Dendritic Cells (DC) Activated by CpG DNA Ex Vivo Are Potent Inducers of Host Resistance to an Intracellular Pathogen That Is Independent of IL-12 Derived from the Immunizing DC[1]

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We used the model of murine leishmaniasis to evaluate the signals enabling Ag-pulsed dendritic cells (DC) to prime a protective Th1 response in vivo. Bone marrow-derived DC (BMDC) that had been activated by TNF-α or CD40 ligation were not able to induce protection against leishmaniasis in susceptible BALB/c mice. In contrast, all mice vaccinated with a single dose of *Leishmania major* Ag-pulsed BMDC stimulated by prior in vitro exposure to CpG-containing oligodeoxynucleotides (ODN) were completely protected, had a dramatic reduction in parasite burden, and developed an Ag-specific Th1 response. Importantly, systemic administration of CpG ODN was not required. Protection mediated by ex vivo CpG ODN-activated and Ag-pulsed DC was solid, as documented by resistance to reinfection with a higher parasite dose, and long-lasting, as immunized mice were still protected against *L. major* challenge 16 wk after vaccination. A significantly increased level of protection could also be elicited in resistant C57BL/6 mice. Surprisingly, IL-12 expression by the immunizing BMDC was not required for induction of host resistance. In contrast, the availability of IL-12 derived from recipient cells was essential for the initial triggering of protective immunity by transferred BMDC. Together, these findings demonstrate that the type of stimulatory signal is critical for activating the potential of DC to induce a Th1 response in vivo that confers complete protection against an intracellular pathogen. Moreover, they show that the impact of activated DC on the initiation of a protective Th cell response in vivo may be independent of their ability to produce IL-12. *The Journal of Immunology,* **2004, 172: 6281–6289.**

Dendritic cells (DC)[4] convey information regarding the nature of a microbial stimulus to T cells and direct the development of polarized Th cell responses. The mechanism by which DC determine the type of adaptive immune response is not well understood. It has been suggested that different DC subsets have an intrinsic tendency to promote either a Th1 or a Th2 response (1). Recently, however, a number of reports indicated that there is a striking plasticity in the ability of a given DC subset to respond to different microbes (2–5), suggesting that the type of DC stimulus is a critical factor leading to DC-mediated polarization of the Th cell response. This idea is supported by the observation that agents typically inducing Th1 or Th2 responses in vivo (certain products of bacteria and helminths) stimulate myeloid DC to prime and activate preferentially Th1 or Th2 cells, respectively (2–4).

A major limitation has been the lack of an in vivo read-out system reflecting the development of Th cells with impact on disease. The criterion usually applied is the in vitro production of different cytokines associated with Th1 or Th2 cells after in vitro (humans and mice) or in vivo (mice) priming, but formal evidence for the generation of Th1 or Th2 cells that are able to mediate effective control of pathogens in vivo is not provided. A mixed pattern of Th1 and Th2 cytokines has been observed in some studies (1), leading to further ambiguity regarding the relevance of the findings for the situation in vivo. Moreover, it has been demonstrated that DC pulsed with a microbial Ag stimulated Th1 cells in vitro, but induced a Th2 response after transfer in vivo, as characterized by the cytokine profile and the lack of protection upon challenge with the pathogen (6).

The ability of DC to release IL-12 in response to microbial stimuli is considered pivotal for the induction of Th1 responses (1). However, this concept has been challenged by the observation that $CD4^+$ T cell responses to an intracellular parasite do not default to a Th2 pattern in the absence of IL-12 (7). Furthermore, although IL-12 expression by DC pulsed with bacterial Ag was shown to be necessary for the development of an optimal Th1 response, it was not an absolute requirement for Th1 cell induction (8). In contrast, we have previously demonstrated that IL-12 production by Langerhans cells, DC of the skin, is essential for the induction of host resistance to *Leishmania major* that correlates with Th1 polarization (9). Together, these findings imply that the immunological characteristics of DC are not necessarily predictive of their in vivo immunizing properties, emphasizing the importance of using in vivo models to assess the DC-mediated induction of physiologically relevant Th1 and Th2 cells with impact on the clinical course of infectious diseases.

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[4] Abbreviations used in this paper: DC, dendritic cell; BMDC, bone marrow-derived DC; CpG/Ag-BMDC, CpG ODN-stimulated and LmAg-pulsed BMDC; LmAg, *L. major* Ag; ODN, oligodeoxynucleotide; TLR, Toll-like receptor; WT, wild type.

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In the present study we used the model of murine leishmaniasis to analyze the signals enabling bone marrow-derived DC (BMDC) to prime a Th1 response in vivo. In this model, infection of susceptible BALB/c mice with *L. major* induces a Th2 response that leads to uncontrolled parasite replication (10). The development of protective immunity in BALB/c mice depends on the IL-12-driven redirection of the immune response toward the Th1 pathway (10). Thus, the model of murine infection with *L. major* is a suitable tool to evaluate the stimuli activating the potential of DC to prime a Th1 response in vivo that influences the course of infection. In this report we show that the type of DC stimulus is a key factor in determining the capacity of DC to promote an effective Th1 response in vivo. In addition, our results demonstrate that the triggering of a protective Th cell response by DC in vivo may be independent of their ability to produce IL-12.

Materials and Methods

Mice, parasites, and reagents

Female BALB/c and C57BL/6 mice were 6–8 wk old at the onset of the experiments and were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany). IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice were generated and backcrossed into the BALB/c background as previously described (11, 12). The knockout mice were provided by Dr. G. Alber (University of Leipzig, Leipzig, Germany). The cloned virulent *L. major* isolate (MHOM/IL/81/FE/BNI) was maintained by passage in BALB/c mice. Promastigotes were grown in vitro in blood agar cultures. For the preparation of parasite lysate, stationary phase promastigotes were subjected to three cycles of rapid freezing and thawing. The CpG oligodeoxynucleotide (ODN) 1668 (5′-TCCATGACGTTCCTGATGCT-3′) and the control AT-rich ODN (non-CpG ODN, 5′-ATTATTATTATTATTAT TAT-3′) were obtained from Qiagen Operon (Cologne, Germany) and were not phosphorothioate-modified. Neutralizing rat anti-mouse IL-12 mAb (IgG2a, hybridoma C17.8) was created in the laboratory of Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). Ab prepared by passing hybridoma culture supernatants over a protein A column and subsequent dialysis in PBS were injected i.p. (1 mg/mouse). Rat IgG control Ab were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Preparation of BMDC

