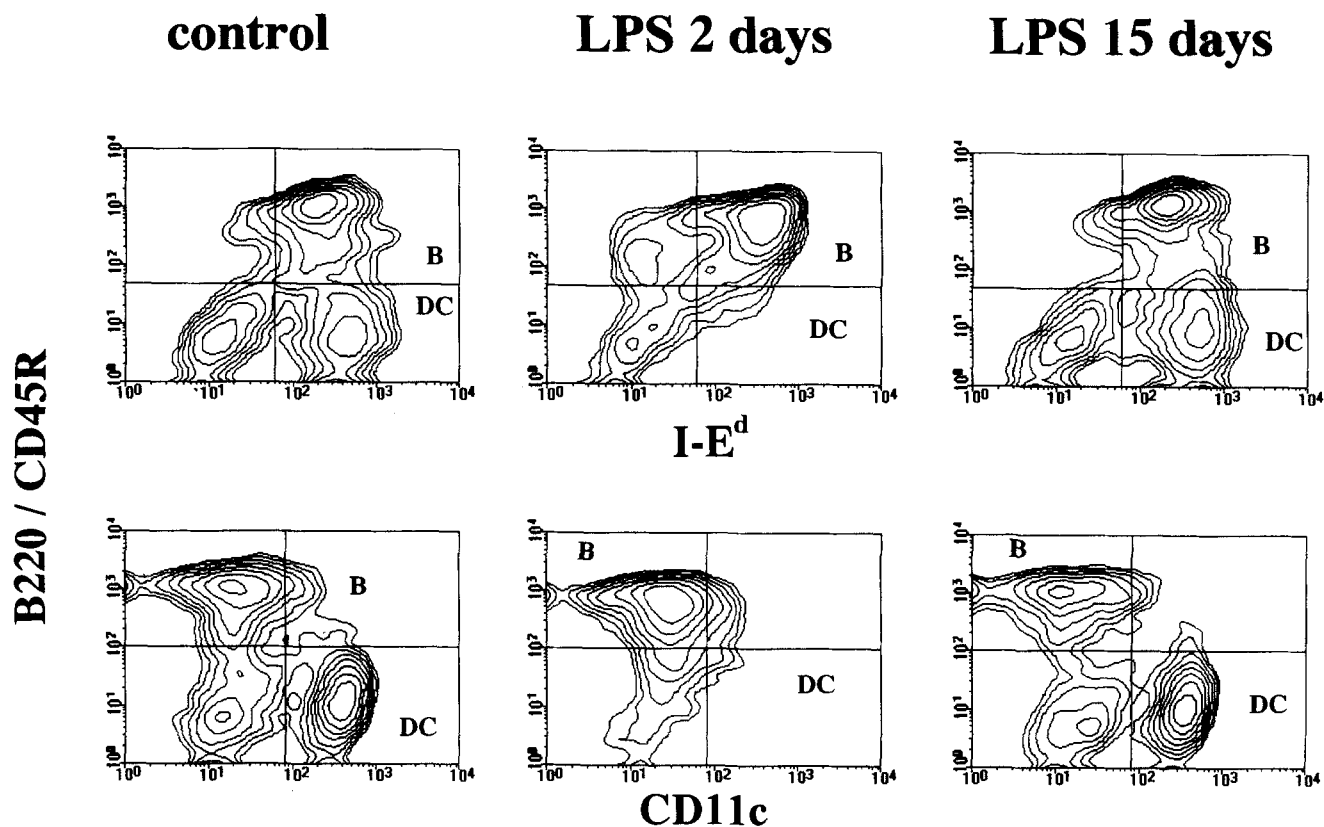


Figure 4. LPS injection results in the loss of N418-positive cells from mouse spleen after 2 d. Two-color immunofluorescence analysis contour plots. Spleen cells from the low buoyant density fraction were stained for red fluorescence with PE anti-B220 mAb and for green fluorescence with anti-I-E^d or anti-CD11c mAb coupled to FITC. Cells were from a pool of two BALB/c mice and injected 48 h or 15 d previously with NaCl (control) or 25 μ g LPS. Boxes on the contour plots define selected populations. DC were identified as B220/CD45R⁻, I-E^{d+} or B220/CD45R⁻, CD11c⁺.

