TLR4 and Toll-IL-1 Receptor Domain-Containing Adapter-Inducing IFN-β, but Not MyD88, Regulate *Escherichia coli*-Induced Dendritic Cell Maturation and Apoptosis In Vivo[1]

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Dendritic cells (DC) are short-lived, professional APCs that play a central role in the generation of adaptive immune responses. Induction of efficient immune responses is dependent on how long DCs survive in the host. Therefore, the regulation of DC apoptosis in vivo during infection remains an important question that requires further investigation. The impact of *Escherichia coli* bacteremia on DCs has never been analyzed. We show here that i.v. or i.p. administration of live or heat-killed *E. coli* in mice induces splenic DC migration, maturation, and apoptosis. We further characterize which TLR and Toll-IL-1R (TIR)-containing adaptor molecules regulate these processes in vivo. In this model, DC maturation is impaired in TLR2$^{-/-}$, TLR4$^{-/-}$ and TIR domain-containing adapter-inducing IFN-β (TRIF)$^{-/-}$ mice. In contrast, DC apoptosis is reduced only in TLR4$^{-/-}$ and TRIF$^{-/-}$ mice. As expected, DC apoptosis induced by the TLR4 ligand LPS is also abolished in these mice. Injection of the TLR9 ligand CpG-oligodeoxynucleotide (synthetic bacterial DNA) induces DC migration and maturation, but only modest DC apoptosis when compared with LPS and *E. coli*. Together, these results suggest that *E. coli* bacteremia directly impacts on DC maturation and survival in vivo through a TLR4-TRIF-dependent signaling pathway. *The Journal of Immunology,* 2005, 175: 839–846.

Dendritic cells (DCs)[3] are professional APCs critical for bridging innate and adaptive immune responses (1). Immature DCs express germline-encoded receptors that recognize pathogen-associated molecular patterns (PAMPs). These receptors allow them to rapidly detect microbial infections. During the past few years, the TLR family has emerged as a major group of signaling receptors for PAMPs (2). Most mammalian species have 10–15 TLRs. TLRs detect multiple PAMPs, including LPS, the major glycolipidic component of Gram-negative bacteria (detected by TLR4), bacterial lipoproteins and lipoteichoic acids (detected by TLR2) and the unmethylated CpG DNA of bacteria and viruses (detected by TLR9). TLRs recruit adaptor molecules that subsequently activate downstream signaling pathways and NF-κB. Four adaptor molecules have been identified: MyD88 (3); Toll-IL-1R (TIR) domain-containing adaptor protein (4); TIR domain-containing adapter-inducing IFN-β (TRIF)/TICAM-1 (TIR domain-containing adaptor-inducing IFN-β) (5); and TRIF-related adapter molecule (TRAM)/TICAM-2 (6). Although the role of the various adaptors in the activation of specific signaling pathways still remains controversial, recent studies suggest that MyD88 and TRIF regulate independent pathways. In this model, TIR domain-containing adaptor protein is implicated in MyD88-dependant signaling, and TRAM (which is highly homologous to TIR) domain-containing adaptor protein) appears to function as a bridge between TLR4 and TRIF (7).

DC activation after TLR activation is characterized by several events: DCs 1) migrate to the T cell area, 2) lose their phagocytic capacity, 3) secrete proinflammatory cytokines, and 4) achieve a mature phenotype. During maturation, DCs increase their ability to present Ags and prime T cells by up-regulating their cell surface MHC II and CD40-, CD80-, and CD86-costimulatory molecules (1). This process is critical for mounting efficient innate immune responses to pathogens as well as for the development of T cell- and B cell-mediated adaptive responses. In addition, it has recently been reported that TLR activation can promote apoptosis of various cell lines (8, 9).

Sepsis is a major health problem (10, 11). During the onset of bacteremia in humans or rodents, massive apoptosis is observed inside lymphoid organs (11). Apoptosis can function as a highly efficient host mechanism to both limit the spread of intracellular pathogens and potentially down-regulate immune responses. In contrast, it is possible that pathogen-mediated induction of apoptosis is a strategy to evade immune responses. Despite the critical importance of DCs in the initiation and regulation of Th1 immune

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Received for publication November 10, 2004. Accepted for publication April 27, 2005.

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[1] This work was supported by grants from the Belgian program on Interuniversity Poles of Attraction (to P.A.I.) initiated by the Belgian State, Prime Minister's Office, Science Policy Programming, the Fonds National de la Recherche Scientifique (Crédit aux chercheurs, Belgium), the Ministère de l'Education Nationale, de la Recherche et de l'Enseignement Supérieur (France), and the Institut National de la Santé et de la Recherche Médicale. M.M. is Senior Research Associated from Fonds National de la Recherche Scientifique. E.M. is Research Associated from Fonds National de la Recherche Scientifique.

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[3] Abbreviations used in this paper: DC, dendritic cell; PAMP, pathogen-associated molecular pattern; TIR, Toll-IL-1R; TRIF, TIR-domain-containing adapter-inducing interferon-β; TRAM, TRIF-related adapter molecule; CpG-ODN, CpG-oligodeoxynucleotide; HK, heat killed; wt, wild type; TICAM, TIR domain-containing adaptor molecule.

0022-1767/05/$02.00

responses against bacteria (12, 13), very little is known on how bacteremia affects DCs in vivo.

Peritonitis continues to be a leading cause of morbidity and mortality (14–16). Although the overall mortality of sepsis is 35% (17), abdominal sepsis is associated with mortality rates of up to 80% (18). Although various bacteria have been identified as causative organisms in peritonitis, *Escherichia coli* belongs to the most common bacteria recovered from peritonitis (up to 60%; Ref. 19) and is the second most frequent bacterium to cause sepsis (up to 20%; Ref. 20).

In this work, we analyze whether *E. coli* bacteremia induce DC migration, maturation, and apoptosis in vivo. We further determine which TLRs and adaptor molecules are required for these effects on DC. Several components of *E. coli* have been reported to impact DCs. LPS induces DC activation (21) and apoptosis (22) in vivo. Bacterial DNA and its synthetic form CpG-oligodeoxynucleotides (CpG-ODN) also induce DC activation in vivo (23). Therefore, we analyzed the ability of *E. coli* bacteremia to induce DC maturation and apoptosis in vivo and compare them with those obtained after LPS and CpG-ODN administrations.