DC were generated from bone marrow progenitors as described by Lutz et al. (13). Briefly, freshly prepared bone marrow cells were cultured in Click RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES buffer, 60 μg/ml penicillin, and 20 μg/ml gentamicin in the presence of 200 U/ml GM-CSF (PeproTech, London, U.K.). Cultures were fed with GM-CSF on days 3, 6, and 8. After 10 days, the nonadherent cells were collected and shown to have a typical DC morphology with a myeloid DC phenotype (MHC class II$^+$, CD80$^+$, CD86$^+$, CD40$^+$, ICAM-1$^+$, CD11c$^+$, B220$^-$, CD8$^-$) and potent APC functions in allogeneic MLR and a proliferation assay with *L. major* Ag (LmAg)-specific T hybridoma cells (data not shown). Cells were resuspended at 1×10^6/ml in culture medium containing 200 U/ml GM-CSF and were pulsed overnight (18 h) with LmAg (equivalent to 30 parasites/cell) in the absence or the presence of well-established inducers of DC maturation (CpG ODN and control non-CpG ODN, 25 μg/ml; anti-CD40 mAb, 5 μg/ml, HM40-3 low endotoxin (BD PharMingen, Heidelberg, Germany); and TNF-α, 500 U/ml (PeproTech)). Thereafter, the cells were washed and resuspended in PBS.

Treatment of mice and analysis of the course of disease

BMDC (5×10^5) were injected i.v. into the tail vein of naive mice. Control mice were treated with PBS. One or 16 wk later, mice were infected intradermally with 2×10^5 (BALB/c mice) or 2×10^6 (C57BL/6 mice) stationary phase promastigotes into the right hind footpad. For reinfection experiments, mice were infected with 5×10^5 parasites into the left hind footpad 10 wk after the primary infection. The course of infection was monitored weekly by measuring the increase in footpad size of the infected vs the noninfected footpad. For determination of the amount of viable parasites in footpads, a limiting dilution assay was used (14).

Determination of cytokine production by lymph node and spleen cells and detection of IgG subclasses in the serum

Lymph nodes draining the infected footpads or spleens were collected before or 5 wk after infection, as indicated, and single-cell suspensions (10^6 lymph node cells/ml; 2×10^6 spleen cells/ml) were cultured in the absence or the presence of LmAg (equivalent to 10^7 parasites/10^6 cells) or *L. major* promastigotes (3×10^6 parasites/10^6 cells) for 72 h. Thereafter, culture supernatants were harvested for the determination of the cytokines IL-2, IL-4, IL-10 and IFN-γ by sandwich ELISA, as described previously (15). The detection limits were 490 pg/ml for IL-2, 3 pg/ml for IL-4, 12.2 pg/ml for IL-10 and 50 pg/ml for IFN-γ. For the detection of *Leishmania*-specific IgG1 and IgG2a Ab in the serum of mice, 96-well plates were coated with LmAg (equivalent to 5×10^5 parasites/well) and supplemented with diluted sera (1/50). After overnight incubation, appropriate conjugates, and substrates were used to detect Ab binding. Relative levels of Ab were determined by measuring the OD in an ELISA reader.

Analysis of IL-12 production by BMDC

BMDC were generated as described above and pulsed with LmAg in the presence or the absence of DC stimuli. Culture supernatants were collected after centrifugation and analyzed for the levels of IL-12p70 by sandwich ELISA, using purified rat anti-mouse IL-12p70 mAb (BD PharMingen) for capture, and a biotin-labeled anti-mouse IL-12p40/p70 mAb (BD PharMingen) for detection. The detection threshold was 488 pg/ml.

Statistical analysis

Student's *t* test was used for statistical analyses.

Results

LmAg-pulsed BMDC exposed to CpG ODN, but not anti-CD40 Ab or TNF-α, induce efficient clinical and parasitological protection against leishmaniasis in BALB/c mice

For assessment of the maturation requirements enabling DC to prime a protective immune response in *L. major*-susceptible BALB/c mice, BMDC were pulsed with LmAg in the absence or the presence of maturation stimuli and subsequently injected i.v. into naive mice. We selected three prototype DC activators. First, CpG-containing ODN, causing direct activation of DC by signaling through a type 1 pattern recognition receptor; second, TNF-α, mediating indirect DC activation by locally induced inflammation; and third, CD40 ligation, a signal provided by T cells in the lymphoid organs. These stimuli induce an enhanced expression of MHC class II and costimulatory molecules by DC and evoke DC cytokine production and migration (16, 17). Although they were shown to promote DC-mediated antitumor immunity (18, 19), it is not known whether they can directly activate the potential of DC to induce an effective Th1 response in vivo. It is important to note that the DC stimuli were not administered systemically, but were solely used for in vitro activation of DC before injection of the cells into mice. The animals were challenged with *L. major* 1 wk later, and the course of disease was monitored. Our results show that BMDC pulsed with LmAg in the absence of activation signals or in the presence of anti-CD40 Ab or TNF-α were unable to induce protection against leishmaniasis (Fig. 1*A*). Treating BMDC with combinations of TNF-α, CD40 ligation, and IL-12 was also ineffective (not shown), indicating that indirect signaling via proinflammatory cytokines and T cell activation is not sufficient to stimulate the development of Th1-promoting DC. However, when DC were pulsed in the presence of a prominent Toll-like receptor (TLR) agonist, CpG ODN, they acquired the ability to induce complete protection of BALB/c mice against an otherwise lethal infection with *L. major*. All mice vaccinated with a single dose of these cells developed an almost imperceptible footpad swelling and did not show any sign of ulceration. Mice immunized with unpulsed CpG ODN-treated BMDC or pulsed BMDC treated with a non-CpG ODN showed a course of disease comparable to that in the PBS-treated control group (Fig. 1*A*), demonstrating that the