LPS injection. Similar data were obtained when syngeneic T lymphocytes were stimulated by SEB in the presence of irradiated spleen cells from both groups (Fig. 5 B). By contrast, spleen cells from control and endotoxin-injected mice induced similar IL-2 production by CD28-independent T cell hybridoma, which has been shown to respond optimally to T cell receptor ligation in the absence of B7-related costimulatory function (Fig. 5 C). The addition of antibodies specific for CD28 overcame the APC defect of spleen cells from LPS-injected mice in the MLR (Fig. 5 D), suggesting that spleen cells from treated animals retained the ability to generate a TCR ligand, but displayed defective costimulatory function.

Injection of LPS Results in Impaired DC Function In Vivo after 48 h. We next compared the in vivo immune response of control and LPS-treated mice after injection of DC-dependent or -independent stimuli. Control and LPS-treated mice were injected with 50 μ g SEB or with 25 μ g anti-CD3 mAb (mainly presented by FcR⁺ B cells or macrophages; 44), and were bled 2 h later to quantify the IL-2 and the IFN- γ produced in the sera. Fig. 6 shows that the injection of the T cell mitogen anti-CD3 mAb resulted in high production of IL-2 and IFN- γ in both groups. By contrast, the secretion of IL-2 and IFN- γ after stimulation

by SEB was reduced in mice injected with endotoxin, as compared to untreated animals.

Discussion

The unique capacity of splenic DC to optimally activate naive T cells has been extensively demonstrated in vitro

Table 1. Effect of LPS on Splenic Cell Populations

	Control*	LPS
DC [‡]	0.84 [§]	0.20
B cells	39.0	49.0
Macrophages	3.3	5.5

*The numbers of spleen cells were: 9×10^7 (control); 1.4×10^8 (LPS-injected 48 h earlier).

[‡]Splenic cell populations were identified as follows: DC, CD11c⁺, 14.4.4⁺; B lymphocytes, CD45R/B220⁺; macrophages, CD45R/B220⁻, CD11b/Mac-1 α ⁺.

[§]Percent of splenic cells.