Materials and Methods

Mice and reagents

Female BALB/c and C57BL/6 mice (6–8 wk old) were purchased from Harlan Sprague Dawley. MyD88$^{-/-}$ (3), TLR2$^{-/-}$ (24), and TLR4$^{-/-}$ (25) mice on the BALB/c background and TRIF$^{-/-}$ mice on the C57BL/6 background (Beutler, Scripps Research Institute; Ref. 5) were bred in the specific pathogen-free animal facility of the Institute of Molecular and Cellular Pharmacology, Nice-Sophia Antipolis (Valbonne, France). The maintenance and care of mice complied with the guidelines of the Institut National de la Santé et de la Recherche Médicale/Centre National de Recherche Scientifique/University of Nice-Sophia Antipolis Ethic Committee for the use of laboratory animals. CpG-ODN 1826 was obtained from Coley Pharmaceutical Group. Highly purified LPS (*E. coli* serotype 0111: B4) was purchased from Invivogen. *E. coli* (strain K514) was grown to stationary phase (8–10 h) in Luria-Bertani medium and further washed (2500 × *g,* 10 min, 4°C) three times in PBS before inoculation into mice. Heat-killed (HK) *E. coli* were obtained by a 60-min incubation at 70°C. We checked that the HK bacteria did not grow after inoculation of an overnight culture in Luria-Bertani medium.

In vivo treatment

Mice were given injections i.v. into the lateral tail vein with indicated doses of LPS, CpG-ODN, or *E. coli* in PBS. Control animals were injected with the same volume of PBS.

MLR

Spleens were cut into very small pieces and incubated (37°C, 30 min) into HBSS (Life Technologies) containing 4000 U/ml collagenase IV (Roche) and 0.1 mg/ml DNase I fraction IX (Sigma-Aldrich Chimie SARL). CD4$^+$ T cells from the spleen were purified by negative depletion of CD8α^+, B220$^+$, CD11b$^+$, and MHC II$^+$ cells using sheep anti-rat Ig-coated Dynabeads (Dynal). The indicated numbers of gamma-irradiated (3000 rads) spleen cells were incubated with 3×10^5 allogeneic CD4$^+$ T cells in DMEM supplemented with L-glutamine (2 mM), heat-inactivated FCS (10%), 2-ME (5×10^{-5} M), penicillin (100 μg/ml), and streptomycin (100 U/ml) in U-bottom 96-well plates. Cell proliferation after 48 h of culture was determined by [^{3}H]thymidine incorporation.

Cytofluorometric analysis

Spleen cells were analyzed by flow cytometry with a FACSCalibur cytofluorometer (BD Biosciences). The cells were first preincubated with saturating doses of 2.4G2 (a rat anti-mouse Fc receptor mAb; American Type Culture Collection) for 10 min to prevent Ab binding to Fc receptor and then labeled with PE-coupled N418 (anti-CD11c), APC-coupled 53-6.7 (anti-CD8α), and fluorescein (FITC)-coupled Abs including L3T4 (anti-CD4), 3.23 (anti-CD40), 16–10A1 (anti-CD80), GL1 (anti-CD86), or 2G9 Ab (anti-IA/IE). All mAbs were purchased from BD Biosciences. Cells were gated according to size and scatter to eliminate dead cells and debris from analysis.

Confocal microscopy

Tissues were fixed for 3 h at 4°C in 1% paraformaldehyde (pH 7.0), washed in PBS, incubated overnight at 4°C in a solution of 30% sucrose under agitation, and washed again in PBS. Tissues were embedded in the Tissue-Tek OCT compound (Sakura) and frozen at −80°C, and 10-μm cryostat sections were prepared. Tissues sections were incubated in 100 mM Tris-HCl, pH 8.0, to eliminate OCT and then in a saturated solution containing 10% normal serum in 100 mM Tris-HCl, pH 8.0. Avidin-biotin sites were saturated using an avidin-biotin blocking kit (Vector). Tissues sections were incubated with any of the following mAbs: Alexa Fluor 488 phalloidin (Molecular Probes); APC-coupled N418 (anti-CD11c); GL1 (anti-CD86); 11-26c.2a (anti-IgD); or 30-H12 (anti-CD90.2, Thy-1.2; BD Biosciences) or MOMA-1 (BMA Biomedicals) in 100 mM Tris-HCl, pH 8.0. Uncoupled mAbs were stained with Alexa Fluor 568-coupled goat anti-rat IgG (Molecular Probes). Slides were mounted in DAKO medium (DakoCytomation). Labeled cells were visualized under a Leica laser scanning confocal microscope (TCS-SP) equipped with a DM-IRBE inverted microscope and an argon-krypton laser.

Immunohistochemistry

Immunohistochemical techniques used in this study were previously described (26). Briefly, spleens were fixed in Immunohistofix (Aphase), embedded in Immunohistowax (Aphase), and sectioned at 3–6 μm. Tissues sections were then treated with 1% blocking reagent (Boehringer) to saturate nonspecific binding sites. The endogenous peroxidase activity was neutralized by adding 3% H_2O_2 in PBS for 30 min. The slides were further treated to visualize apoptotic cells using TUNEL (Boehringer, Mannheim) and costaining with alkaline phosphatase substrates (SK-5300, blue precipitate; Vector Laboratories). A blocking kit (Vector Laboratories) was used to saturate the excess of biotin. Slides were incubated with the N418-biotinylated Ab (10 μg/ml in PBS-0.5% blocking reagent), washed, and then incubated with avidin-biotin-peroxidase complexes (Vectastain ABC kit) revealed with a solution of AEC tablets (Sigma-Aldrich). Digitalized images were captured using a charge-coupled device color camera (Ikegami Tsushinki).

Results

Live and HK E. coli *induce DC migration and maturation in vivo*

To model the potential effect of bacteremia on DCs in lymphoid organs, live or HK *E. coli* (5×10^7 bacteria) were injected i.v. into BALB/c mice. This treatment induced the migration of splenic DCs (CD11c^{high} cells) to T cell zones as shown by confocal microscopy on spleen sections from wild-type (wt) BALB/c mice (Fig. 1*A* and data not shown for HK *E. coli*). This result parallels previous findings using LPS (21). DCs migrate from red pulp and marginal zone to T cell-rich areas surrounding the central arteriole within a 60-h time frame. Marginal metallophilic macrophages (MOMA-1$^+$ cells) are localized in the inner part of the marginal zone and form a discontinued ring delineating the border between red pulp and white pulp. DC migration to T cell zones occurs through areas of the marginal zone containing fewer MOMA-1$^+$ cells (Fig. 1, *A* and *B*). Similar migration profiles are observed after i.v. administration of 10^5–10^8 bacteria, as well as with 50 μg of the TLR9 ligand CpG-ODN (data not shown). We also observed that CD11c$^+$ cells, localized in the T cell area, up-regulate the expression of CD86 on inoculation of HK and live *E. coli* and LPS (Fig. 1*C*) as well as with HK and CpG-ODN (data not shown). In addition, high levels of constitutive CD86 were expressed on the CD11c$^-$ MOMA-1$^+$ macrophage population in the marginal zone, as shown previously (27). Similar results with regard to DC migration and maturation were also seen in C57BL/6 mice (data not shown).