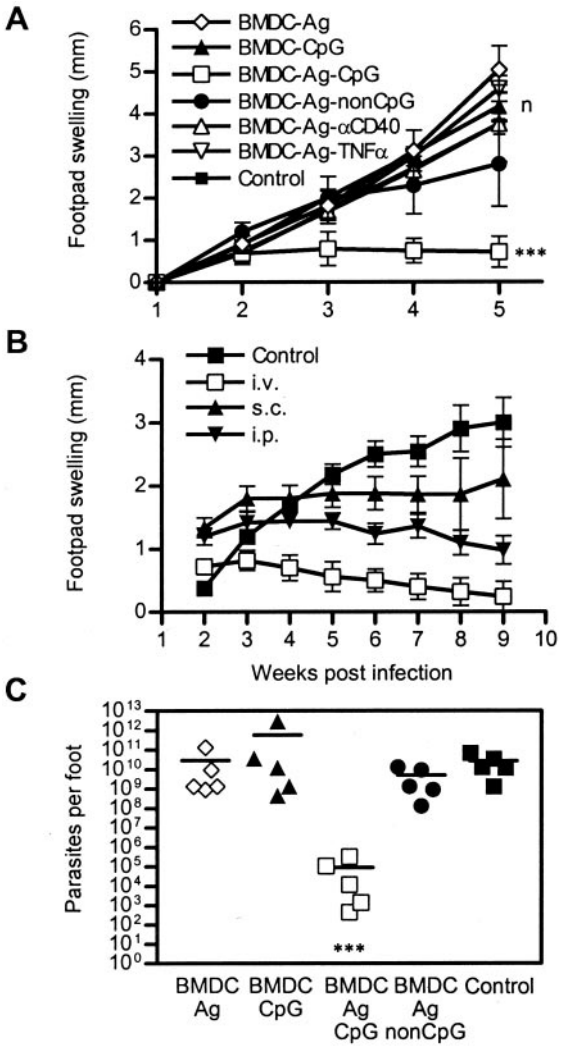

FIGURE 1. LmAg-pulsed BMDC stimulated with CpG ODN induce protection against leishmaniasis. *A*, BALB/c mice were immunized i.v. with DC that had been treated in vitro as indicated and were infected with *L. major* 1 wk later. *B*, BALB/c mice were immunized i.v., s.c., or i.p. with BMDC that had been pulsed with LmAg and stimulated with CpG ODN. After 1 wk, mice were challenged with *L. major*. Control mice (*A* and *B*) received PBS before infection. The increase in size of the infected compared with the noninfected footpad was measured weekly. Results are expressed as the mean ± SEM ($n = 5$). *C*, The parasite burden in the footpads of individual mice of the most relevant groups shown in *A* was determined 5 wk after infection. The results are representative of at least three independent experiments. ***, $p < 0.0005$ compared with the PBS-treated control group. n, severe footpad necrosis.

development of resistance was dependent on both ex vivo DC stimulation by CpG motifs and LmAg presentation by DC. Only i.v. administration of CpG ODN-stimulated BMDC delivering LmAg induced complete protection, whereas s.c. or i.p. injection was much less efficient (Fig. 1*B*). Delivery of peptide-pulsed DC via the i.v. route is also being used for the treatment of cancer patients in clinical trials (20, 21).

To determine whether the clinical cure observed in BALB/c mice vaccinated i.v. with CpG-activated and LmAg-pulsed BMDC (CpG/Ag-BMDC) corresponded with effective eradication of the pathogen, we analyzed the parasite burden in the footpads of the most relevant experimental groups. The results revealed a striking correlation between parasite numbers and clinical outcome (Fig. 1*C*). The number of parasites was reduced ~10^5-fold in mice in the protected group (treated with CpG/Ag-BMDC) compared with unprotected control mice. Together, these findings demonstrate that stimulation of Ag-pulsed BMDC with a type 1 microbial signal is necessary and sufficient to confer a resistance-promoting phenotype to DC, thus enabling them to induce protective anti-*Leishmania* effector mechanisms in BALB/c mice.

Protection induced by LmAg-pulsed and CpG ODN-stimulated BMDC is solid and long-lasting

Another important aspect was whether mice that resolved the primary infection were able to resist a second challenge. Therefore, cured mice were rechallenged with 5×10^5 parasites (a 2.5-fold higher number than that used for primary infection) 10 wk after the first challenge. The results in Fig. 2*A* show that a solid immunity had been established by immunization with a single dose of CpG/Ag-BMDC, as the swelling after secondary infection was even lower than that observed after primary challenge. Again, the parasite burden in footpads of rechallenged mice was ~10^5-fold lower than that in those of naive mice infected with the same inoculum (Fig. 2*B*). The protected mice were followed for >20 wk after secondary challenge, and no signs of disease were observed.

Furthermore, treatment with CpG/Ag-BMDC was shown to confer protection even after extended time intervals between vaccination and infection with *L. major*. The course of lesion development in mice that had been infected 16 wk after prophylactic immunization with CpG/Ag-BMDC (Fig. 2*C*) was comparable to that observed after an interval of only 1 wk (Fig. 1*A*). Compared with the unprotected control group, the mean number of parasites in the footpads was 10^3- to 10^4-fold lower in mice that had been vaccinated with CpG/Ag-BMDC 16 wk before infection (Fig. 2*D*). Thus, CpG-activated BMDC delivering LmAg are able to induce solid and long-lasting protection against leishmaniasis. Moreover, we showed that CpG/Ag-BMDC are highly efficient. Treatment of BALB/c mice with 3–5 $\times$ 10^5 cells was sufficient to confer maximal levels of resistance and induce complete cure of skin lesions (data not shown).

Mice vaccinated with LmAg-pulsed and CpG ODN-stimulated BMDC develop a Th1 immune response after L. major *infection*

To determine whether the protection induced by CpG/Ag-BMDC was associated with a Th1-like cytokine profile, cells from lymph nodes draining the lesions were collected 5 wk after infection, and the secretion of IL-2, IFN-γ, and IL-4 was analyzed. The highest levels of Ag-stimulated IFN-γ and IL-2 production (150- and 13-fold higher than the control group, respectively) and very low levels of IL-4 release were observed for cells from protected mice that had been vaccinated with CpG/Ag-BMDC (Fig. 3*A*). Consistent with the idea that Ab isotype switching is a surrogate marker of Th1 or Th2 development, serum from these protected animals contained low levels of *Leishmania*-specific IgG1 and high levels of parasite-specific IgG2a Ab and exhibited the highest IgG2a/IgG1 ratio (1.499), which was 4 times higher than that of the control group (0.366; Fig. 3*B*). Thus, both cytokine and Ab profiles indicate that vaccination with CpG/Ag-BMDC induced a shift toward a Th1-dominated immune response after *L. major* infection.

