TLR9-Dependent Activation of Dendritic Cells by DNA from *Leishmania major* Favors Th1 Cell Development and the Resolution of Lesions[1]

Faihaa Hkima Abou Fakher, Nicolas Rachinel, Martine Klimczak, Jacques Louis, and Noëlle Doyen[2]

In its vertebrate host, *Leishmania* encounters cells that express TLRs. Using genetically resistant C57BL/6 mice deficient in either TLR2, 4, or 9, we show in this study that only TLR9-deficient mice are more susceptible to infection with *Leishmania major*. TLR9-deficient mice resolved their lesions and controlled parasites growth with much lower efficiency than wild-type C57BL/6 mice. The absence of TLR9 also transiently inhibited the development of curative Th1 response. In an attempt to analyze the possible basis for such aberrant response in TLR9$^{-/-}$ mice, we have studied the importance of TLR9 for the activation of dendritic cells (DCs) by *L. major*. Results show that DCs in the draining lymph nodes are activated following infection with *L. major*. Furthermore, bone marrow-derived DCs as well as DCs freshly isolated from the spleen of C57BL/6 mice can be activated by either heat-killed or live *L. major* in vitro. In sharp contrast, *L. major* failed to activate DCs from TLR9$^{-/-}$ mice. Noteworthily, activation of DCs was abolished either following treatment of the parasites with DNase or after acidification of the endosomal compartment of DCs by chloroquine, pinpointing the DNA of *L. major* as the possible ligand of TLR9 leading to the activation of DCs. Results showed that DNA purified from *L. major* was indeed capable of activating DCs in a strictly TLR9-dependent manner. Moreover we showed that the *L. major* DNA-induced TLR9 signaling in DCs condition these cells to promote IFN-γ production by CD4$^+$ T cells. *The Journal of Immunology,* 2009, 182: 1386–1396.

The immunological control of infection with *Leishmania major* in mice has been shown to depend upon Th1 CD4$^+$ T cells and the IFN-γ they produce. IL-12, a cytokine produced largely by APCs such as dendritic cells (DCs)[3] and macrophages, contributes to immunity against *L. major* by both inducing and maintaining IFN-γ-producing Th1 cell responses (1, 2). DCs appear to be the main source of IL-12 in response to *Leishmania* parasites (3, 4). To date, the greatly enhanced susceptibility of MyD88-deficient mice to infection with *L. major* (5, 6) is the strongest evidence for a role of the TLR/IL-1R signaling pathway in host resistance to this pathogen. In addition to their loss of resistance to *L. major*, MyD88$^{-/-}$ mice display impaired proinflammatory cytokine synthesis in response to infection that was correlated with decreased NO synthase 2 expression and reduction in the synthesis of IL-12 and TNF-α by macrophages and diminished IFN-γ synthesis by CD4$^+$ T cells (7).

Numerous studies indicate that the capacity of DCs to produce IL-12 is directly conditioned by the recognition of pathogen products, and TLRs are the best characterized molecules involved in such recognition (8, 9). However, possibly because mice deficient for each of the known TLRs have not yet been systematically screened, the enhanced susceptibility of MyD88$^{-/-}$ mice to *L. major* has not yet been ascribed to any individual TLR/IL-1R family member. Although the possibility remains that one yet to be identified TLR accounts for the MyD88-mediated resistance to *L. major*, an alternative hypothesis to be considered would be that several TLRs act in concert in determining the control of the pathogen.

Only few reports have documented the recognition of molecules from *Leishmania* by a particular TLR. The binding of lipophosphoglycan from *Leishmania* by TLR2 has been described (10). Lipophosphoglycan from *L. major* has also been shown to induce the up-regulation of TLR2 on human NK cells and to activate these cells, resulting in increased destruction of the parasites (11). Nevertheless, results from our laboratory do not support a role for TLR2 in resistance to infection with *L. major* in mice because TLR2-deficient mice were as resistant as wild-type C57BL/6 mice (N. Doyen, unpublished results). In contrast, TLR4 has been shown to contribute to an efficient control of parasite growth during the innate phase of the immune response to *L. major*. Furthermore, no evidence for direct interaction between *L. major*-derived molecules and TLR4 could be obtained (12).

More recently it has been shown that the early NK cell response to infection with *Leishmania donovani* was dependent upon the expression of IL-12 by myeloid DCs (mDCs), which required TLR9 (13). Moreover, in this study a TLR9-dependent activation of plasmacytoid DCs (pDCs) by *L. donovani* was also observed but

Unité des Réponses Précoces aux Parasites et Immunopathologie, Département de Parasitologie et Mycologie, Institut Pasteur, Paris, France

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[2] Address correspondence and reprint requests to Dr. Noëlle Doyen, Institut Pasteur, Department of Parasitology and Mycology, Unité des Réponses Précoces aux Parasites et immunophysiopathologie, 28 Rue du Docteur Roux, 75015 Paris, France, E-mail address: ndoyen@pasteur.fr

[3] Abbreviations used in this paper: DC, dendritic cell; BM, bone marrow; BMDC, BM-derived DC; DC1, Th1-inducing DC; HPRT, hypoxanthine phosphoribosyltransferase; mDC, myeloid DC; ODN, oligodeoxynucleotide; pDC, plasmacytoid DC; poly:IC, polyinosinic-polycitidylic acid.

was not required for IL-12 production and NK cell activation. The absence of detectable parasites in pDCs was contrasting with the ability of mDCs to internalize parasites, suggesting distinct mechanisms of TLR9-dependent activation between these two types of DCs that could also have been elicited by separated ligands from the parasites.

Activation of pDCs or natural IFN-producing cells by several viruses, such as the HSV1 and HSV2 β-herpesviruses and the murine cytomegalovirus, has been shown to require TLR9 (14, 15). Evidence also exists for a role of TLR9 in the manifestation of innate immunity against Gram-negative bacteria in a model of pneumonia (16) and for resistance to infection with *Mycobacterium tuberculosis* in mice (17). These effects of TLR9 signaling appear to be mediated by mDCs or pDCs, the later primarily expressing TLR9, and to a lesser extent by macrophages and B cells. In addition to *Leishmania*, some results suggest a role for TLR9 in the triggering of innate immune response to protozoan parasites. For example, TLR9 is required for the development of the Th1-type inflammatory responses that follows oral infection with *Toxoplasma gondii* in mice from some inbred strains (18) and is also implicated in the control of parasitemia during infection with *Trypanosoma cruzi* (19). The hemozoin pigment of *Plasmodium* or some parasite DNA associated with it results in signaling through TLR9 (20, 21).

During the course of studies aimed at assessing the importance of various TLRs in the resistance to *L. major* characterizing genetically resistant C57BL/6 mice, we observed that mice deficient in TLR9 exhibit an enhanced susceptibility to *L. major* even though they ultimately healed their lesions. This increased susceptibility was paralleled by a transient Th2 cell maturation. DCs could be at the basis of this aberrant phenotype because their activation by *L. major* was not observed in the absence of TLR9. Interestingly, DNA from *L. major* was identified as the specific ligand responsible for the parasite-induced TLR9-dependent activation of DCs.

Materials and Methods

Mice, reagent, and parasite

Six to 8-wk-old female C57BL/6 mice were purchased from Charles River Laboratories. MyD88$^{-/-}$ (22) and TLR9$^{-/-}$ (23), backcrossed to the C57BL/6 background for 10 generations, were provided by S. Akira (Osaka University, Osaka, Japan). OT-II mice, transgenic for the TCR recognizing the I-A^b restricted peptide corresponding to the residues 323–339 of OVA peptide (24), were bred on a C57BL/6 background. All mice were bred in our facilities and housed under specific pathogen-free-conditions. All animal experiments were conducted according to guidance from institutional committees for animal care.

Promastigotes of *L. major* LV39 were grown from amastigotes isolated from skin lesions of nude mice and propagated in vitro in M199 medium supplemented by 10% of FCS. Killed or live *L. major* promastigotes were in stationary phase; the killed *L. major* promastigotes were heat killed at 56°C for 30 min. LPS and polyinosinic-polycytidylic acid (poly:IC) were purchased from Sigma-Aldrich. Synthetic OVA$_{323–339}$ (ISQAVHAAH AEINEAGR) peptide from Neosystem (Strasbourg, France). CpG oligodeoxynucleotide (ODN) 1826; TCCATGACGTTCCTGACGTT) from Sigma-Proligo. Genomic DNA from mammalian kidney or ovary and from *L. major* parasite strain LV39 were prepared by proteinase K digestion followed by phenol/chloroform extractions and ethanol precipitation.