To confirm results observed using immunohistochemistry, flow cytometry was used to analyze cell surface expression of MHC II, CD40, CD80, and CD86 on splenic CD11c^{high} cells 9 h after administration of HK and live *E. coli*, LPS (50 μg), and CpG-ODN (50 μg) (Fig. 1*D*). The effects observed on DC maturation were similar whether *E. coli* were administered through the i.p. or i.v. route, and the kinetics was similar to that seen after i.v injection of

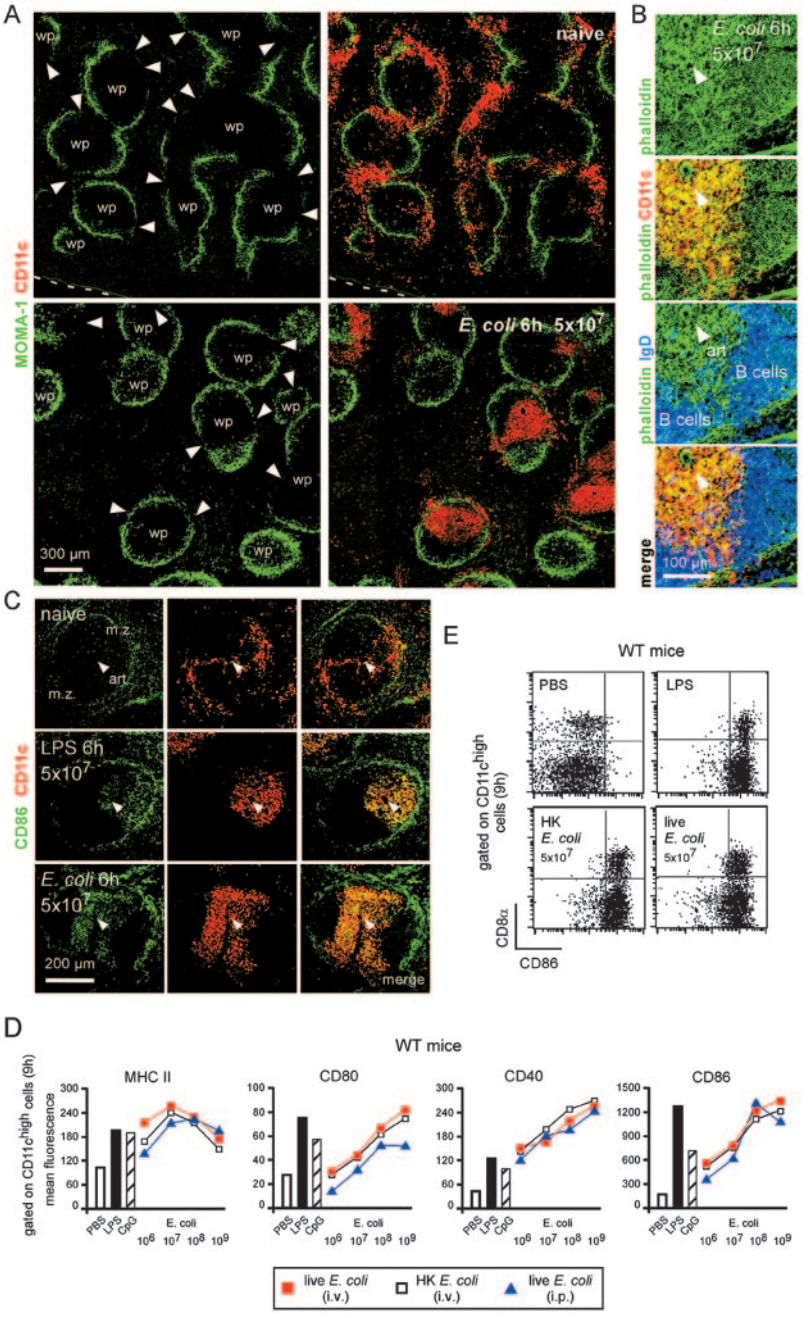

FIGURE 1. *E. coli* induces DC migration and maturation in vivo. BALB/c mice were injected i.v. (or i.p. as indicated) with PBS, LPS (50 μg), CpG-ODN (50 μg), and various numbers of live or HK *E. coli* (as indicated in the figure). Six (*A–C*) and nine (*D* and *E*) hours after treatment, mice were sacrificed, and spleens were harvested. *A—C,* Cryosections of spleens were incubated with Alexa Fluor 488 phalloidin, APC-coupled anti-CD11c mAb, anti-IgD mAb, anti-CD86 mAb, and anti-MOMA-1 mAb, as specified. Uncoupled mAbs were stained with Alexa Fluor 568-coupled goat anti-rat IgG. These images are representative of three independent experiments. *D* and *E*, Pooled spleen cells from three individuals in each group were stained with PE-labeled anti-CD11c, APC-coupled anti-CD8α, and the following FITC-coupled mAbs: anti-CD40; anti-CD80 anti-CD86; and anti-MHC II. These results are representative of four independent experiments. wp, White pulp; mz, marginal zone; art, central arteriole. Arrowheads indicate the localization of marginal zone (*A*) and central arteriole (*B* and *C*).

LPS or CpG with peak expression of maturation markers observed at 6–12 h postinjection. Moreover, all CD11c^{high} DC subsets were affected (Fig. 1*E* for CD86; data not shown for CD40, CD80, and MHC II). Interestingly, because the administration of live and HK *E. coli* induces comparable up-regulation of costimulatory molecules on DCs, this suggests that structural components of *E. coli* are sufficient to induce complete DC maturation.

It has been recently found that commercial LPS preparations are frequently contaminated by lipoprotein and other bacterial components that interact with TLR2 (28). For these reasons, we injected highly purified *E. coli* LPS in all experiments. In addition, to prevent mice mortality after *E. coli* injections, we immunized them with an *E. coli* strain (K514) exhibiting a low virulence. However, short term experiments monitoring DC migration and maturation after injection of a virulent strain (018:K1) gave similar results (data not shown).

E. coli*-induced DC maturation is dependent on TLR2, TLR4, and TRIF in vivo*

To characterize the receptors and the intracellular signaling pathways involved in *E. coli*-induced DC migration and maturation, we inoculated wt, TLR2$^{-/-}$, TLR4$^{-/-}$, MyD88$^{-/-}$, and TRIF$^{-/-}$ mice with 5×10^7 live *E. coli*, LPS (50 μg), and CpG-ODN (50 μg) (Fig. 2). Because DCs from wt BALB/c or C57BL/6 immunized recipients undergo comparable migration and maturation (data not shown), only data obtained with wt BALB/c mice are presented.