In the unprotected groups of mice, the levels of IgG2a and IgG1 in the serum did not always correlate with the relative amounts of IFN-γ and IL-4 released by lymph node cells. This is probably related to the high parasite burden in these mice and may reflect differences in the systemic immune response compared with the local cytokine production in the lymph nodes draining the lesions. Considering the high levels of IgG1 detected in unprotected mice immunized with Ag-pulsed BMDC or Ag-pulsed BMDC treated with non-CpG ODN, the low IL-4 levels were unexpected. It is

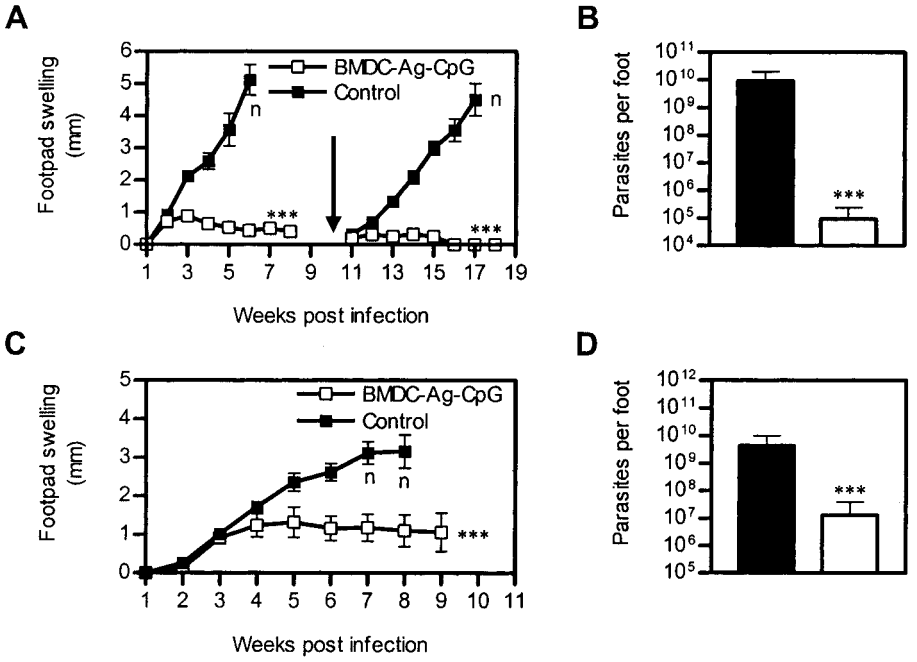

FIGURE 2. LmAg-pulsed BMDC stimulated with CpG ODN confer protection against a second challenge with *L. major* and have a long-lasting effect. *A*, BALB/c mice cured from primary infection after protective treatment with CpG/Ag-BMDC were rechallenged 10 wk after the first infection (arrow), and lesion development was monitored weekly. *B*, Parasite numbers in footpads of the groups of mice shown in *A* were evaluated 5 wk after secondary infection. *C*, BALB/c mice were immunized with CpG/Ag-BMDC and infected with *L. major* 16 wk later. The footpad swelling was measured weekly. *D*, Parasite numbers in footpads of the groups of mice shown in *C* were evaluated 9 wk after infection. Results in *A* and *C* are expressed as the mean ± SEM ($n = 5$). The results are representative of three (*A* and *B*) or two (*C* and *D*) independent experiments. ***, $p < 0.0005$ compared with the PBS-treated control group. n, severe footpad necrosis.

possible that the small amounts of IL-4 produced in the lymph nodes of those mice were sufficient to allow IgG1 class switching. An alternative explanation is that the levels of IL-4 detected in the culture supernatants may not have strictly reflected the cytokine response in vivo because of IL-4 consumption (22).

A single injection of CpG ODN-stimulated BMDC carrying LmAg induces a primary T cell response that is biased toward the Th1 type

To analyze the immune status of mice after i.v. treatment with BMDC and before the challenge with parasites, we determined the levels of cytokines secreted by splenocytes. Although immunization with BMDC induced a robust production of all the cytokines analyzed, significant differences were observed depending on the in vitro treatment of BMDC before their injection into mice (Fig. 4). Adoptive transfer of LmAg-pulsed BMDC induced significant levels of IFN-γ, but, at the same time, maximal levels of IL-4 and IL-10 (Fig. 4*A*, □). Additional treatment of BMDC with non-CpG ODN caused the development of a mixed Th1- and Th2-type response, as indicated by intermediate levels of IFN-γ, IL-4, and IL-10 (Fig. 4*A*, ▨). Notably, only the stimulation of LmAg-loaded BMDC with CpG ODN led to the production of maximal amounts of IFN-γ and minimal levels of IL-4 and IL-10 (Fig. 4*A*, ■). When splenocytes were cultured for prolonged periods in the absence of

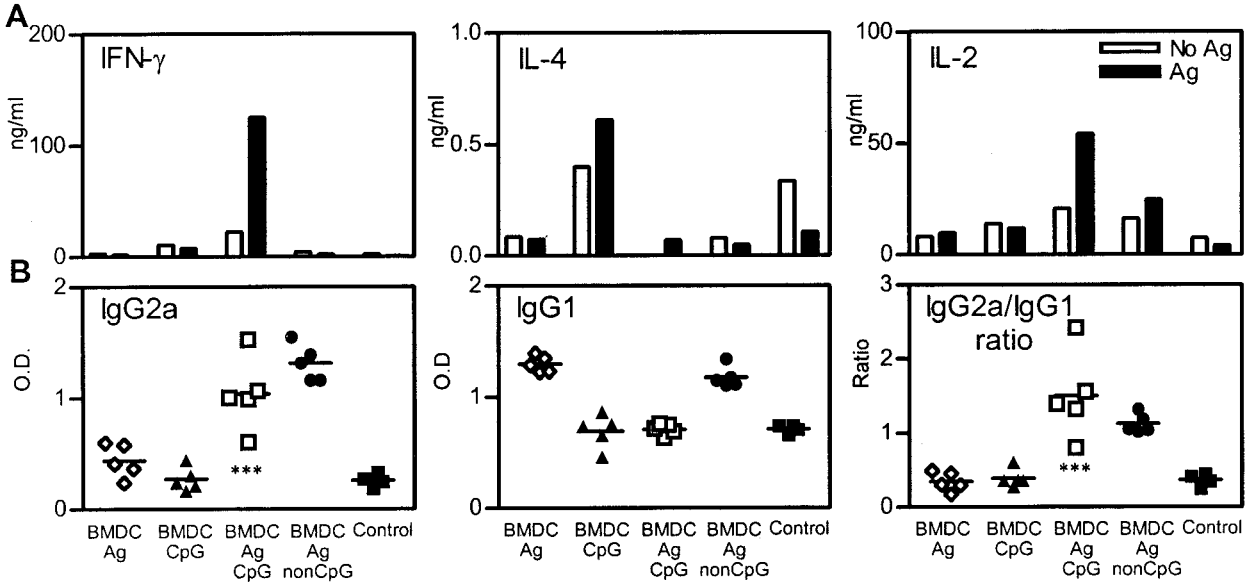

FIGURE 3. BALB/c mice immunized with CpG ODN-activated BMDC carrying LmAg develop a Th1 immune response upon challenge with *L. major*. *A*, Lymph node cell suspensions prepared 5 wk after infection from mice of the most relevant groups shown in Fig. 1*A* ($n = 5$) were pooled and incubated for 72 h in the absence or the presence of LmAg. Supernatants were assayed for the production of IL-2, IFN-γ, and IL-4 by ELISA. *B*, Sera of the same animals were analyzed individually for the levels of anti-*Leishmania* IgG1 and IgG2a Ab by ELISA. Bars indicate the mean. The results are representative of at least two independent experiments. ***, $p < 0.0005$ compared with the PBS-treated control group.