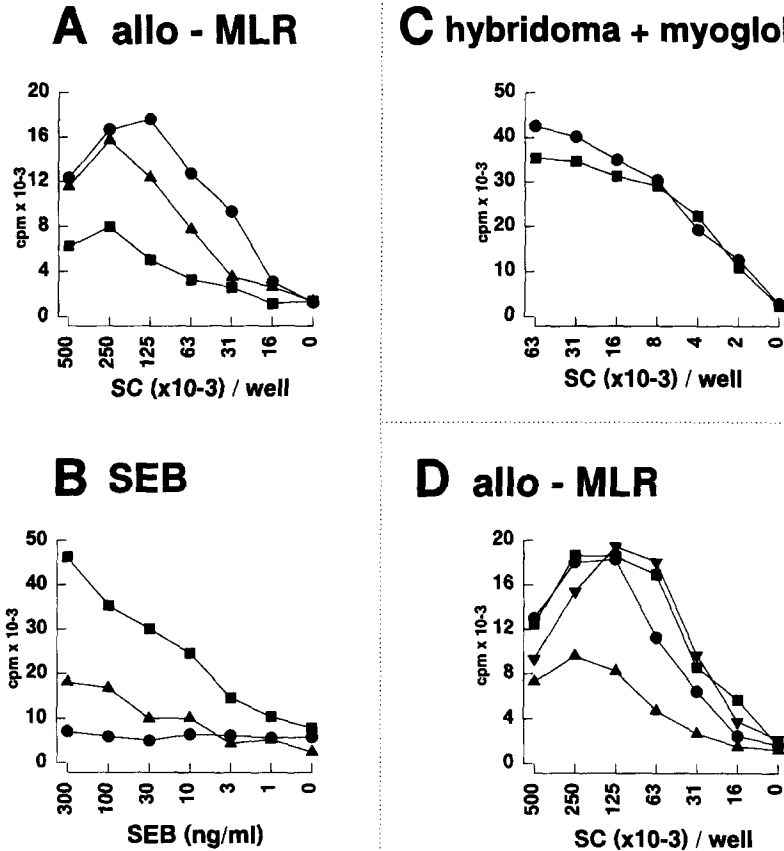


Figure 5. LPS administration results in decreased capacity to sensitize naive T lymphocytes in vitro 2 d after injection. (A) Various numbers of γ -irradiated (3,000 rads) spleen cells from control (circles) or LPS-injected (squares) BALB/c mice or both (triangles) were cultured with 3×10^5 T cells from CBA mice. IL-2 production was quantitated from the 48-h culture supernatants. (B) γ -Irradiated spleen cells from control (squares) or LPS-injected (triangles) mice were cultured with 2×10^5 syngeneic T cells in the presence of serial dilutions of SEB. As a control, T cells alone were cultured with serial dilutions of SEB (circles). IL-2 production was quantitated from the 24-h culture supernatants. (C) Various numbers of γ -irradiated spleen cells from control (circles) or LPS-treated (squares) mice were cultured with 3×10^4 T cell hybridoma 13-26-8 and 100 $\mu\text{g/ml}$ sperm whale myoglobin, and the IL-2 content was evaluated from the 24-h culture supernatants. (D) Various numbers of γ -irradiated spleen cells from control or LPS-treated mice were cultured with 3×10^5 T cells from CBA mice in the presence of antibodies to CD28 or control antibodies. IL-2 production was quantitated from the 48-h culture supernatants. ●, control spleen cells + control mAb; ■, control spleen cells + αCD28 mAb; ▲, LPS-treated spleen cells + control mAb; ▼, LPS-treated spleen cells + αCD28 mAb.

and in vivo. In particular, purified DC, pulsed extracorporeally with an antigen, induce specific cellular and humoral responses when injected into syngeneic animals (22, 23). The adjuvant properties of these cultured DC may depend on a process of maturation that occurs "spontaneously" in vitro during the purification steps (26-30). Whether a similar maturation occurs in vivo is still unknown, and it is of major interest to define the conditions that are required for the induction of primary responses in situ. The data presented herein strongly suggest that LPS induces splenic DC maturation in situ, as assessed by upregulation of immunostimulatory properties and downmodulation of processing capacity. These functional changes correlate with a rapid migration of DC to T cell areas, and are followed by the loss of most splenic DC 48 h after LPS injection.

The identification of a specific costimulatory signal such as CD28 has focused attention on the functional heterogeneity of APCs. Indeed, optimal activation of naive T cells requires TCR occupancy by antigen/MHC complexes and additional signal(s), such as CD80, CD86, CD40, etc. The expression of CD80 and CD86 varies among the APC populations and seems to correlate with their immunostimulatory properties. DC appear to have some constitutive costimulatory function in several sites in situ, but more typ-

ically, this function rapidly develops upon in vitro culture (26). Steinman's group has defined two populations of splenic DC that differ by their localization and phenotype. DC in the T cell regions express MHC class II and B7-2, and are recognized by M342 and NLDC-145 mAbs. DC in the marginal zone are M342⁻ and resemble freshly isolated DC that become M342⁺ upon culture. These authors suggest that DC from the marginal zone may give rise to M342⁺ cells in T cell areas, and this hypothesis is supported by our data showing that DC migrate from the marginal zone to the T cell area and concomitantly mature into M342⁺, NLDC-145⁺ potent accessory cells that express increased levels of class II, B7-1, and B7-2 molecules. We interpret the redistribution of the N418 marker in the spleen 6 h after LPS injection as a migration of DC from the marginal zone to T cell area, although it could result from the loss of a subset of DC present in peripheral areas in spleens and from the recruitment of a distinct subset in the central white pulp. Our hypothesis, however, is supported by the observation that N418⁺ cells are gradually detected in regions located from the periphery to the center of the white pulp 1.5-4 h after LPS injection (data not shown). The phenotypic changes observed 6 h after LPS injection correlate with increased stimulatory properties, as