Although LPS-induced DC migration is inhibited in TLR4$^{-/-}$ mice (as expected), LPS-activated DCs migrate normally in TRIF$^{-/-}$ or MyD88$^{-/-}$ mice (Fig. 2*A*). This suggests that multiple or distinct intracellular signaling pathways promote DC migration in vivo. Moreover, no differences could be observed after CpG-ODN or *E. coli* injection in all mice tested (data not shown).

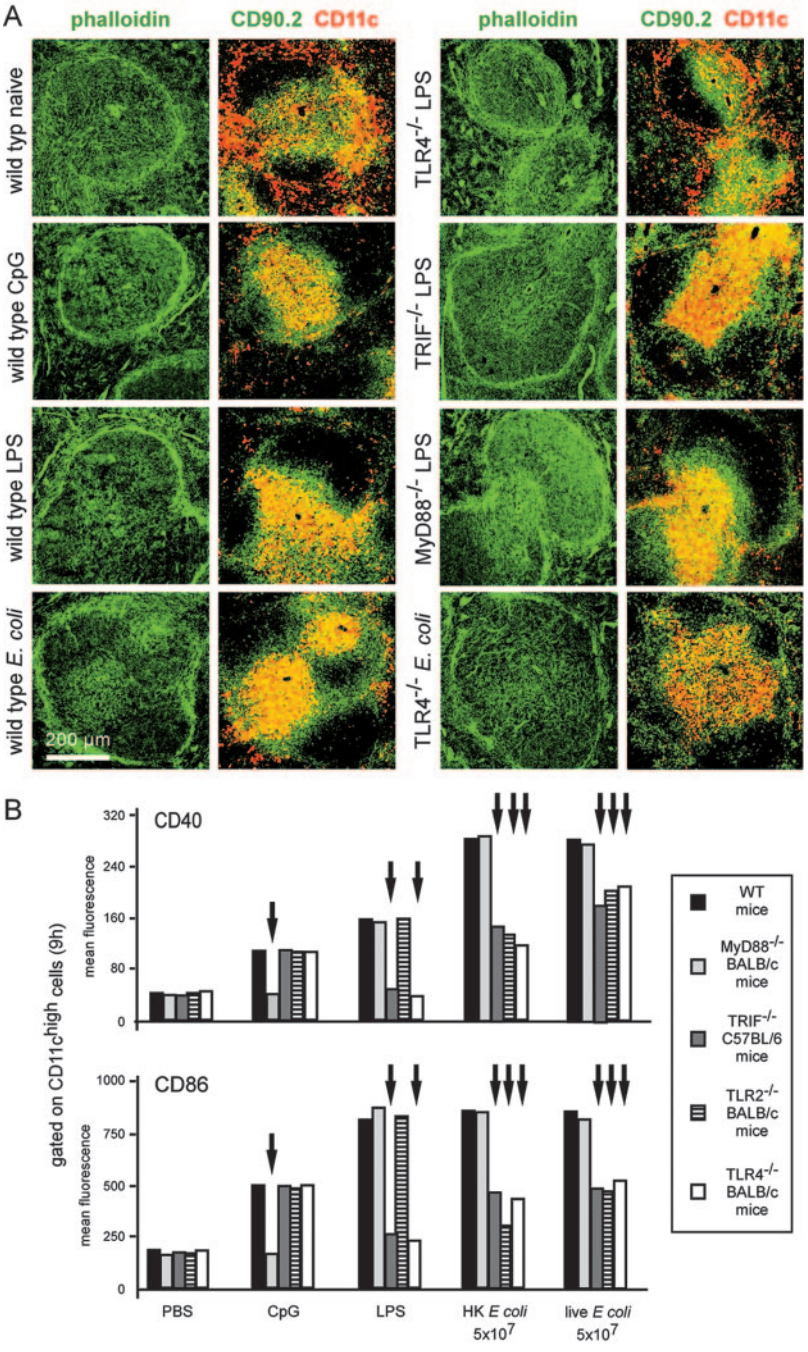

FIGURE 2. *E. coli*-induced DC maturation depends on TLR2, TLR4, and TRIF in vivo. wt, MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ BALB/c mice and TRIF$^{-/-}$ C57BL/6 mice were injected i.v. with PBS, CpG-ODN (50 μg), LPS (50 μg), and live or HK *E. coli*, as specified. Six (*A*) and nine (*B*) hours after treatment, mice were sacrificed, and spleens were harvested. *A*, Cryosections of spleens were incubated with Alexa Fluor 488 phalloidin, APC-coupled anti-CD11c mAb, and anti-CD90.2 (Thy-1.2) mAb. Anti-CD90.2 mAb was stained with Alexa Fluor 568-coupled goat anti-rat IgG. These images are representative of two independent experiments with three mice per group. *B*, Pooled spleen cells from three individuals in each group were stained with PE-labeled anti-CD11c, APC-coupled anti-CD8α, and the FITC-coupled mAbs anti-CD40 and anti-CD86. Data represent the fluorescence of cells gated for high CD11c expression. These results are representative of four independent experiments.

Interestingly, we further found that live *E. coli*-induced DC maturation partially depends on TLR2, TLR4, and TRIF but not MyD88 (Fig. 2*B*), similar results were seen in mice inoculated with HK *E. coli* (data not shown). As controls for these experiments, LPS-induced DC maturation is abolished in TLR4$^{-/-}$ (2) as well as TRIF$^{-/-}$ mice (29) but is intact in MyD88$^{-/-}$ mice (3). Also, CpG-ODN-mediated DC activation is impaired in MyD88$^{-/-}$ mice (2).

It should be mentioned that wt and deficient mice display similar distribution of CD11c^{high} cells in spleen. The ratio of CD11^{+}CD8^{-} vs CD11c^{+}CD8^{+} spleen cells when analyzed by flow cytometry appears conserved. However, we have frequently observed a higher frequency of CD11c^{high} cells in spleen of MyD88$^{-/-}$ mice when compared with wt mice (data not shown).