Inoculation with L. major*, lesion monitoring, and parasite burden*

For infection, mice were inoculated s.c. into the footpad with 3×10^6 stationary phase *L. major* promastigotes. The size of the resulting lesions was assessed weekly by measuring the footpad swelling with a metric caliper (Kroeplin) and comparing it with the footpad thickness before infection. Each week after infection, the tissue parasite burden was detected by limiting dilution analysis (25). Moreover, C57BL/6 mice were inoculated i.v. with 3×10^6 stationary phase *L. major* promastigotes. Spleens were harvested 8 days postinoculation.

Generation of bone marrow (BM)-derived DCs (BMDCs)

BM cells were isolated by flushing femurs and tibia with PBS. After treatment with RBC lysis buffer (Sigma-Aldrich), BM cells were cultured in complete RPMI 1640 supplemented with GM-CSF (26) from the J558L cell line supernatant. Expanded BM culture at day 8 contained 76–80% CD11c$^+$CD11b$^+$ cells.

Purification of draining lymph node DCs, splenic pDCs, and conventional mDCs

DCs were isolated from lymph nodes of 8–12 naive or *L. major*-infected mice at various times after infection. Lymph nodes were digested with collagenase type 4 (0.5 mg/ml) and DNase type I (40 μg/ml) (Boehringer-Mannheim) for 30 min, and then a positive selection was performed using an anti-CD11c Ab coupled with magnetic beads (Miltenyi Biotec). After selection, the cell suspension contained 70 to 90% CD11c$^+$ cells.

Splenic pDCs (CD11c^{low} B220$^+$) and mDCs (CD11c^{high}B220$^-$) were isolated from C57BL/6 or TLR9$^{-/-}$ mice treated with B16 FLT3L cells producing FLT3 ligand given by Dr. M. Moser (Université Libre de Bruxelles, Gosselies, Belgium). After collagenase type 4 (0.5 mg/ml) (Worthington) treatment and RBC lysis, total spleens cells were depleted for B and T cells with anti-CD19 and anti-CD3 Abs, respectively. pDCs were enriched from these depleted spleen cells by positive selection on microbeads (allophycocyanin; Miltenyi Biotec) after staining with anti-B220-allophycocyanin Abs. mDCs retained in the effluent were purified using an anti-CD11c Ab coupled with magnetic beads (Miltenyi Biotec).

Stimulation of DCs

BMDCs, spleen pDCs, or mDCs were cultured in 6-well plates (3×10^6/ml) at 37°C and 5% CO_2 using completed RPMI 1640. Cells were activated for 4–12 h with killed *L. major* or live *L. major* promastigotes in parasites/DCs at a ratio of 5:1, *L. major* genomic DNA (80, 20, or 2 μg/ml), 20 μg/ml CpG ODN 1826, 1 μg/ml LPS, 25 μg/ml poly:IC, or mammalian DNA (80, 20, or 2 μg/ml). In some experiments, killed parasites were digested with DNase at 1 mg/ml for 30 min at 37°C before their addition to DCs. In some experiments, cells were treated with chloroquine at 20 μM for 1 h before activation. Cells were harvested for RNA extraction.

FACS analysis

For surface phenotyping and cell sorting, the following Abs from BD Pharmingen were used: FcγIII/II receptor (2.4G2), anti-CD11c-allophycocyanin or -PE (HL3), anti-CD40-FITC (3/23), anti-CD86-FITC (GL2), anti-CD3-allophycocyanin (145-2CII), anti-CD4-allophycocyanin or -PerCP (RM4-5), anti-CD19-allophycocyanin (ID3), anti-CD45R/B2200-FITC or -allophycocyanin (RA3-6B2), and anti-CD11b-allophycocyanin (M1/70). For intracellular staining, anti-IFN-γ-allophycocyanin (XMG1.2) and rat IgG1κ-allophycocyanin (R3-34) isotype were used. Anti-mPDCA-1 mAb was from Miltenyi Biotec. All data were acquired on a four-color FACSCalibur cytometer (BD Biosciences) and analysis was done with Cell Quest Pro software.

Real-time RT-PCR

RNA was extracted from DCs or from lymph nodes using a MicroRNeasy extraction kit (Qiagen). A trace of genomic DNA was removed with an RNase free-DNase set. RNA (2 μg) was reverse transcribed using (200 U) Moloney murine leukemia virus reverse transcriptase (SuperScript II; Invitrogen). Subsequent real-time PCR was performed on an ABI PRISM 7000 sequence detector (Applied Biosystems) using *Taq* polymerase (TaqMan Universal master mix; Applied Biosystems/Roche), 20 ng of cDNA as template, forward and reverse primers, and the FAM-labeled probe for the target gene. The mRNA expression levels were normalized to the hypoxanthine phosphoribosyltransferase (HPRT) gene and calculated as the *n*-fold difference of the expression in activated cells compared with its naive counterpart.

Percentage of CD4$^+$ T cells producing IFN-γ in draining lymph nodes

Single cell suspensions were prepared from draining popliteal lymph nodes of four C57BL/6 and TLR9-deficient mice obtained 48 h and 17 and 20 days after infection with 3×10^6 stationary phase *L. major* promastigotes in each footpad. Lymph node cells (3×10^6 cells/ml) were cultured with $1.5–3 \times 10^7$ heat-killed *L. major* promastigotes for 24 h and then incubated for another 4 h with (10 μg/ml) brefeldin A (Sigma-Aldrich). After CD4

staining of the cells, intracellular IFN-γ staining was performed using a BD Cytofix/Cytoperm kit (BD Biosciences) and anti-IFN-γ Abs.

Statistical analysis

Statistical analysis was performed with Student's *t* test. Quantitative data are expressed as mean ± SD unless otherwise stated.

T cell proliferation assay

Spleen of naive transgenic mice (OT-II) was enriched in T cells by depletion of $CD19^+$ B cells and $CD11c^+$ and $CD11b^+$ cells. $CD4^+$ T cells were purified by positive selection with anti-CD4-allophycocyanin and anti-allophycocyanin microbeads (Miltenyi Biotec). Harvested cell suspension contained 95% $CD4^+$ T cells. BMDCs (10^4) from C57BL/6 and $TLR9^{-/-}$ mice were stimulated with killed *L. major* promastigotes (five parasites per cell), *L. major* DNA at 10 μg/ml, CpG at 1 μg/m1, and LPS at 10 ng/ml and pulsed with 0.5 μg of $OVA_{323–339}$ for 6 h (triplicate cultures). After washing, the activated BMDCs were cocultured with $CD4^+$ T cells (10^5) for 72 h in a 96-well tissue culture plate (200 μl/well).

$CD4^+$T cells from mice i.v injected with *L. major* were purified as described above. BMDCs (10^4) from C57BL/6 and $TLR9^{-/-}$ mice were stimulated in vitro for 6 h with killed *L. major* promastigotes (five parasites per cell) as source of Ag. After washing, T cells (10^5) were cocultured with stimulated BMDCs for 96 h. All cocultures were pulsed for 16 h with 1 μCi of [H^3]thymidine.

For both experiments, pulsed cells were harvested on a glass filter and analyzed using a liquid scintillation beta counter. Supernatants were collected for IFN-γ titration by ELISA from BD Biosciences.

Results

The expression of a resistant phenotype and the profile of the cytokine response to infection with L. major *are influenced by TLR9*

To study the importance of TLR9 signaling on the development of cutaneous lesions following infection with *L. major*, the footpad lesions were compared over time between TLR9-deficient and wild-type C57BL/6 mice infected s.c. with 3×10^6 *L. major* promastigotes. $TLR9^{-/-}$ mice developed significantly larger lesions than C57BL/6 mice. The lesions in TLR9-deficient mice were still important at day 42 after infection, whereas healing was complete in C57BL/6 mice and only began in $TLR9^{-/-}$ mice (S1.A online supplemental Fig. 1*A*).[4] Furthermore, although 14 days after the inoculation of *L. major* the footpad lesions of $TLR9^{-/-}$ and C57BL/6 mice contained similar numbers of viable parasites, from day 21 after infection significantly larger numbers of parasites were observed in lesions of $TLR9^{-/-}$ mice. For example, at day 28 after infection the numbers of viable parasites in lesions from $TLR9^{-/-}$ mice were four orders of magnitude higher than those seen in lesions of wild-type C57BL/6 mice. At day 42 after infection, the parasite load started to decrease in $TLR9^{-/-}$ mice (S1.B online supplemental Fig. 1*B*). Similar differences were observed in the numbers of viable parasites in draining lymph nodes between $TLR9^{-/-}$ and wild-type mice (data not shown). These results confirm those obtained by Liese et al. (27) and clearly show that TLR9-deficient mice resolved their lesions and controlled parasite growth with much lower efficacy than wild-type mice.