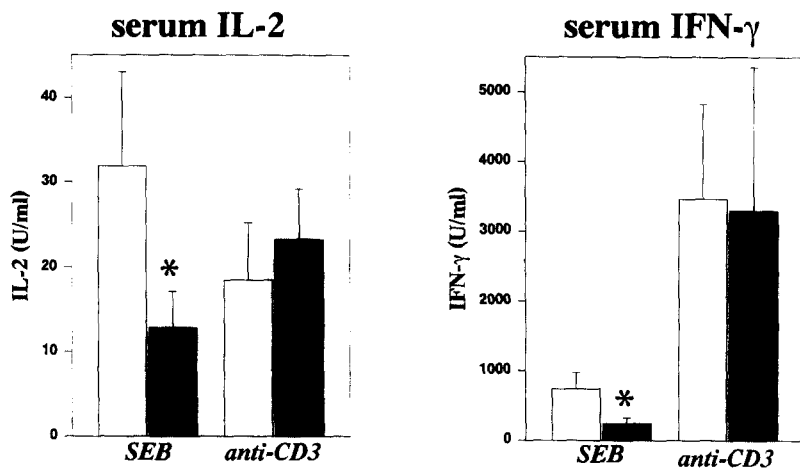


Figure 6. Injection of LPS results in impaired DC function in vivo after 48 h. BALB/c mice injected 48 h previously with NaCl or 25 μ g LPS were stimulated by an intravenous injection of 50 μ g SEB or anti-CD3 mAb. 2 h later, the sera were assayed individually for IL-2 and IFN- γ production, as described in Materials and Methods. Each bar represents the mean \pm SD. Similar results were obtained in three experiments. The groups (*) are significantly different from control (Student's *t* test, $P < 0.05$, $n = 5$). \square , Control; \blacksquare , LPS 2 d.

assessed by enhanced capacity to induce IL-2 secretion by naive T cells in the presence of SEB. By contrast, the capacity of DC to process native protein such as insulin diminishes after LPS injection, as shown by a decreased ability to trigger IL-2 production by an insulin-specific T cell hybridoma. Collectively, these data strongly suggest that immature DC present in the marginal zone mature after migration to T cell area.

It is noteworthy that recent studies (45) have shown that the NLDC-145 mAb recognizes a receptor, termed DEC-205, with a multilectin domain structure that favors efficient capture and presentation of carbohydrate-bearing antigens. The observation that mature DC lose their capacity to process antigens but display increased expression of DEC-205 is intriguing and suggests that DC maintain their capacity to selectively bind carbohydrates. Since these common constituents of microbial cell walls have structures that are distinct from those of carbohydrates of eukaryotic cell surfaces, mature DC may discriminate self from infectious nonself.

The loss of splenic DC 2 d after endotoxin administration probably reflects a physical disappearance of these cells rather than downmodulation of DC-specific markers, as suggested by the loss of several molecules (class II, CD11c, NLDC-145, and M342), the movement of N418⁺ cells early after injection, and the corresponding functional deficiency. In vitro, we showed that the capacity to sensitize naive T cells, a unique property of DC, is impaired in mice that were injected with LPS 2 d earlier. By contrast, spleen cells from treated mice retain the capacity to activate a costimulatory-independent T cell hybridoma, as well as naive T cells in the presence of an anti-CD28 mAb that mimics B7 engagement. In vivo, activation of T cells by DC-dependent T cell mitogen (SEB) is impaired in LPS-treated mice, as assessed by IL-2 and IFN- γ secretion. By contrast, similar levels of both cytokines are released in the sera of mice after the injection of a DC-independent stimulus such as anti-CD3 mAb. These observations strongly suggest that the inability to induce primary responses in vitro and in vivo results from the selective loss of cells from the den-

dritic lineage, which displays some costimulatory function in vivo in the absence of intentional stimulation.

The disappearance of DC could be caused by cell exhaustion, migration out of the spleen, or death (by induction of apoptosis?). Fas ligand and TNF have indeed been found to induce apoptosis in several cell types (46, 47). A similar loss of DC was induced by LPS in mice displaying defective Fas ligand (our unpublished observations), however, suggesting that the induction of programmed cell death via Fas/FasL is not involved in this phenomenon. Alternatively, mature DC could migrate from the T cell area to the liver, where they would be eliminated, or to the celiac nodes, although there is some evidence that DC do not recirculate from blood to lymph (48). It would be of major interest to analyze whether this sequence of events is regulated by DC/T interaction in the presence of antigen. The injection of LPS and the antigen in mice expressing a TCR transgene will help to analyze whether antigen-specific, MHC-restricted interaction between DC and T lymphocytes would lead to reciprocal regulation of function and, in particular, may prevent a rapid loss of DC.

The loss of DC after endotoxin administration could be related to the marked depression of cell-mediated immune functions, often associated with septic shock. Indeed, several lines of evidence suggest that the depression in the patient's immune function induced by traumatic injury underlies the development of infection and/or sepsis (49–51). The analysis of DC phenotype and function in these patients would help to evaluate the role of DC in the pathogenesis of sepsis.