E, coli *induces splenic DC apoptosis in vivo*

Forty-eight hours after *E. coli* and LPS (50 μg) administration, spleens of recipients exhibited reduced frequencies of CD11c^{high} cells (Fig. 3*A*). This loss correlated with a reduction in the ability of total spleen cells from treated mice to activate allogenic CD4^{+} T cells in vitro (Fig. 3*B*). Using immunohistochemical analysis, we further show that the loss of splenic DCs correlates (20 h after bacterial injection) with the appearance of apoptotic, TUNEL^{+} CD11c^{+} inside T cell zones (Fig. 3*C*). We obtained similar results after LPS injection (data not shown and Ref. 22). Interestingly, CpG-ODN that induced DC migration and maturation at levels similar to those observed with LPS and *E. coli* induced fewer DC to undergo apoptosis (Fig. 3, *A* and *C*) and did not impair the APC ability of spleen cells (Fig. 3*B*).

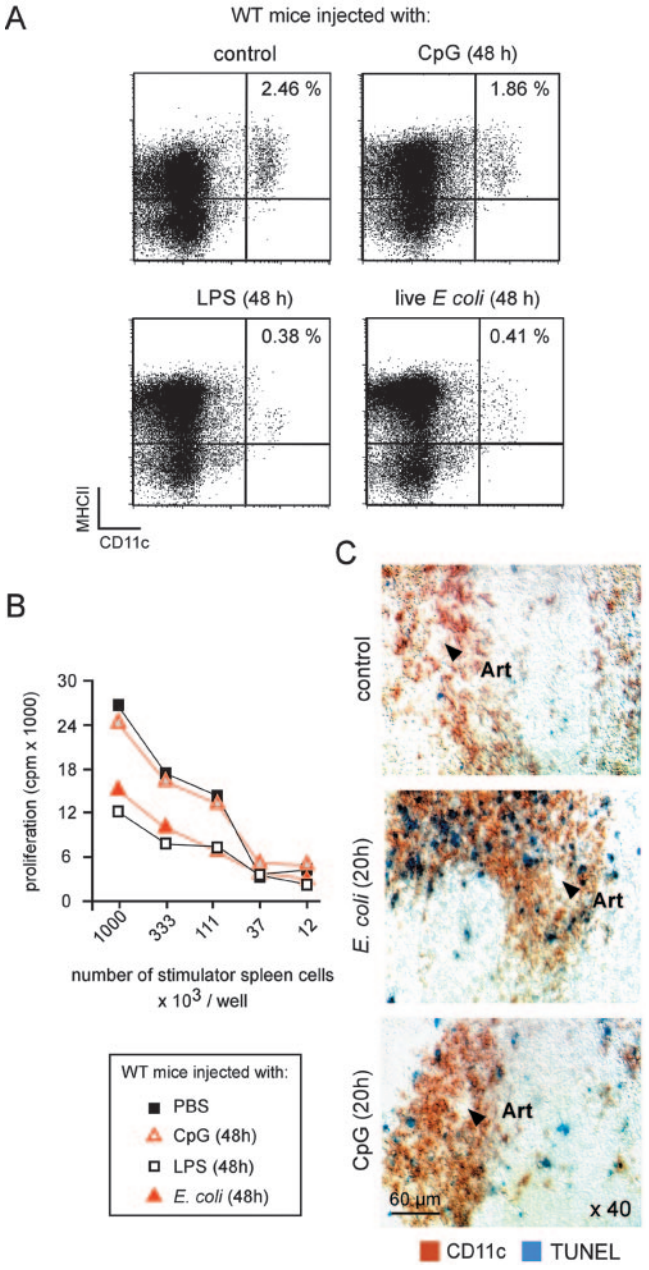

FIGURE 3. *E. coli* induces splenic DC apoptosis in vivo. BALB/c mice were injected i.v. with PBS, LPS (50 μg), CpG-ODN (50 μg), or 5×10^7 live *E. coli*, as specified in the figure. Mice were sacrificed at different time points after injection, and spleens were harvested. *A*, Pooled spleen cells from three individuals in each group were stained with PE-labeled anti-CD11c and with FITC-coupled anti-MHC II. *B*, Spleen cells from 48-h-injected wt BALB/c mice with PBS, LPS, CpG-ODN, or live *E. coli* were irradiated and used as APCs to stimulate 3×10^5 purified $CD4^+$ T cells from C57BL/6 mice. T cell proliferation was assessed after 48 h of culture as described in *Materials and Methods*. Results are expressed as counts per minutes of [^{3}H]thymidine incorporation. These results are representative of two independent experiments. *C*, Alkaline phosphatase (TUNEL, blue) and peroxidase (CD11c, red) staining of embedded spleens sections from PBS or 20-h live *E. coli* and CpG-ODN-injected BALB/c mice. Art, central arteriole. Arrowheads indicate the localization of the central arteriole.

Next, we monitored the kinetics of splenic DC loss and found that splenic DC subsets are differentially affected by *E. coli*, LPS, and CpG-ODN injections (Fig. 4). In all settings, $CD4^-CD8^+CD11c^{high}$ underwent the most significant loss (Fig. 4*B*).

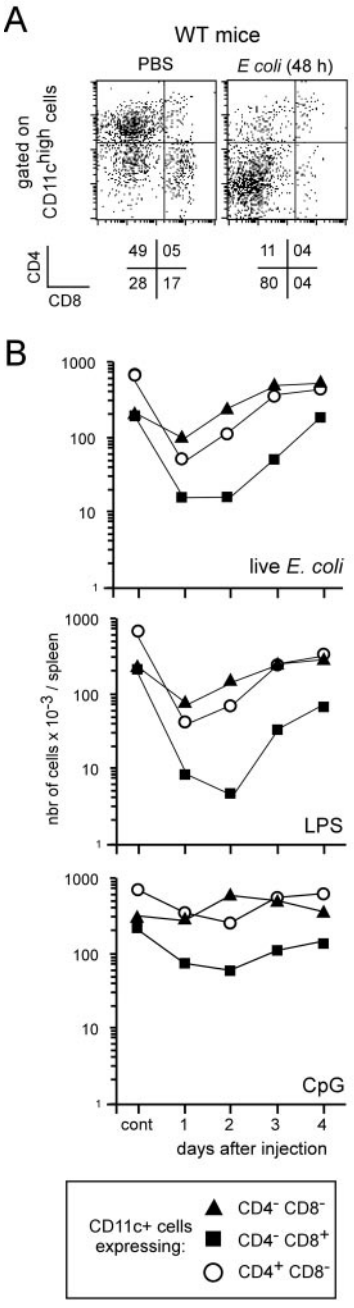

FIGURE 4. *E. coli* induces splenic DC apoptosis in vivo. BALB/c mice were injected i.v. with PBS, LPS (50 μg), CpG-ODN (50 μg), or 5×10^7 live *E. coli*, as specified in the figure. Mice were sacrificed at different time points after injection, and spleens were harvested. Pooled spleen cells from three individuals in each group were stained with PE-labeled anti-CD11c, APC-coupled anti-CD8α, and with FITC-coupled anti-CD4.