It is now well known that BALB/c mice develop an aberrant Th2 response after infection with *L. major* that underlies their susceptibility and that resistant mice mount a polarized Th1 response that underlies their ability to resolve their lesions (2, 28). Therefore, we investigated whether or not the relative susceptibility to *L. major* of TLR9-deficient mice was correlated with an alteration of the Th response characteristic of the otherwise resistant C57BL/6 mice. Therefore, the expression of mRNA specific for Th1-associated (IFN-γ and IL-12) and Th2-associated (IL-4 and IL-10) cytokines in the draining lymph nodes was compared between $TLR9^{-/-}$ and wild-type C57BL/6 mice following infection with *L. major*. Analysis of the kinetics of IFN-γ mRNA expression during the course of infection in TLR9-deficient and C57BL/6 mice revealed a significant increase in the expression of this cytokine, reaching maximal levels at the time of the peak in lesion development (Fig. 1*A*). Noticeably however, the levels of IFN-γ transcripts in lymph nodes of TLR9-deficient mice were drastically lower than those of wild-type mice at the time of maximal lesion development. Determining the number of IFN-γ-producing $CD4^+$ T cells by intracellular staining for IFN-γ confirmed the results obtained by measuring mRNA levels because, compared with wild-type C57BL/6 mice, the number of IFN-γ-producing $CD4^+$ T cells was significantly lower in the draining lymph nodes of

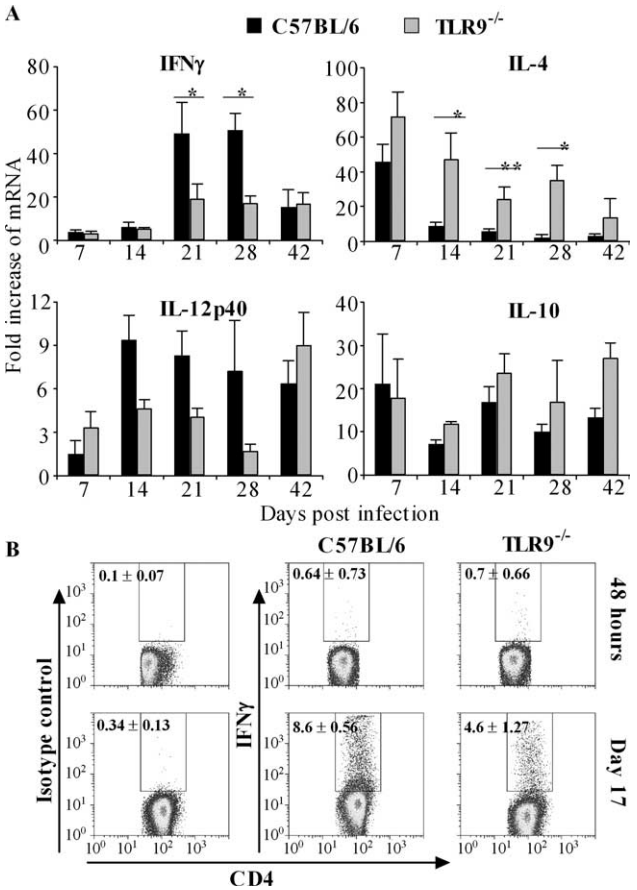

FIGURE 1. Cytokine mRNA expression in draining lymph nodes from C57BL/6 and $TLR9^{-/-}$ mice infected with *L. major*. Every 7 days postinfection, total mRNA from the draining lymph nodes of four mice per group was extracted. *A*, IFN-γ, IL-4, IL-12p40, and IL-10 mRNA were determined by real-time PCR and normalized to the constitutively expressed HPRT gene. Levels of mRNA expression were calculated as the fold difference with the expression in lymph node cells of noninfected mice from the corresponding strain. The data represent the mean and SD of the relative expression in four mice. Significant difference between C57BL/6 and $TLR9^{-/-}$ was indicated as follows: *, $p < 0.05$; **, $p < 0.01$. Data are representative of two independent experiments. *B*, Intracellular IFN-γ production by $CD4^+$ T cells was determined by flow cytometry after gating lymphocyte $CD4^+$ T cells. Briefly, $CD4^+$ T cells were harvested from popliteal lymph nodes of infected C57BL/6 and $TLR9^{-/-}$ mice 48 h and day 17 postinfection by *L. major*. Control group corresponds to $CD4^+$ T cells from C57BL/6 or $TLR9^{-/-}$ labeled with the isotype-matched control for IFN-γ (R3-34). The presented dot plots are from one of three independent experiments with similar results. Numbers in the dot plots represent the mean percentage ± SD of IFN-γ-producing $CD4^+$ T cells in the three independent experiments. Significant difference ($p < 0,05$) between C57BL/6 and $TLR9^{-/-}$ has been observed at day 17 post infection.

[4] The online version of this article contains supplemental material.

TLR9$^{-/-}$deficient mice infected with *L. major*. Results presented in Fig. 1*B* show that 17 days after infection there are three times more IFN-γ-producing CD4$^+$ cells in C57BL/6 (9,23%) than in TLR9$^{-/-}$ (3,33%) mice. The presented FACS dot plots are from one of three independent experiments that gave similar results. The numbers represent the mean percentage ± SD of IFN-γ-producing CD4$^+$ T cells from the three independent experiments.

From the second week of infection the expression of IL-12 mRNA, a cytokine favoring Th1 cell maturation, was lower in TLR9$^{-/-}$ mice than in wild-type mice (Fig. 1*A*). Although a similar increase in IL-4 mRNA expression was seen in mice from both strains 1 wk after infection, from day 14 after infection significantly higher levels of IL-4 mRNA expression were observed in draining lymph nodes of TLR9$^{-/-}$ mice (Fig. 1*A*). A similar increase in IL-10 transcripts was seen in mice from both strains (Fig. 1*A*) Thus, it appears that infection with *L. major* in TLR9-deficient mice favor, at least transiently, the maturation of Th2 responses inhibits the development of a curative Th1 response.

Reduced maturation of DCs in draining lymph nodes of TLR9-deficient mice infected with L. major

On the one hand DCs are specialized in the initiation of adaptive T cells response (29, 30) and the development of T cell subsets, and on the other hand DCs express a broad range of TLRs including TLR9. Thus, the possibility existed that the observed effect of the absence of TLR9 on the quality of the T cell response to infection with *L. major* resulted from an inability of *L. major* to induce the functional maturation of DCs in the absence of TLR9 signaling.

To investigate the capacity of infection with *L. major* to induce maturation of DCs in vivo, the expression of CD40, CD80, and CD86 was measured by flow cytometry on DCs purified from draining lymph nodes of C57BL/6 and TLR9$^{-/-}$ mice 24, 48, and 17 days after inoculation with 3×10^6 parasites in each footpad. Results from one representative experiment in Fig. 2*A* illustrate an up-regulation of the expression of the costimulatory molecule CD80, which persists at 48 h on DCs from wild-type mice, and a significantly lower up-regulation of CD80 on TLR9$^{-/-}$ DCs. Fig. 2*B*, showing the mean fluorescence intensity of DCs expressing CD40, CD80 and CD86. illustrates quantitatively the reduced up-regulation of CD40 and CD86 in lymph node DCs from TLR9$^{-/-}$ mice infected with *L. major*. These results suggest that the TLR9 deficiency partially impaired or delayed DCs maturation. Similar numbers of DCs in lymph nodes of wild-type and TLR9-deficient mice indicate that the migration of DCs is not impaired in TLR9$^{-/-}$ mice (data not shown). These results are in agreement with previous observations by using MyD88$^{-/-}$ mice infected with *Leishmania* (31).