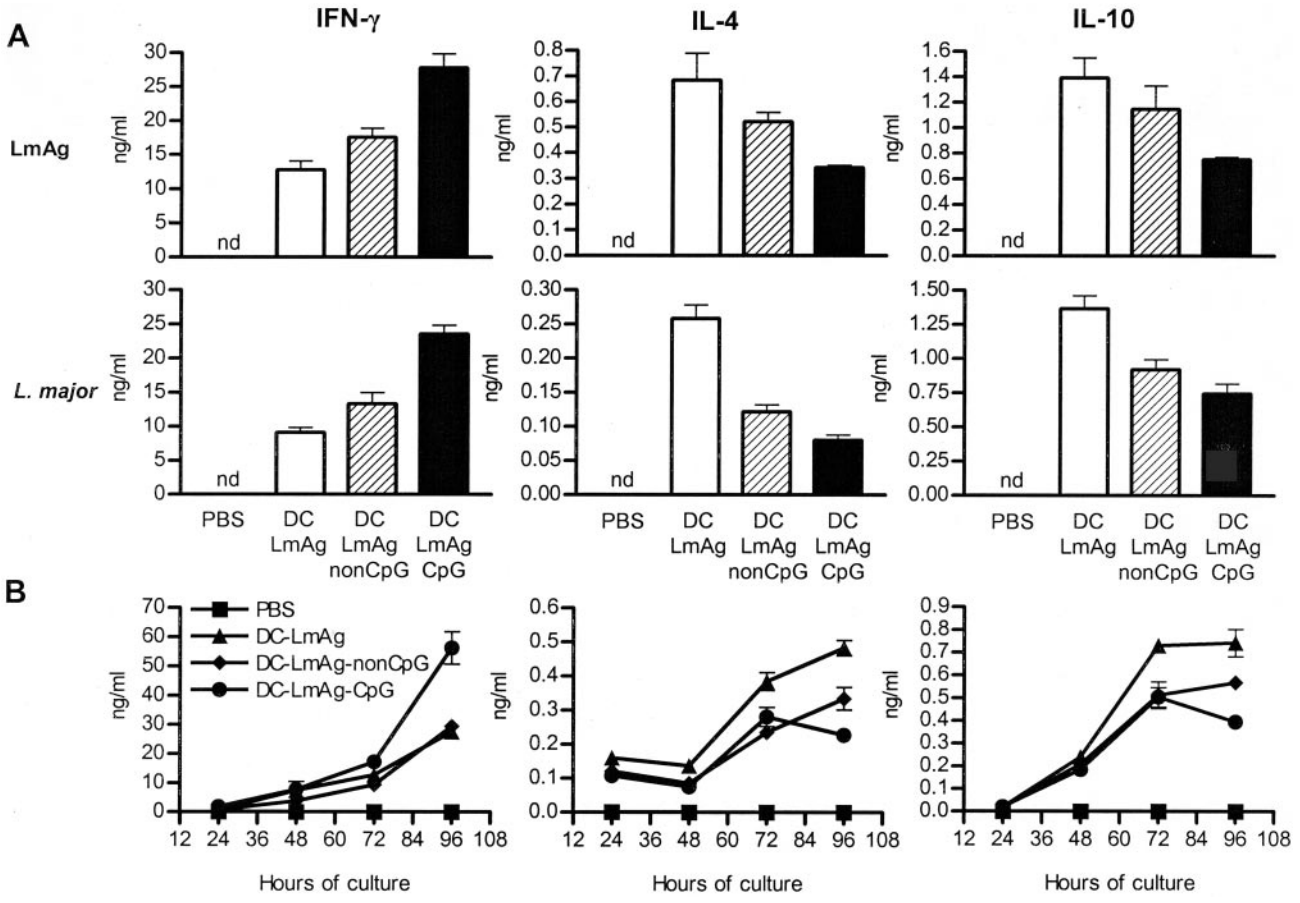

FIGURE 4. A single injection of Ag-loaded and CpG-activated BMDC induces a primary immune response strongly biased toward the Th1 type. Naive BALB/c mice were immunized i.v. with 5×10^5 BMDC that had been treated as indicated. PBS-injected animals were used as controls. After 7 days, mice were sacrificed, and single-cell suspensions of the spleens were cultured in the presence of LmAg or *L. major* parasites (*A*) or in the absence of Ag (*B*). Supernatants were collected at 72 h of culture (*A*) or the indicated time points (*B*), and the levels of IFN-γ, IL-4, and IL-10 were determined by ELISA. Data represent the mean ± SEM of triplicate cultures from five mice per group. nd, not detectable.

LmAg or *L. major* parasites, a similar pattern of ex vivo cytokine production was observed (Fig. 4*B*, 96 h values). These results demonstrate that immunization with LmAg-pulsed BMDC triggers both Th1- and Th2-type cytokines, but further stimulation of the cells with CpG ODN ex vivo results in the induction of a cytokine profile that is strongly biased toward the Th1 type.

A protective effect of LmAg-pulsed and CpG ODN-activated BMDC is also observed in resistant C57BL/6 mice

C57BL/6 mice are genetically resistant to cutaneous leishmaniasis. They develop a Th1 response, and skin lesions heal spontaneously within 5–8 wk of *L. major* infection (10). In many respects, the clinical and immunological features of human cutaneous leishmaniasis can be reproduced more closely in C57BL/6 than in BALB/c mice. We therefore analyzed whether CpG-activated BMDC delivering LmAg have a protective effect in C57BL/6 mice. Immunization of C57BL/6 mice with CpG/Ag-BMDC induced a significant reduction in the lesion development (Fig. 5*A*), with a lower maximal peak and faster healing compared with the PBS-treated control group. Treatment with BMDC alone mediated an initial effect, but the size of the lesions in those mice was comparable to that in the control group after 4 wk of infection. As expected, immunization with BMDC that had been exposed to CpG in the absence of LmAg had no effect. Surprisingly, in contrast to BALB/c, C57BL/6 mice immunized with Ag-pulsed BMDC showed a significant reduction in lesion size, although it was less pronounced than that induced by CpG-activated BMDC bearing LmAg (Fig. 5*A*). This finding may be explained by the genetic resistance of C57BL/6 mice and their ability to mount a Th1 response without immune intervention. In susceptible BALB/c mice, in contrast, effective vaccination requires a shift from the naturally developing Th2 pattern toward a Th1 response that appears to depend on further stimulation of Ag-loaded BMDC. When the parasite load in footpads of C57BL/6 mice was analyzed, a most significant decrease was observed only after vaccination with CpG/Ag-BMDC (Fig. 5*B*). Compared with PBS-treated controls, mice treated with Ag-pulsed BMDC showed a 10-fold reduction, whereas those vaccinated with CpG/Ag-BMDC had an ~100-fold lower number of parasites at the site of infection. These results demonstrate that activation of DC with CpG strongly enhances their ability to induce a protective immune response to *L. major* in C57BL/6 mice.