E. coli-*induced splenic DC apoptosis is reduced in TLR4$^{-/-}$ and TRIF$^{-/-}$ mice*

To determine whether TLR2, TLR4, MyD88, or TRIF is involved in DC apoptosis, wt mice or mice bearing a targeted deletion for each of these molecules were injected with LPS (50 μg), CpG-ODN (50 μg), or live *E. coli* (5×10^7). DC numbers were monitored by flow cytometry (Fig. 5, *A* and *C*) and confocal microscopy (Fig. 5, *B* and *D*).

As expected from the results shown in Fig. 2, LPS-induced DC loss was prevented in TLR4$^{-/-}$ mice and reduced in MyD88$^{-/-}$ and TRIF$^{-/-}$ mice (Fig. 5, *A* and *B*). LPS-mediated DC maturation

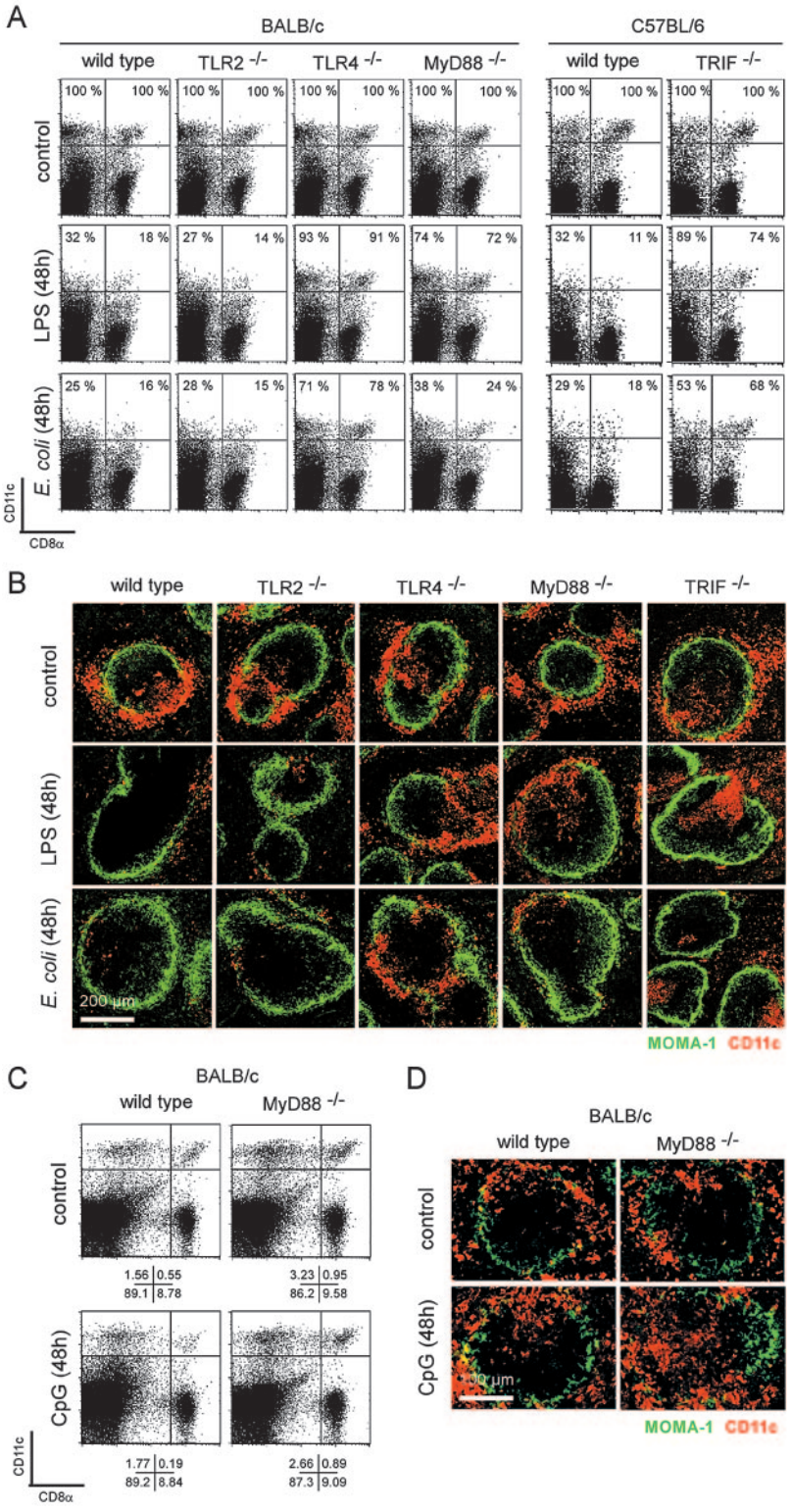

FIGURE 5. *E. coli*-induced splenic DC apoptosis is reduced in TLR4$^{-/-}$ and TRIF$^{-/-}$ mice. wt, MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$ BALB/c mice and wt, TRIF$^{-/-}$ C57BL/6 mice were injected i.v. with PBS, LPS (50 μg), CpG-ODN (50 μg), or live *E. coli* (5×10^7), as specified. Mice were sacrificed 48 h after treatment, and spleens were harvested. *A* and *C*, Spleen cells from three mice per group were pooled and stained with anti-CD11c-PE and anti-CD8α-APC (for *A*, numbers shown on dot plots represent the percentages of CD11c$^+$CD8α^- cells and CD11c$^+$CD8α^+ cells among spleen cells from LPS- and *E. coli*-injected mice as compared with control mice of the same genetic background). These results are representative of four independent experiments. *B* and *D*, Cryosections of spleens were incubated with APC-coupled anti-CD11c mAb and anti-MOMA-1 mAb visualized with Alexa Fluor 568-coupled goat anti-rat IgG. These images are representative of two independent experiments with three mice per group.

was not inhibited in MyD88$^{-/-}$ mice (Fig. 2*B*), indicating that distinct signaling pathways regulate LPS-mediated DC maturation and apoptosis. Furthermore, we found that *E. coli*-induced DC apoptosis is strongly reduced in TLR4$^{-/-}$ as well as TRIF$^{-/-}$ mice but not in TLR2$^{-/-}$ or MyD88$^{-/-}$ mice (Fig. 5, *A* and *B*). Finally, limited loss of DC was found after CpG-ODN stimulation; this modest reduction was totally abrogated in MyD88$^{-/-}$ mice (Fig. 5, *C* and *D*).