Activation by L. major *of dendritic cells derived in vitro from BM progenitors requires TLR9*

We also studied the importance of TLR9 for the activation of DCs by *L. major* in vitro using BMDCs that can be obtained in large quantities. BMDCs from either C57BL/6 or TLR9$^{-/-}$ mice were exposed to either heat-killed or live *L. major* promastigotes in vitro. After various times of culture, the expression of costimulatory molecules and the production of selected cytokines by DCs were determined. Results clearly show that incubation of wild-type BMDCs with both heat-killed and live *L. major* promastigotes resulted in an up-regulation of CD40 and CD86 (Fig. 3) and CD80 (data not shown). In sharp contrast, BMDCs from TLR9$^{-/-}$ mice did not exhibit up-regulation of costimulatory molecules following incubation with *L. major*. As shown in Fig. 4*A*, stimulation with either killed or live *L. major* promastigotes induced the generation

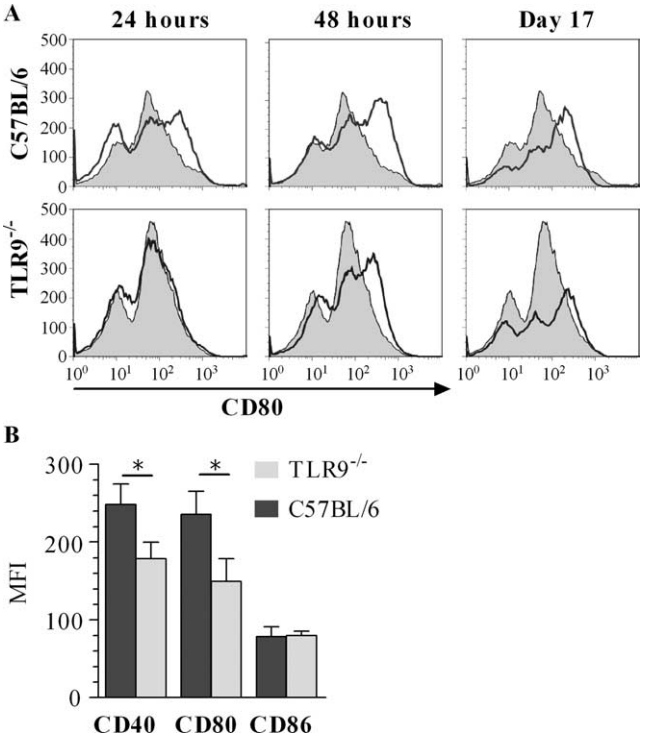

FIGURE 2. Costimulatory molecule expression on freshly isolated popliteal dendritic cells from C57BL/6 or TLR9$^{-/-}$ mice. DCs have been isolated after popliteal lymph node (obtained from eight mice per group) digestion by collagenase and a positive selection with an anti-CD11c Ab coupled with magnetic beads. *A*, Flow histogram showing cell surface expression of CD80 on CD11c$^+$ cells harvested at various time points following infection with 3×10^6 *L. major* promastigotes per footpad. Filled and open histograms represent the staining of DCs from naive and *L. major*-infected mice, respectively. *B*, Comparison of the mean fluorescence intensity (MFI) obtained following labeling of the costimulatory molecules CD40, CD80, and CD86 between C57BL/6 and TLR9$^{-/-}$ DCs purified from the draining lymph nodes 24 h after infection. Bars represent the MFI in three independent experiments. Statistical analysis (*t* test) has been performed (∗, $p < 0.05$).

of IL-12p40, IL-6 and IFN-β transcripts in BMDCs from C57BL/6 mice but not in BMDCs from TLR9$^{-/-}$ mice. To determine whether or not the observed lack of expression of costimulatory molecules and of cytokine production by BMDCs from TLR9-deficient mice in response to *L. major* was the result of a state of unresponsiveness of these cells to any activating stimulus, we tested the capacity of these cells to become activated by stimuli not requiring TLR9 expression. This hypothesis was negated by results in Fig. 4*B* showing that BMDCs from TLR9$^{-/-}$ mice and C57BL/6 mice were similarly activated by poly:IC and LPS, ligands of TLR3 and TLR4 respectively but not by CpG, a ligand of TLR9 (see also Fig. 3).

The above results demonstrating the TLR9 dependence of the capacity of *L. major* to induce maturation of BMDCs combined with the well-known nature of the ligand interacting with TLR9 prompted us to assess whether or not DNA from *L. major* could be the ligand interacting with TLR9. Furthermore, observations by others had shown that the DNA from other Trypanosomatidae, i.e., *Trypanosoma brucei* and *T. cruzi* and *Babesia bovis*, stimulates cytokine production by APCs and proliferation of B cells in a TLR9-dependent manner (19, 32). The results depicted in Figs. 3 and 4*C* support this hypothesis because they reveal that DNA purified from *L. major* induced a significant activation of BMDCs from C57BL/6 but had no effect on TLR9-deficient BMDCs.

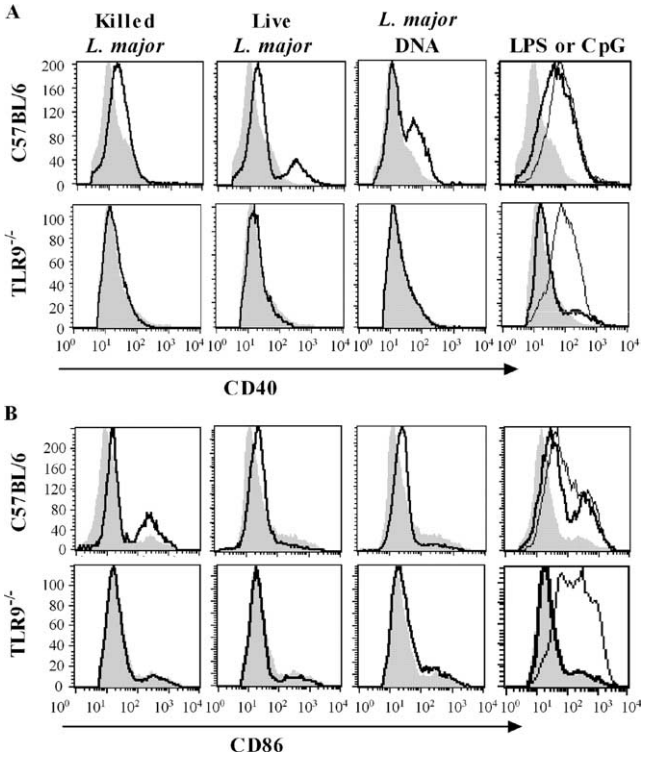

FIGURE 3. Surface expression of costimulatory molecules on BMDCs from C57BL/6 and TLR9$^{-/-}$ mice following activation. Flow histograms show cell surface expression of CD40 (*A*) and CD86 (*B*) on BMDCs stimulated with killed or live *L. major* promastigotes or *L. major* DNA, as well as CpG and LPS as control ligands. Cells were harvested 24 h after stimulation and analyzed by FACS. Filled and open histograms represent the staining of the unstimulated and stimulated BMDCs, respectively; only LPS is represented with a thin line. Histograms are from one of three independent experiments with three distinct isolates of BMDC.

Additional support for this hypothesis is provided by results showing that treatment with DNase greatly impaired the capacity of killed *L. major* promastigotes to activate wild-type BMDCs and that treatment of BMDCs with chloroquine impaired their activation by *L. major* DNA (Fig. 4*D*). Noteworthily, DNA isolated from different mammalian organs was unable to induce maturation of wild-type BMDCs (Fig. 4*E*), suggesting that the observed activation was specific for *L. major* DNA and not the consequence of recognition of self-DNA possibly present in the cultures.

Activation of distinct subsets of splenic dendritic cells by L. major *also requires TLR9*

The next set of experiments was designed to assess the capacity of DCs freshly isolated from lymphoid organs to become activated by *L. major* and to determine the TLR9 dependence of this activation. Thus, two different populations of DCs purified from the spleen of mice treated with FLT3 ligand, resulting in the expansion of DCs in vivo, were next tested for their capacity to become activated by *L. major*.

Eighty-five percent of cells in the first subpopulation were pDCs (CD11c^{low}B220^{+}; 52% of these cells expressed PDCA-1 (murine pDC Ag-1)). Eighty percent of the cells in the second subpopulation were mDCs (CD11c^{high}B220^{-}; 53% of these cells were CD11b^{-} and 26% expressed CD11b). Cells from these two populations, i.e., pDCs and mDCs, obtained from either C57BL/6 or TLR9$^{-/-}$ mice, were exposed to either killed or live *L. major* promastigotes or parasite DNA and, after 24 h, analyzed for the expression of the costimulatory surface molecules CD86 or CD40.