IL-12 production by BMDC exposed to various stimuli

After having demonstrated that CpG stimulation of LmAg-pulsed BMDC is a requirement for activating their potential to mediate protection against leishmaniasis and trigger a Th1-type immune response, it was of interest to analyze whether the induction of IL-12 release by these cells was responsible for the effect. BMDC were exposed to various stimuli, and the formation of bioactive IL-12p70 was determined. As shown in Fig. 6, significant IL-12 synthesis was observed only after BMDC stimulation with CpG.

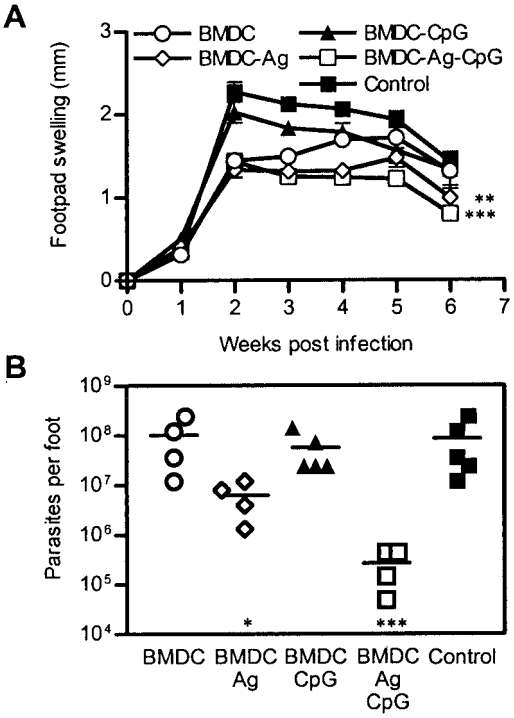

FIGURE 5. Treatment with LmAg-pulsed and CpG ODN-activated BMDC reduces the clinical manifestations of leishmaniasis and the parasite burden in resistant C57BL/6 mice. *A*, BMDC were treated as indicated and injected i.v. into naive mice. One week later, animals were infected with *L. major* injected into one hind footpad, and the increase in size of the infected compared with the noninfected footpad was measured weekly. Results are expressed as the mean ± SEM ($n = 5$). *B*, The mice were killed 6 wk after infection, and the parasite load in footpads of individual mice was analyzed. The results are representative of two independent experiments. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$ (compared with the PBS-treated control group).

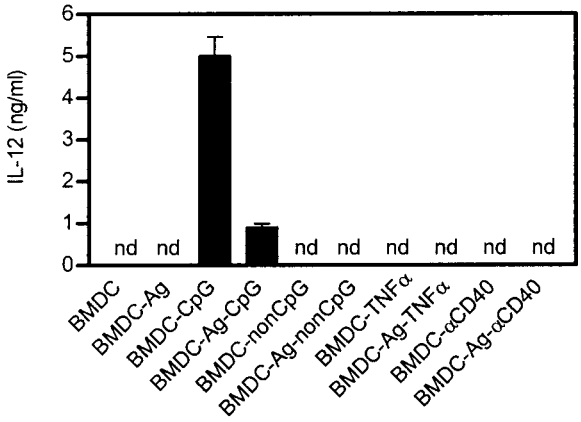

FIGURE 6. IL-12 production by BMDC after LmAg pulsing and/or treatment with various stimuli in vitro. Supernatants of cultures of BMDC that had been treated as indicated were assayed for IL-12p70 levels by ELISA. The results are representative of three independent experiments. nd, not detectable.

Interestingly, LmAg pulsing diminished the production of IL-12 by CpG-treated BMDC, but it was required to enable DC to mediate protection (see above, Fig. 1).

Expression of IL-12 by donor BMDC is not required for the induction of protective immunity

To analyze the role of the Th1-polarizing cytokine IL-12 in the initiation and development of a protective T cell response in mice vaccinated with CpG/Ag-BMDC, we compared the protective potential of BMDC obtained from IL-12-deficient mice with that of BMDC from wild-type (WT) mice. BMDC from WT BALB/c mice or from IL-12p35$^{-/-}$ or IL-12p40$^{-/-}$ BALB/c mice were loaded with LmAg in the presence or the absence of the stimulator CpG ODN. Subsequently, the cells were injected into WT BALB/c recipients, and 1 wk later, mice were challenged with *L. major* parasites. As expected, mice that had been treated with BMDC pulsed with LmAg in the absence of CpG activation developed large lesions comparable to those of control animals, whereas mice immunized with CpG/Ag-BMDC were protected (Fig. 7*A*). Interestingly, when mice were treated with CpG/Ag-BMDC from IL-12p35- or IL-12p40-deficient donors, the protection levels were similar to those observed after vaccination with CpG/Ag-BMDC from WT mice (Fig. 7, *A* and *B*). Determination of parasite numbers in the footpads confirmed this finding, as CpG/Ag-BMDC from WT and IL-12-deficient mice induced an equivalent reduction of the parasite load (~10^5-fold; data not shown). These results demonstrate that the protection induced by CpG-activated BMDC carrying LmAg is not dependent on the ability of donor BMDC to secrete IL-12.

Availability of IL-12 in the recipients is necessary for BMDC-mediated protection against L. major *infection*

Next we investigated the role of IL-12 produced by recipient cells. IL-12-deficient mice were treated with CpG/Ag-BMDC from WT mice and were infected with *L. major* 1 wk later. The results in Fig. 7*C* show that CpG/Ag-BMDC from WT mice were unable to confer protection to *L. major* in IL-12$^{-/-}$ mice, as opposed to their potent resistance-promoting effect in WT recipient mice. This finding indicates that the production of IL-12 by recipient cells is critical for the control of infection in CpG/Ag-BMDC-vaccinated mice. To discern whether the presence of IL-12 is required during the initiation or maintenance of antileishmanial immunity after DC-mediated vaccination, a neutralizing anti-IL-12 mAb was administered to WT recipient mice 1 day before treatment with CpG/Ag-BMDC. Control mice received isotype-matched Ab or PBS. Mice were infected 1 wk later, and the course of disease was monitored. Strikingly, neutralization of IL-12 at the time of BMDC-based immunization completely abrogated the potential of BMDC to induce protection (Fig. 7*D*), demonstrating that the availability of IL-12 derived from recipient cells is required for initiating the development of a protective immune response.