Our data extend previous studies showing that LPS affects DC populations. Groeneveld and co-workers showed that the number of interdigitating cells was severely decreased in the mouse 48 h after LPS administration (52). More recently, MacPherson et al. reported that injection of LPS in rats caused an increased release of intestinal DC into lymph (53). Similarly, Austyn's group showed that injection of LPS induced a loss of DC from the heart, kidneys, and epidermal Langerhans cells (54). By contrast, LPS aerosolized has been shown to recruit DC into the conducting

airways (55). Of note, a recent report indicates that inflammatory cytokines or exogenous bacterial products modulate the cell-surface phenotype and the immunostimulatory function of human cultured DC (56).

Migration patterns of DC in the mouse have been extensively studied. Skin painting mice with picryl chloride resulted in an increase in the number of DC in lymph nodes that initiate primary antigen-specific stimulation *in vitro* and *in vivo* (57). Donor-derived DC have been shown to move from the nonlymphoid organs to the recipient's spleen, where they trigger allograft rejection (20). Labeled mature DC transferred intravenously into a syngeneic host localized in the spleen and the homing was T dependent (58). After subcutaneous footpad inoculation, DC accumulated in the popliteal nodes (48). These data show that DC migrate from nonlymphoid tissues to T cell areas of lymphoid organs, and our results suggest that this flux may correlate with a functional maturation.

A large array of cytokines and/or inflammatory mediators (including TNF- α , IL-1 β , IL-6, IL-12, and GM-CSF) is secreted in mice after LPS injection. We have tested the effect of two cytokines that have been shown to affect DC viability and/or maturation *in vitro* or *in vivo* (59–61). The injection of recombinant TNF- α induced migration of DC to the T cell area, followed by loss of these cells, whereas GM-CSF did not affect the DC population (De Smedt, T., and M. Moser, manuscript in preparation). Simultaneous injection of neutralizing anti-TNF- α mAb and LPS, however, did not prevent DC movement, suggesting that additional factors induced by LPS may promote DC migration *in vivo*. Experiments are in progress to test whether IL-1, which has been shown to induce changes in DC function *in vitro* (62) and promote DC migration *in vivo* (59), is involved in this phenomenon.

A direct effect of endotoxin on DC cannot be excluded, however, since treatment of DC by LPS has been shown to lead to increased expression of IL-1, IL-6, and IL-12 mRNA by these cells (63), as well as to upregulate gene expression and induce release of TNF- α in DC clones (64).

The cell population(s) involved in this phenomenon remain(s) to be identified. The injection of LPS in SCID mice results in similar changes in DC (data not shown),

suggesting that functional T and B lymphocytes are not required for this phenomenon.

LPS endotoxin from Gram-negative bacteria possesses both harmful and beneficial effects. LPS triggers a host immune response resulting in activation of various cell types and production of multiple cytokines that mediate resistance to the bacteria. An exaggerated inflammatory response to bacterial products, however, may cause irreversible tissue damage leading to death when homeostasis is disrupted. Therefore, it is tempting to speculate that the inhibition of DC function in endotoxemia may constitute a feedback aimed at maintaining the homeostasis of the immune system by inhibiting the function of the most efficient APC *in vivo*. Such a mechanism would be beneficial to the host by preventing harmful effects of an exaggerated inflammatory response, as well as to the bacteria, by limiting the pathogen-specific immune response. The selective loss of DC after endotoxin administration may provide some insights in bacterial-host relations, and in particular, suggest that bacteria may derive some evolutionary advantage to retain a wall component that is strongly immunogenic, since high doses of LPS may reduce host resistance to the spread of bacteria.

In conclusion, our data support the hypothesis that a critical signal for the initiation of immune responses is provided by microbial infection, as proposed by Janeway (65). LPS induces costimulatory activity for CD4⁺ T cells on B lymphocytes and macrophages (66), and triggers maturation and migration of DC *in vivo* (this paper). The upregulation of DC function by microbial products and their subsequent movement to T cell areas result in colocalization of T lymphocytes and mature DC that have encountered microbial antigens. Since immature DC have been shown to take up and process antigen, a property that can be lost in mature DC, it is tempting to speculate that most DC in T cell areas would present infectious non-self-antigens. The positive and negative regulation of antigen presentation by microbial agents may help to gain an understanding of how the immune system efficiently eliminates pathogens while avoiding self-reactivity and controls potentially harmful inflammatory responses.

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Address correspondence to M. Moser, Laboratoire de Physiologie Animale, Université Libre de Bruxelles, Rue des Chevaux 67, B-1640 Rhode-Saint-Genèse, Belgium.

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