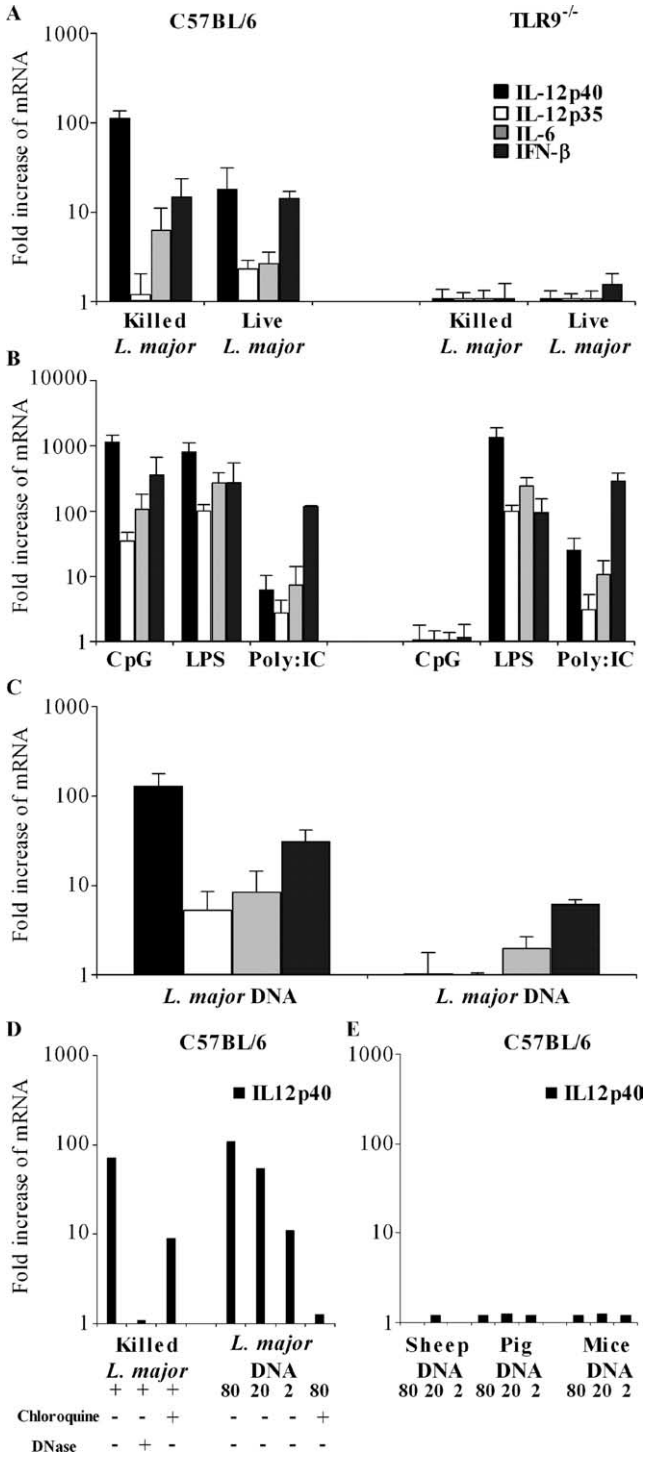

FIGURE 4. Cytokine mRNA expression in C57BL/6 and TLR9$^{-/-}$ BMDCs. *A* and *B*, C57BL/6 and TLR9$^{-/-}$ BMDCs were stimulated in vitro for 6 h with either killed or live *L. major* promastigotes (*A*) or CpG, LPS or poly:IC (*B*). *C*, *L. major* DNA (80 μg/ml). *D* and *E*, C57BL/6 BMDCs were stimulated with either killed *L. major* promastigotes *or L. major* DNA (*D*) or with sheep, pig, or mice DNA (*E*). In the indicated cultures the stimuli were treated with DNase or the BMDCs were treated with chloroquine before stimulation. Expression of the indicated cytokines was determined by real time RT-PCR. The mRNA expression levels were normalized to the expression of the HPRT gene and calculated as the *n*-fold difference with the expression in similarly cultured but not stimulated BMDCs. The results are expressed as the mean and SD of the calculated relative expression of four independent experiments (*A–C*) and two separate experiments (*D* and *E*).

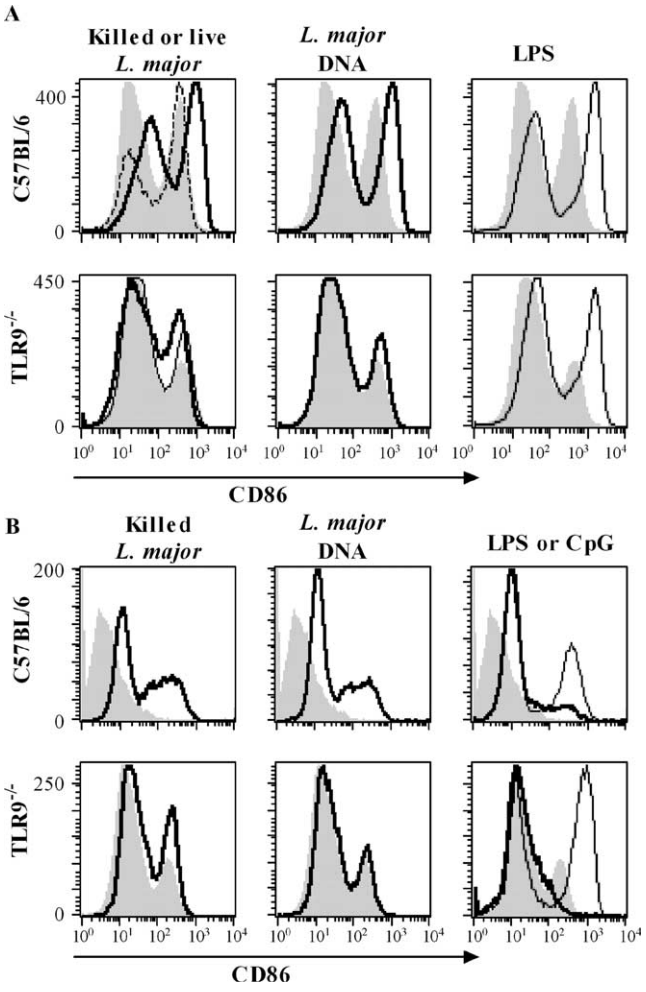

FIGURE 5. Expression of costimulatory CD86 molecules on C57BL/6 and TLR9$^{-/-}$ DCs freshly isolated from the spleen. pDCs (*A*) and mDCs (*B*) were harvested following 24 h of exposure in vitro to killed or live *L. major* promastigotes, *L. major* DNA, CpG, or LPS and analyzed by FACS. Filled and open histograms represent the staining of the unstimulated and stimulated DCs, respectively. Expression of CD86 following stimulation with live *L. major* (*A*) is represented by a dashed line and LPS (*A* and *B*) by a thin line. Results are from one of three independent experiments using three different isolates of splenic DCs that gave similar profiles.

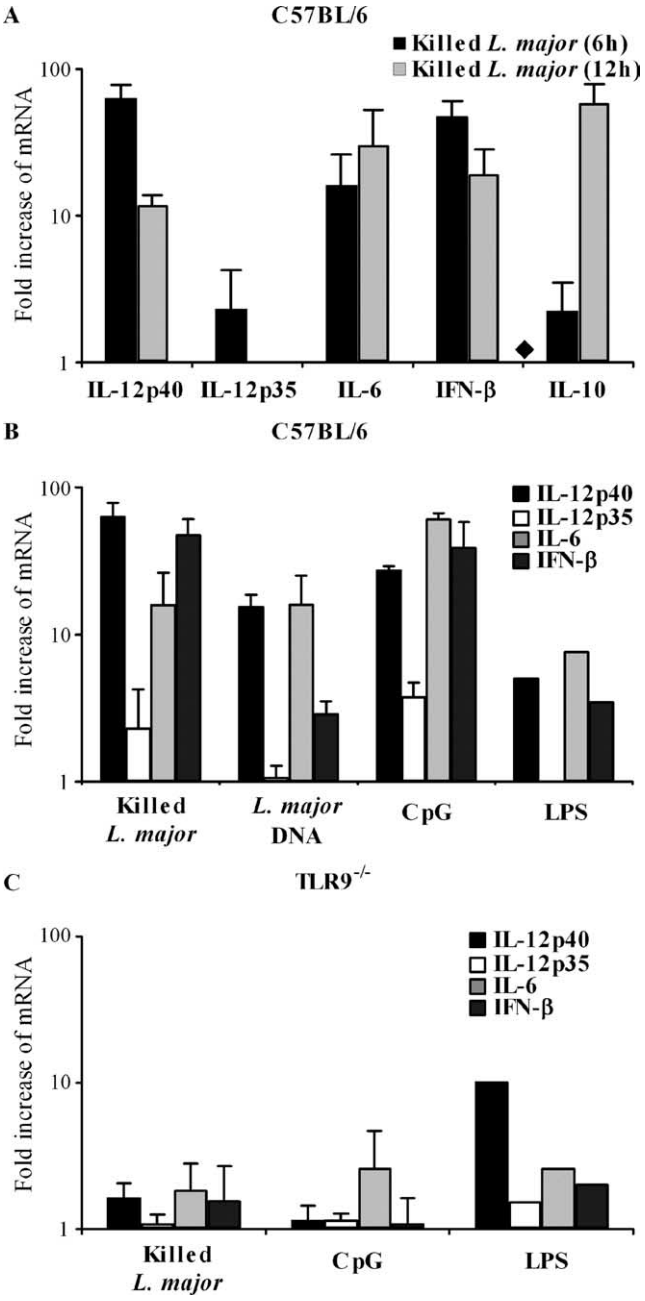

FIGURE 6. Analysis of cytokine mRNA expression in response to stimulation with *L. major* in C57BL/6 and TLR9$^{-/-}$ spleen pDCs. *A* and *B*, C57BL/6 pDCs were stimulated in vitro with killed *L. major* promastigotes for 6 or 12 h (*A*) or killed *L. major* promstigotes, *L. major* DNA (80 μg/ml), CpG, or LPS during 6 h (*B*). *C*, pDCs purified from TLR9$^{-/-}$ mice were stimulated with the indicated stimuli for 6 h. Expression of mRNA for the indicated cytokines was determined by real-time RT-PCR as described in Fig. 4. The results represent the means and SD of the calculated relative expression of three independent experiments.