Discussion

The present study revealed three key findings. First, it describes a novel strategy to induce highly effective and solid immunity against an intracellular pathogen using the unique Ag-presenting potential of DC, and thus provides a promising new tool for DC-based vaccination and immunotherapy. Second, it has important implications for the understanding of in vivo Th1 polarization by DC, by demonstrating that the type of DC stimulus is a critical factor determining the capacity of DC to direct a Th cell response that mediates protection. Third, it shows that the role of IL-12 in DC-mediated priming and differentiation of T cells depends on the type of DC and that the impact of activated DC on the initiation of a protective Th cell response in vivo may be independent of their ability to produce IL-12.

Our results demonstrate that a very high level of protection against a normally lethal challenge with *L. major* can be achieved by immunization of BALB/c mice with a single dose of BMDC

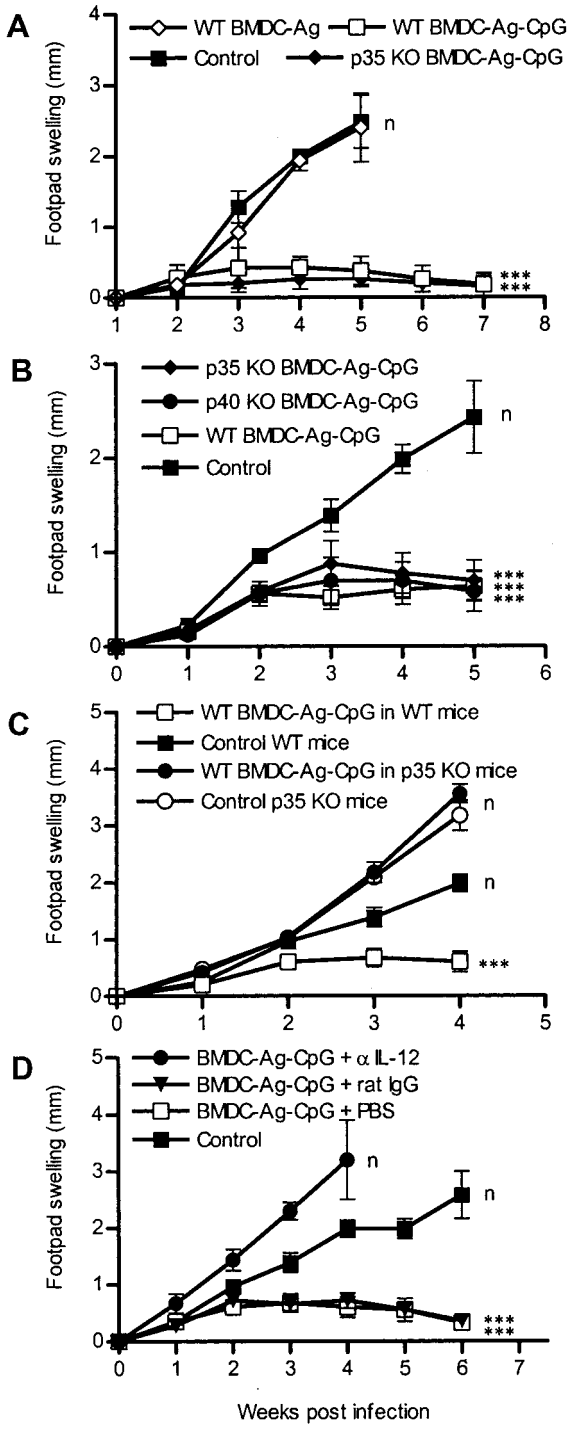

FIGURE 7. The protective effect of CpG ODN-activated BMDC delivering LmAg is not dependent on IL-12 expression by donor BMDC, but requires the availability of recipient-derived IL-12 at the time of BMDC-based vaccination. *A*, WT BALB/c mice were immunized with Ag-pulsed BMDC or CpG/Ag-BMDC derived from WT or IL-12p35-deficient BALB/c mice. *B*, WT BALB/c mice were immunized with CpG/Ag-BMDC from WT, IL-12p35$^{-/-}$ or IL-12p40$^{-/-}$ BALB/c mice. Controls (*A* and *B*) received PBS. *C*, WT or IL-12p35$^{-/-}$ BALB/c mice were treated with CpG/Ag-BMDC from WT BALB/c mice. Control WT or IL-12p35$^{-/-}$ mice did not receive cells. *D*, One day before i.v. immunization with CpG/Ag-BMDC from WT mice, WT BALB/c mice were treated i.p. with 1 mg of anti-IL-12p70 mAb, 1 mg of rat IgG, or PBS. Control mice were treated i.v. with PBS and received neither cells nor Ab. One week later, mice were infected with *L. major*, and footpad swelling was determined weekly. ***, $p < 0.0005$ compared with the control group. n, severe footpad necrosis.

that had been pulsed with LmAg and activated with CpG ODN ex vivo. Complete and durable protection of *L. major*-susceptible BALB/c mice was observed consistently ($n > 50$, seven independent experiments). Such a substantial protection against leishmaniasis has to date been reported only for DNA vaccination or when IL-12 was used as an adjuvant and administered repeatedly (10, 23, 24). Given their exquisite capability to activate T cells, DC are being considered as natural adjuvants for the treatment of tumors and the prophylaxis of infectious diseases (25). We reported previously that Ag-pulsed Langerhans cells, DC of the skin, can protect susceptible mice from leishmaniasis (9, 15). However, Langerhans cells pulsed with LmAg did not confer complete protection, and, of major concern with regard to the potential clinical use of DC, it is very difficult to obtain large numbers of pure Langerhans cells. These constraints prompted us to explore alternative approaches. The protocol described in the present report provides the basis for the development of more powerful strategies of DC-based vaccination. It allows the standardized generation of sufficient amounts of appropriately activated DC from precursors in the blood or bone marrow. In addition, it documents the importance of the type of signal used for DC activation.