Discussion

Systemic LPS administration has been used in previous studies to visualize DC maturation and apoptosis in vivo (21, 22). However, to our knowledge, these data have never been validated in a more physiological model, such as a bacterial infection. In this study, we demonstrate that *E. coli* bacteremia induces DC migration, maturation, and apoptosis with magnitudes and kinetics similar to that of LPS in vivo. Although all DC subsets mature in response to *E.*

coli or LPS, apoptosis preferentially affects CD8α^+ DCs that are described to preferentially induce Th1 responses (12, 13). It is conceivable that CD8α^+ DCs are more sensitive to apoptosis because they express higher levels of TLR4 (30). Along similar lines, preferential apoptosis of CD8α^+ DCs has recently been described in lymph nodes during polymicrobial sepsis (31). Thus, DC apoptosis can be a general consequence of bacterial infection. In the model of polymicrobial sepsis (31), DC maturation is not observed before DC apoptosis.

As a possible consequence of DC apoptosis, we observed that *E. coli* bacteremia decreased the ability of total spleen cells to stimulate naive T cells during MLR. It is possible that the DC apoptosis that we observed could be a contributing factor to the immunosuppression associated with sepsis (32, 33). In agreement with this idea, reduction in DC numbers has been correlated in several systems with a reduced ability to mount T cell responses in vivo (13, 21). Moreover, DC loss has been associated with a decreased ability of recipient mice to produce systemic IL-12 in response to LPS injection (34). This suggests, perhaps not surprisingly, that massive DC apoptosis can specifically impair antibacterial Th1 responses.

The impact of TLRs, TRIF, and MyD88 in *E. coli*, LPS, and CpG-ODN-induced DC maturation and apoptosis is summarized in Table I. *E. coli*-induced DC maturation is reduced in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice, demonstrating that both TLRs are important PAMP receptors for this pathogen. It also suggests that TLR2 and TLR4 participate in detection and control of *E. coli* infection. The requirement for TRIF in LPS-induced DC maturation has been described previously (29), but the role of TRIFs in *E. coli* bacteremia-induced DC maturation is a novel observation. This suggests a possible role for TRIF in the development of adaptive immune response against bacteria.

Although LPS-mediated DC activation requires signals emanating from TLR4 and the downstream binding of TIRAP, TRAM, TRIF, and MyD88 adaptor proteins (2), little has been described regarding the roles of these receptors and signaling molecules involved in LPS-induced DC apoptosis in vivo. However, it is known that the death receptor Fas is not involved in this process (21). We show here that LPS-induced splenic DC apoptosis in vivo depends on TLR4, TRIF, and MyD88 adaptor proteins. In addition, we demonstrate that the TLR4-mediated TRIF-dependent signaling pathway contributes to the control of DC apoptosis during *E. coli* bacteremia. Several recent in vitro studies using macrophages, bone marrow-derived DCs, and other cell lines show a direct role of TLR4 (8, 9), MyD88 (35), and TRIF (9) on apoptosis. Another in vitro study using TLR3 ligands suggests that TRIF regulates three distinct signaling pathways leading to the induction of the IFN-β promoter, NF-κB activation, and apoptosis via a FADD/caspase-8-dependent pathway (36).

CpG-ODN injection promoted DC migration and maturation but induced less DC apoptosis compared with LPS and *E. coli*; this was seen by several different assays (Figs. 3*A*, 5*C*, and 5*D*). CpG-ODN induced DC apoptosis is completely abrogated by MyD88 deficiency. Our in vivo results are in agreement with a recent work (37) showing that in vitro stimulation with LPS- but not CpG-induced apoptosis of bone marrow-derived DC. This study concluded that the ability of CpG to up-regulate Bcl-2, a potent anti-apoptotic molecule, counteracted the induction of programmed cell death of bone marrow-derived DC. Thus, the weak ability of CpG-ODN to induced DC apoptosis suggests a limited implication of bacterial DNA in *E. coli*-induced DC apoptosis and may explain its important role as a potent adjuvant in vivo.

Taken together, our results provide new insight into how Gram-negative bacteremia modulates DCs function and survival. Coevolution between bacteria and host immune systems has selected the emergence of PAMPs receptors to initiate innate immunity to these pathogens and control infection. However, during septicemia, bacteria subvert this detection system and induce massive DC apoptosis. This phenomenon most likely impairs or reduces the initiation and/or development of adaptive immune responses.

Table I.

	Migration	*Maturation*	*Apoptosis*
CpG			
Wild-type mice	+++	+++	+
TLR2$^{-/-}$ mice	+++	+++	+
TLR4$^{-/-}$ mice	+++	+++	+
TRIF$^{-/-}$ mice	+++	+++	+
MyD88$^{-/-}$ mice	+++	−	−
LPS			
Wild-type mice	+++	+++	+++
TLR2$^{-/-}$ mice	+++	+++	+++
TLR4$^{-/-}$ mice	+	−	+
TRIF$^{-/-}$ mice	+++	−	+
MyD88$^{-/-}$ mice	+++	+++	++
Live *E. coli*			
Wild-type mice	+++	+++	+++
TLR2$^{-/-}$ mice	+++	+	+++
TLR4$^{-/-}$ mice	+++	+	++
TRIF$^{-/-}$ mice	+++	+	++
MyD88$^{-/-}$ mice	+++	+++	+++

Acknowledgments

We thank Drs. P. M. Dubois and C. A. Benedict for criticism of this manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245–252.
2. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nat. Rev. Immunol.* 4: 499–511.
3. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11: 115–122.
4. Fitzgerald, K. A., E. M. Palsson-McDermott, A. G. Bowie, C. A. Jefferies, A. S. Mansell, G. Brady, E. Brint, A. Dunne, P. Gray, M. T. Harte, et al. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature* 413: 78–83.
5. Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S. O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, K. Crozat, S. Sovath, J. Han, and B. Beutler. 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 424: 743–748.
6. Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda, and S. Akira. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat. Immunol.* 4: 1144–1150.
7. Palsson-McDermott, E. M., and O'Neill, L. A. 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 113: 153–162.
8. Haase, R., C. J. Kirschning, A. Sing, P. Schrottner, K. Fukase, S. Kusumoto, H. Wagner, J. Heesemann, and K. Ruckdeschel. 2003. A dominant role of Toll-like receptor 4 in the signaling of apoptosis in bacteria-faced macrophages. *J. Immunol.* 171: 4294–4303.
9. Ruckdeschel, K., G. Pfaffinger, R. Haase, A. Sing, H. Weighardt, G. Hacker, B. Holzmann, and J. Heesemann. 2004. Signaling of apoptosis through TLRs critically involves Toll/IL-1 receptor domain-containing adapter inducing IFN-β, but not MyD88, in bacteria-infected murine macrophages. *J. Immunol.* 173: 3320–3328.
10. Angus, D. C., and R. S. Wax. 2001. Epidemiology of sepsis: an update. *Crit. Care Med.* 29: S109—S116.
11. Oberholzer, A., C. Oberholzer, R. M. Minter, and L. L. Moldawer. 2001. Considering immunomodulatory therapies in the septic patient: should apoptosis be a potential therapeutic target? *Immunol. Lett.* 75: 221–224.
12. Maldonado-Lopez, R., and M. Moser. 2001. Dendritic cell subsets and the regulation of Th1/Th2 responses. *Semin. Immunol.* 13: 275–282.
13. Jung, S., D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, et al. 2002. In vivo depletion of CD11c$^+$ dendritic