As shown in Fig. 5*A*, killed and live *L. major* promastigotes up-regulated the expression of CD86 and CD40 (data not shown) by C57BL/6 but not TLR9$^{-/-}$ pDCs. Noteworthily, live parasites were less potent in activating pDCs from C57BL/6 mice than killed *L. major* parasites. Parasite DNA also up-regulated the expression of CD86 on pDCs from C57BL/6 mice but not on TLR9$^{-/-}$ pDCs. As expected, wild-type and TLR9$^{-/-}$ pDCs were similarly activated by LPS. As shown in Fig. 5*B*, the expression of the costimulatory molecules CD86 and CD40 (data not shown) by mDCs also increased in response to stimulation with killed parasites, *L. major* DNA, CpG, and LPS. In contrast, although responding well to stimulation by LPS, mDCs from TLR9$^{-/-}$ mice failed to up-regulate the expression of costimulatory molecules following stimulation by either *L. major* parasite DNA or CpG. Thus, both mDCs and pDCs can be activated by the DNA from *L. major*, and this activation appears slickly TLR9 dependent.

We have also analyzed and compared the cytokine expression by pDCs and mDCs purified from the spleen of FLT3-treated C57BL/6 and TLR9$^{-/-}$ mice in response to stimulation with *L. major* promastigotes (killed or live) or parasite DNA. Kinetics analysis of the expression of IL-12p40 and IFN-β mRNA in pDCs in response to these stimuli revealed higher levels of transcripts at 6 h compared with 12 h (Fig. 6*A*). In contrast, higher levels of IL-6 and IL-10 transcripts were seen 12 h after activation, suggesting that the expression of these cytokines is differentially controlled. The increased levels of IL-12p40 and IL-6 transcripts after stimulation with CpG were of the same order of magnitude as those seen in response to stimulation with *L. major* and parasite DNA. A lower level of IFN-β expression was observed after stimulation

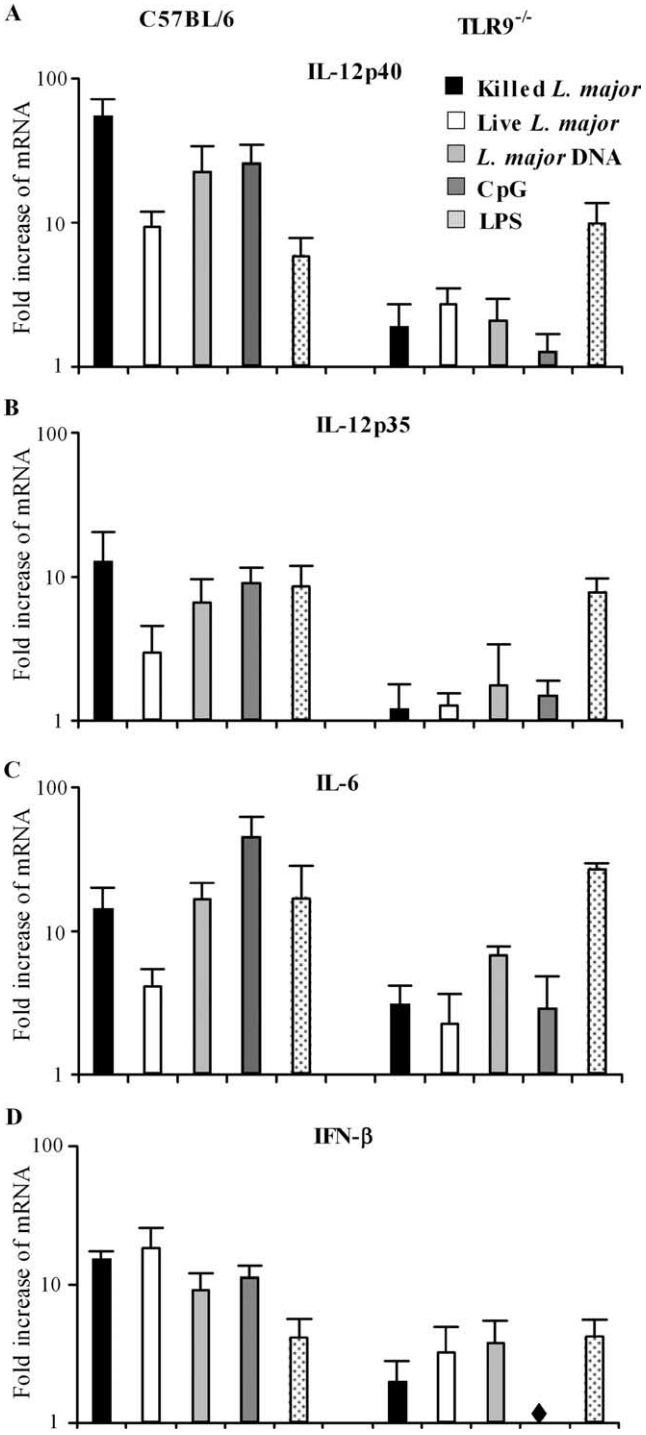

FIGURE 7. Analysis of cytokines mRNA expression in response to stimulation with *L. major* in C57BL/6 and TLR9$^{-/-}$ spleen mDCs. mDCs were stimulated in vitro for 6 h by killed or live *L. major* promastigotes, *L. major* DNA (80 μg/ml), CpG, or LPS. Cytokine mRNA expression for IL-12p40 (*A*), IL-12p35 (*B*), IL-6 (*C*), and IFN-β (*D*) were determined by real-time RT-PCR as described in Fig. 4. The result represent the means and SD of the calculated relative expression of three independent experiments.