cells abrogates priming of CD8$^+$ T cells by exogenous cell-associated antigens. *Immunity* 17: 211–220.
14. Bosscha, K., K. Reijnders, P. F. Hulstaert, A. Algra, and C. van der Werken. 1997. Prognostic scoring systems to predict outcome in peritonitis and intraabdominal sepsis. *Br. J. Surg.* 84: 1532–1534.
15. Ohmann, C., Q. Yang, T. Hau, and H. Wacha. 1997. Prognostic modelling in peritonitis: Peritonitis Study Group of the Infection Society Europe. *Eur. J. Surg.* 163: 53–60.
16. Christou, N. V., P. S. Barie, E. P. Dellinger, J. P. Waymack, and H. H. Stone. 1993. Surgical Infection Society intra-abdominal infection study: prospective evaluation of management techniques and outcome. *Arch. Surg.* 128: 193–199.
17. Wheeler, A. P., and G. R. Bernard. 1999. Treating patients with severe sepsis. *N. Engl. J. Med.* 340: 207–214.
18. Holzheimer, R. G., K. H. Muhrer, N. L'Allemand, T. Schmidt, and K. Henneking. 1991. Intraabdominal infections: classification, mortality, scoring and pathophysiology. *Infection.* 19: 447–452.
19. Lorber, B., and R. M. Swenson. 1975. The bacteriology of intra-abdominal infections. *Surg. Clin. North Am.* 55: 1349–1354.
20. Krcmery, V., S. Spanik, M. Mrazova, J. Trupl, S. Grausova, E. Grey, E. Kukuckova, M. Sulcova, I. Krupova, and P. Koren. 2002. Bacteremias caused by *Escherichia coli* in cancer patients: analysis of 65 episodes. *Int. J. Infect. Dis.* 6: 69–73.
21. De Smedt, T., B. Pajak, E. Muraille, L. Lespagnard, E. Heinen, P. De Baetselier, J. Urbain, O. Leo, and M. Moser. 1996. Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J. Exp. Med.* 184: 1413–1424.
22. De Smedt, T., B. Pajak, G. G. Klaus, R. J. Noelle, J. Urbain, O. Leo, and M. Moser. 1998. Antigen-specific T lymphocytes regulate lipopolysaccharide-induced apoptosis of dendritic cells in vivo. *J. Immunol.* 161: 4476–4479.
23. Jakob, T., Walker, P. S., Krieg, A. M., Udey, M. C, and Jonathan, C. V. 1998. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* 161: 3042–3049.
24. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity* 11: 443–451.
25. Hoshino, K, O. Takeuchi., T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J. Immunol.* 162: 3749–3752.
26. Pajak, B., T. De Smedt, V. Moulin, C. De Trez, R. Maldonado-Lopez, G. Vansanten, E. Briend, J. Urbain, O. Leo, and M. Moser. 2000. Immunohistowax processing, a new fixation and embedding method for light microscopy, which preserves antigen immunoreactivity and morphological structures: visualisation of dendritic cells in peripheral organs. *J. Clin. Pathol.* 53: 518–524.
27. Muraille, E., C. De Trez, B. Pajak, M. Brait, J. Urbain, and O. Leo. 2002. T cell-dependent maturation of dendritic cells in response to bacterial superantigens. *J. Immunol.* 168: 4352–4360.
28. Hirschfeld, M., Y. Ma, J.H. Weis, S. N. Vogel, and J. J. Weis. 2000. Repurification of lipopolysaccharide eliminates signaling through both human and murine Toll-like receptor 2. *J. Immunol.* 165: 618–622.
29. Hoebel, K., E. M. Janssen, S. O. Kim, L. Alexopoulou, R. A. Flavell, J. Han, and B. Beutler. 2003. Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by TRIF-dependent and TRIF-independent pathways. *Nat. Immunol.* 4: 1223–1229.
30. Edwards, A. D., S. S. Diebold, E. M. Slack, H. Tomizawa, H. Hemmi, T. Kaisho, S. Akira, and C. Reis e Sousa. 2003. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8α^+ DC correlates with unresponsiveness to imidazoquinolines. *Eur. J. Immunol.* 33: 827–833.
31. Efron, P. A., A. Martins, D. Minnich, K. Tinsley, R. Ungaro, F. R. Bahjat, R. Hotchkiss, M. Clare-Salzler, and L. L. Moldawer. 2004. Characterization of the systemic loss of dendritic cells in murine lymph nodes during polymicrobial sepsis. *J. Immunol.* 173: 3035–3043.
32. Volk, H. D., P. Reinke, and W. D. Docke. 2000. Clinical aspects: from systemic inflammation to "immunoparalysis." *Chem. Immunol.* 74: 162–177.
33. Efron, P., and L. L. Moldawer. 2003. Sepsis and the dendritic cell. *Shock* 20: 386–401.
34. Wysocka, M., S. Robertson, H. Riemann, J. Caamano, C. Hunter, A. Mackiewicz, L. J. Montaner, G. Trinchieri, and C. L. Karp. 2001. IL-12 suppression during experimental endotoxin tolerance: dendritic cell loss and macrophage hyporesponsiveness. *J. Immunol.* 166: 7504–7513.
35. Colino, J., and C. M. Snapper. 2003. Two distinct mechanisms for induction of dendritic cell apoptosis in response to intact *Streptococcus pneumoniae. J. Immunol.* 171: 2354–2365.
36. Han, K. J., X. Su, L. G. Xu, L. H. Bin, J. Zhang, and H. B. Shu. 2004. Mechanisms of the TRIF-induced interferon-stimulated response element and NF-κB activation and apoptosis pathways. *J. Biol. Chem.* 279: 15652–15661.
37. Hou, W.-S., and Van Parijs, L. 2004. A Bcl-2-dependent molecular timer regulates the lifespan and immunogenicity of dendritic cells. *Nat. Immunol.* 5: 583–589.