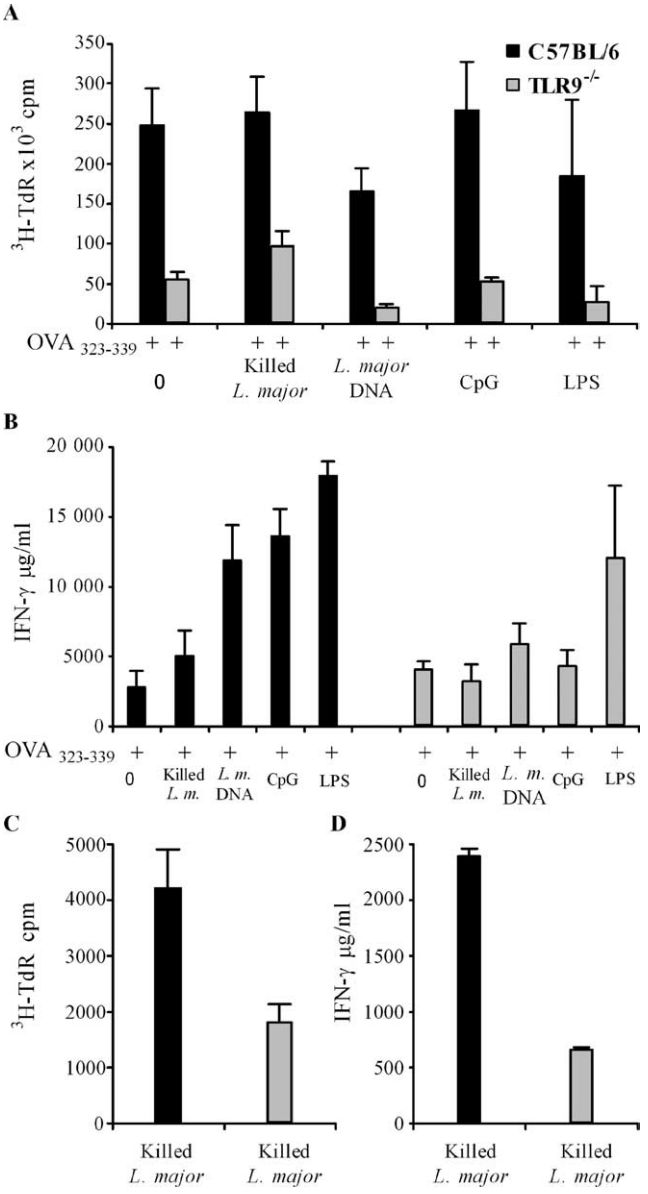

FIGURE 8. Analysis of the accessory function of BMDCs from C57BL/6 and TLR9$^{-/-}$ in the induction of a specific T cell response and promotion of IFN-γ production. BMDCs (10^4 cells/well) were stimulated or not in vitro for 6 h with either killed *L. major* (Killed *L.m.*), *L. major* DNA (*L.m.* DNA), CpG, or LPS and pulsed with 0.5 μg of the peptide $OVA_{323-339}$. After washing, CD4$^+$ T cells (10^5) from OT-II mice were added for 72 h. *A* and *B*, Proliferation of CD4$^+$ T cells was evaluated by ^{3}H-labeled TdR incorporation (*A*) and in IFN-γ production was measured in the supernatants of cultures by ELISA (*B*). The ability of BMDCs from C57BL/6 and TLR9$^{-/-}$ mice to trigger a *L. major*-specific T cell response was compared. BMDCs (10^4) from C57BL/6 and TLR9$^{-/-}$ were stimulated with killed *L. major* as a source of Ag. CD4$^+$ T cells from C57BL/6 mice injected i.v. with *L. major* were added for 96 h. *C* and *D*, Proliferation of T cells (*C*) and IFN-γ production (*D*) were analyzed. Data represent the mean and SD of triplicates and are from one of two independent experiments with similar results. Neither significant proliferation (5000 or 50 cpm) nor IFN-γ production (20 pg/ml) was observed in cultures of T cells alone stimulated with the OVA peptide or killed. *L. major*.

with parasite DNA. Strikingly, neither stimulation with *L. major* nor CpG was able to increase the expression of IL-12, IL-6, or IFN-β transcripts in pDCs from TLR9$^{-/-}$mice, confirming the role of TLR9 in these processes (Fig. 6*C*). Although pDCs are known to be relatively poorly activated by LPS, possibly because of low expression of TLR4, pDCs from wild-type and TLR9-deficient mice exhibited some increased expression of the various cytokines in response to LPS (Fig. 6, *B* and *C*).

Stimulation of mDCs, the subpopulation of DCs expanding preferentially in the spleen of mice treated with FLT3, from C57BL/6 mice with *L. major* promastigotes (killed or live) or DNA purified

from parasites also resulted in a significant increase of cytokine mRNA expression. Results in Fig. 7 reveal a large increase in transcripts for several cytokines, including, IL-12p40, IL-12p35, IL-6, and IFN-β. Interestingly, stimulation with either killed parasites or DNA from *L. major* led to higher levels of transcripts for most cytokines in activated mDCs than stimulation with live *L. major* parasites. In sharp contrast, mDCs from TLR9$^{-/-}$ mice did not exhibit significantly increased cytokine mRNA expression in the presence of either *L. major* promastigotes (killed or live) or parasite DNA (Fig. 7). As expected, results from control experiments showed that mDCs from TLR9$^{-/-}$ mice fail to respond to CpG whereas mDCs from C57BL/6 and TLR9-deficient mice respond equally well to LPS (Fig. 7).

Together, these results indicate that, in response to stimulation with *L. major*, the expression of cytokines, mostly proinflammatory, by freshly isolated DCs requires the integrity of TLR9.

The ability of L. major *and/or* L. major *DNA to cast DCs for promoting IFN-γ-producing T cell Th1 phenotype is TLR9 dependent*

The findings above demonstrating the TLR9 dependence of the activation of DCs by *L. major* and/or parasite DNA prompted us to directly assess whether or not this activation effectively rendered DCs capable of promoting Th1 cell maturation in vitro in a TLR9-dependent manner. Transgenic mice on a C57BL/6 background with CD4$^+$ T cells expressing a TCR specific for a *L. major* Ag are not available. Therefore, we assessed the effect of prior incubation of either wild-type or TLR9-deficient BMDCs with either *L. major* and/or parasite DNA on their capacity to induce, in the presence of the relevant epitope ($OVA_{323–339}$), the response of CD4$^+$ T cells (OT-II CD4$^+$ cells) specific for this peptide unrelated to *L. major*. Results in Fig. 8*A* show that the magnitude of the specific proliferative response of OT-II CD4$^+$ cells was significantly higher in the presence of C57BL/6 BMDCs compared with TLR9-deficient BMDCs. However, conditioning of BMDCs with either *L. major*, *L. major* DNA, CpG, or LPS before pulsing with the specific peptide had no effect on the magnitude of the T cell proliferative response. In contrast, production of IFN-γ by activated OT-II CD4$^+$ cells was significantly enhanced when BMDCs were treated with either *L. major*, *L. major* DNA, CpG, or LPS. In contrast, only treating TLR9-deficient BMDCs with LPS resulted in higher production of IFN-γ by responding OT-II CD4$^+$ T cells, the other stimuli having no effect (Fig. 8*B*). Similar results were obtained using DCs (CD11c$^+$ cells) freshly isolated from the spleens of FLT3-treated mice (data not shown).

Finally, we compared the ability of wild-type and TLR9-deficient DCs to trigger the production of IFN-γ by CD4$^+$ cells specific for *Leishmania* Ags. CD4$^+$ T cells obtained from the spleens of mice 8 days after i.v injection with *L. major* proliferated more and produced significantly more IFN-γ upon restimulation with *L.* major in vitro in the presence of wild-type DCs than when restimulated using TLR9-deficient DCs (Fig. 8, *C* and *D*).

Discussion

These data confirm and extend previous observations revealing the importance of TLR9 for the expression of a fully resistant phenotype by C57BL/6 mice infected with *L. major* (27). Indeed, C57BL/6 mice deficient in TLR9 resolved their lesions significantly later than wild-type mice and controlled parasite growth with much lower efficiency. Because the absence of TLR9 significantly although transiently inhibited the development of a protective Th1 response, we particularly studied the importance of *L. major*-induced TLR9 signaling for the acquisition by DCs of a Th1-inducing DC (DC1) functional phenotype. DCs, either derived from BM progenitors in vitro or freshly isolated from the spleens of mice treated with FLT3 ligand, required TLR9 expression to become activated by *L. major* and express inflammatory cytokines. Strong evidence was obtained that DNA was the parasite ligand responsible for the TLR9-dependent maturation of DCs to express a DC1 phenotype. Finally, stimulation of DCs with *L. major* strongly enhanced the production of IFN-γ by specifically activated T cells reactive to an unrelated Ag in vitro, and this effect was strictly dependent upon the expression of TLR9 by DCs and observed in the absence of NK cells in the culture system.

A possible involvement of TLR signaling in the development of resistance to infection with *L. major* was first evoked by observations showing that treatment of BALB/c mice with CpG-ODN, a ligand of TLR9, before infection redirected Th1 cell maturation and protective immunity in these otherwise highly susceptible mice. This effect of CpG-ODN was mediated by extended IL-12 and IFN-γ production and associated with maintained expression of the IL-12β2-chain (33, 34). Further evidence for a role of TLR signaling in resistance to infection with *L. major* derives from observations showing that mice from a resistant background deficient in the adapter protein MyD88 are susceptible (5). An impairment in resistance to *L. major* was subsequently reported in mice deficient in TLR4 (12). Interestingly, in this study, using mice from a resistant background carrying an homozygous mutation of the *tlr4* gene (TLR4$^{e/e}$) it was observed that even as soon as 1 day after the inoculation of *L. major* the growth/survival of parasites in the cutaneous lesions was drastically increased in mutant mice compared with wild-type controls. These results might suggest that TLR4 controls innate responses operating during the very early phase of parasite establishment, i.e., before the development of an adaptive T cell response. Noteworthily, at later times of infection an enhanced arginase activity was seen in mutant mice. The increase of this enzyme leading to the production of compounds essential for parasite proliferation in macrophages of mutant mice suggest that TLR4 signaling could favor the effector function of macrophages harboring parasites. Of interest, we failed to detect differences in the course of diseases between wild-type C57BL/6 and constructed TLR4 knockout mice (our unpublished observation). Although the reasons for these discrepancies are not known, the possibility exists that an additional mutation carried by the mice TLR4$^{e/e}$ with a "natural" mutation of the *tlr4* gene plays a role in the observed effect on the severity of disease.

Although older reports indicated that the NK cell-derived IFN-γ was unlikely to participate in the early regulation of visceral leishmaniasis in mice (35), evidence for a protective function of NK cells during visceral leishmaniasis exists (36). In this context, it has been shown recently that NK cell activation in mice infected with *Leishmania infantum* required IL-12 production by mDCs that was dependent upon TLR9 signaling (13). During the course of our study, the TLR9 signaling requirement for the activation of NK cells in mice infected with *L. major* was also reported (27). However, the causal linkage between a TLR9-dependent production of IL-12 by DCs responding to *L. major* and the activation of NK cells was more difficult to demonstrate. Our presently reported results showing that the parasite burden in C57BL/6 mice is similar to that in TLR9-deficient mice during at least the first 14 days after infection suggest that the absence of TLR9 does not influence the early containment of parasites normally resulting from the activity of NK cells (37, 38), previously identified as the earlier source of IFN-γ in this model system (1). Rather, it appears that a deficiency in TLR9 signaling results in a redirection, at least transient, of the effector Th1 response normally triggered in resistant C57BL/6 mice toward a Th2 phenotype, with a pronounced reduction of IFN-γ production and an increased expression of IL-4.

This shift in cytokine expression toward a Th2 phenotype in TLR9-deficient mice was parallel to an impaired expression of IL-12 in draining lymph nodes. Thus, it appears that the defective ability of TLR9-deficient mice to control, as efficiently as wild-type mice, the parasite growth between days 21 and 42 after infection with *L. major* proceeds from a deferred development of an effector Th1 response. Given the recent results showing that TLR9 signaling is essential for the NK cells response in murine cutaneous leishmaniasis (27) and the delay in Th1 cell maturation observed in TLR9-deficient mice infected with *L. major*, it appears that NK cells, although possibly influential in shaping Th1 cell development, are not essential. In some studies NK cells have been shown to exert anti-*Leishmania* effector activities, particularly during the early phase of infection (37, 38). In other reports, however, the importance of NK cells for the control of cutaneous leishmaniasis could not be demonstrated (39). Some of these discrepancies could result from the use in these studies of mice from different inbred strains exhibiting various levels of NK cell responsiveness. Furthermore, the role of NK cells in influencing the maturation of protective Th1 cells in the murine model of infection with *L. major* is also still a matter of debate. Nevertheless and in contrast to observations made in viral model systems of infection showing an important role for the IFN-γ produced by NK cells, the IFN-γ derived from NK cells was convincingly shown not to be required for Th1 cell differentiation in C57BL/6 mice infected with *L. major* (40).

We further show here that stimulation with *L. major* and/or parasite DNA of DCs presenting an immunogenic peptide to specific T cells significantly amplified the production of IFN-γ without significantly affecting the magnitude of the T cell proliferative response. This effect on IFN-γ production by specific T cells via the stimulation of DCs with *L. major* DNA was also observed after stimulation with CpG and was strictly dependent upon the expression of TLR9 by DCs. In a similar vein, $CD4^+$ T cells purified from the spleen of mice infected with *L. major* produced significantly more IFN-γ upon specific stimulation in the presence of wild-type DCs than TLR9-deficient DCs. These results also indicate that the effect of conditioning the Ag-presenting DCs through TLR9-mediated signals on IFN-γ production by specifically stimulated T cells occurs independently from the participation of NK cells.

An impaired maturation of DCs with a resulting damage in functional activities has been described during infection with some protozoan parasites (41, 42). However, uptake of *L. major* has been shown to efficiently prime human DCs for IL-12 secretion, a finding consistent with the results in other reports documenting the effectiveness of *L. major* in inducing up-regulation of MHC class II and costimulatory molecule expression and the production of inflammatory cytokines by DCs (43). The results in this report clearly confirm activation of murine DCs by *L.* major in vivo and interestingly show that similar levels of activation of DCs, either derived from BM progenitors in vitro or freshly isolated from spleens, are induced in vitro by heat-killed and live *L. major* promastigotes. In the mammalian host, the professional phagocytic cells, mainly the macrophages, are the primary targets of *Leishmania*. Contrasting with the observed activation of DCs, it appears that *L. major* deteriorates some antimicrobial defense mechanisms of macrophages and impairs their capacity to promote protective Th1 cell development. A transient inhibition of the microbicidal properties of macrophages would clearly favor the intracellular differentiation of the invading promastigotes into the hydrolase-resistant amastigotes (43). A prominent effect of *L. major* on macrophages is the selective inhibition of IL-12 production with a practically intact secretion of other cytokines (44). By reducing IL-12 production from the probably larger source of this cytokine during the early phase of infection, it has been proposed that *L. major* limits tissue damage extension that could ultimately eliminate the parasites and interrupt transmission (43).

It has been shown that after their inoculation into mice, the majority of parasites are rapidly destroyed by a yet poorly understood mechanism (25, 45, 46). This initial destruction results in the liberation of various parasite components, including a significant amount of DNA (particularly after inoculation of high number of *L. major* promastigotes in the footpad) that, after interacting with TLR9, is shown in the present work to be capable of activating DCs to a DC1 functional phenotype. The parasite DNA-induced TLR9 signaling possibly results in the building of a Th1 response specific for *L. major* epitopes from Ags released simultaneously with DNA during the early nonimmune phase of parasite destruction. A higher frequency of these Th1 cells and/or an increased production of IFN-γ by these cells, resulting in an enhanced activation of macrophages harboring amastigotes, could account for the greater resistance of wild-type C57BL/6 mice compared with syngeneic TLR9-deficient mice.

Results from our study clearly indicate that DNA from *L. major* is likely the ligand resulting in TLR9 signaling. First, heat-killed *L. major* promastigotes were able to induce the TLR9-dependent maturation of DCs; second, treatment of *L. major* preparations with DNase abolished their activation properties on DCs; third, modifying the pH of the endosomal compartment with chloroquine inhibited the effect of *L. major* and/or parasite DNA on DCs maturation. It is known that treatment of target cells with chloroquine interferes with the interactions between TLR9 and its well-characterized ligand CpG (47). Strikingly, the presently described *L. major* DNA-induced activation of DCs appears specific for the parasite DNA, because we failed to observe similar effects after stimulation with purified DNA from either mice, sheep, pig, or fish, regardless of the concentrations used. These vertebrate DNA, although possibly capable of binding to TLR9, might not lead, because of their sequence and structure, to the conformational changes likely required for the recruitment of signaling adaptor molecules. A higher proportion of the stimulatory sequence in protozoan parasites DNA than in vertebrate DNA could account for these differences in TLR9 signaling. Testing this hypothesis requires determination of the substantial conformational changes necessary for the recruitment of signaling adaptor molecules (48).

The intracellular localization of TLR9 could more likely be at the basis for the failure of these vertebrate DNA to induce TLR9 signaling. In this context, it has been shown on the one hand that a chimeric TLR9 anchored at the cell surface could respond to otherwise nonstimulatory mammalian DNA (49) and, on the other hand, that efficient translocation of vertebrate DNA into the endosomal compartment enables activation of cells via the TLR9 pathway (49). A recent elegant study revealed that in psoriatic skin an antimicrobial peptide formed a complex with self-DNA that reached the endocytic compartment of pDC, which normally does not respond to self-DNA, inducing IFN type I production through TLR9 signaling (50). In this particular context it has been reported that the DNA from other protozoan parasites (*B. bovis*, *T. cruzi*, and *T. brucei*) as from *Escherichia coli* stimulated B lymphocyte proliferation and activated macrophages (32) and that DNA from *T. cruzi* stimulated BM-derived macrophages and splenic DCs in a TLR-dependent manner (19). Therefore, the possibility that an unknown molecule is associated with *L. major* DNA, allowing its efficient translocation into the TLR9-rich endocytic compartment, is currently being investigated in our laboratory. Noteworthily, it

has been recently shown that the DNA sugar backbone 2′-deoxyribose is a prime determinant for DNA-TLR9 interaction (51). Indeed, a base-free phosphodiester acted as a basal TLR9 agonist. Strikingly, not only CpG motifs but any DNA base linked via the phosphodiester sugar backbone increased both TLR9 affinity and activation, because upon forced endosomal translocation not only CpG-ODNs but also phosphodiester non-CpG ODNs triggered important TLR9-dependent activation of DCs. In this context, it is of particular interest that at high concentrations *T. cruzi* DNA-mediated macrophage activation was not inhibited following methylation. These results support the likelihood that our failure to activate DCs with mice, sheep, pig, or fish DNA resulted from a poor uptake of these DNAs by DCs.

Admittedly, this study is limited to the assessment of the role of TLR9 signaling in mice genetically resistant to infection with *L. major*. Further studies are clearly needed to determine whether or not a deficiency in TLR9 signaling by *L. major*, possibly resulting from a down-regulation of TLR9 expression, coincides with the development of an aberrant Th2 response and of severe and uncontrolled lesions in mice from genetically susceptible strains.

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Disclosures

The authors have no financial conflict of interest.

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