Neither TNF-α treatment nor CD40 ligation enabled LmAg-pulsed BMDC to mediate protection against leishmaniasis. This observation is consistent with several reports showing that DC exposed to these stimuli in the absence of microbial signals secrete low levels of IL-12 (18, 19, 26) and do not exhibit Th1-polarizing activity in vitro (4, 26). In contrast, TLR ligands such as LPS and CpG ODN were shown to be potent inducers of IL-12 production by DC (2, 3, 18, 19, 27, 28), which, in the case of CpG ODN, can be further enhanced by CD40 ligation (27). The present study provides a fundamental extension of these previous findings because it demonstrates that CpG ODN directly activates the ability of DC to instruct the differentiation of fully competent Th1 cells in vivo that confer complete protection against an intracellular pathogen. Strikingly, systemic administration of CpG ODN was not required. In this report, CpG ODN were not used as a vaccine adjuvant for injection into mice. It was sufficient to expose BMDC to CpG ODN during LmAg pulsing ex vivo before use of the cells for vaccination. Thus, the approach described in this study differs substantially from those reported previously. Systemic treatment of mice with CpG alone (29) as well as its administration as an adjuvant in vaccine formulations (30–33) have been shown to protect against leishmaniasis and other intracellular infections (17) by establishing a Th1-like milieu. The present report demonstrates for the first time that the direct effect of CpG ODN on the myeloid DC subset is sufficient to activate their potential to promote complete resistance and that a cooperative effect on various DC subsets, as previously suggested by others (33), is not required. Therefore, our findings elucidate the mechanism of CpG ODN functions. A single injection of LmAg-pulsed BMDC that had been activated with CpG ODN ex vivo mediated maximal levels of protection. In fact, the specific targeting of DC according to the protocol described in this study may have considerable advantages, as bacterial DNA has been shown to activate potentially autoreactive T cells (34) and promote septic shock resulting from an overshooting cytokine release by macrophages (35). Furthermore, the effective dose window of CpG ODN upon direct inoculation as an adjuvant was demonstrated to be narrow, with higher doses leading to decreased protection (31). This was not observed with the BMDC-based vaccine approach described in this study. Together, these findings emphasize the relevance of directly targeting DC in intervention strategies aimed at infections associated with cellular immune responses.

Understanding the mechanisms by which DC determine the class of T cell immune response is of central importance to harness the processes that lead to effective immunity. It has recently been shown that murine myeloid $CD8^-$ DC, previously thought to induce Th2 responses, also promote Th1 responses when stimulated with appropriate microbial signals (3, 5, 36), and that CpG ODN and other bacterial products predispose both $CD8^+$ and $CD8^-$ DC to secrete IL-12 (27). Both murine plasmacytoid DC and myeloid DC are able to induce effector Th1 or Th2 cells in vitro depending on the dose of Ag presented and the presence of microbial stimuli (37). It has also been reported that the selective priming of T cells producing IFN-γ or IL-4 in vitro by DC subsets from mouse spleen is primarily determined by the type of microbial signal used for activating the DC, and that the intrinsic capacity of different DC subsets to polarize Th cell development is only weak (5). A similar plasticity is emerging for human DC (4, 38); human plasmacytoid DC, initially proposed to promote Th2 responses, are now known to drive Th1 differentiation after virus infection (38). These observations support the concept that the nature of the microbial stimulus determines Th polarization by a given DC subset. However, it has not yet been evaluated whether a physiologically relevant Th1 response with impact on disease is induced by activated DC in vivo. In the present study the essential new finding is that an effective pathogen-restricting Th1 response can readily be induced in vivo by CpG-activated murine myeloid DC presenting Ag from that pathogen, but not by Ag-pulsed DC treated with other stimuli. Notably, short term exposure of BMDC to CpG ODN for enhanced maturation after basal stimulation with GM-CSF was sufficient to trigger the immunomodulatory and protective potential of DC.

An unexpected finding of the present study was that the level of IL-12 released by BMDC did not correlate with their capacity to mediate protection against leishmaniasis, as the protective BMDC population activated with CpG ODN and pulsed with LmAg did not express high amounts of IL-12. The LmAg-induced reduction of the IL-12 production by CpG-activated BMDC was a consistent finding. Whether this is an LmAg-specific effect or a more general phenomenon related to interference of Ag with TLR ligation of DC remains an open question. Interestingly, LmAg-loaded BMDC stimulated with LPS produced IL-12 levels comparable to those released by CpG-activated and Ag-pulsed BMDC, but were unable to mediate protection (data not shown), also indicating that IL-12 derived from the vaccinating donor DC may not be the critical parameter for induction of protective immunity. This suggestion was confirmed by the finding that IL-12 deficiency did not impair the ability of Ag-pulsed BMDC to confer resistance. The protective efficacy of Ag-loaded BMDC from IL-12p35$^{-/-}$ or IL-12p40$^{-/-}$ mice was virtually identical with that of BMDC obtained from WT mice, excluding also a role of the related cytokine IL-23, which shares the p40 subunit with IL-12. These results were in sharp contrast to our recent study with epidermal Langerhans cells as the source of DC. Ag-pulsed Langerhans cells from IL-12-deficient mice, as opposed to those from WT mice, completely failed to mediate protection against *L. major* (9). Thus, our data point to the importance of the type of DC used for vaccination approaches. They suggest fundamental differences in the immunological mechanisms underlying immunization with distinct DC populations. Indeed, several pieces of evidence demonstrate that ex vivo DC differ from in vitro generated DC with regard to various biological properties (37, 39, 40), including the dependence on IL-12 for Th1 induction (37).

The essential role of IL-12 in the establishment and maintenance of immunity to *L. major* is well recognized (10, 24, 41, 42). Therefore, it was not surprising that treatment of IL-12-deficient mice with CpG-activated BMDC delivering LmAg did not result in protection, demonstrating that IL-12 released by recipient cells is required. This is in agreement with a report showing that recipient IL-12 production is necessary for Th1 development induced by transferred BMDC (8). Importantly, our data document that the availability of recipient IL-12 is essential for the initiation of a protective immune response by DC, as transient neutralization of IL-12 activity in WT recipient mice at the time of BMDC-mediated immunization and T cell priming completely abrogated the potential of DC to induce protection. Together with the finding that donor BMDC-derived IL-12 was not required, these results suggest that CpG activation enables Ag-pulsed BMDC to trigger antileishmanial immunity via mechanisms that are not dependent on their ability to release IL-12 and possibly involve other Th-polarizing cytokines (37, 43), but rely on their induction of IL-12 in cells of the vaccinated recipient mice. The immunizing DC delivering LmAg may trigger recipient IL-12 production by direct or indirect interaction with resident DC and/or other cell types, such as polymorphonuclear leukocytes. Neutrophils produce a series of proinflammatory cytokines, including IL-12, and it has recently been demonstrated that the cross-talk between DC and neutrophils after pathogen encounter leads to Th1 cell induction (44). Other factors, derived from neutrophils or DC, may synergize in promoting a Th1 response and ultimately immunity to infection. Moreover, the bidirectional interaction between NK cells and DC might support the development of type 1 immune responses (45, 46). Although the source of recipient-derived IL-12 and the mechanisms of its induction in the model of DC-based vaccination against leishmaniasis described in this study remain to be defined, our data clearly demonstrate that the ability of DC to produce IL-12 in response to microbial stimuli is not the only parameter determining their immunomodulatory and protective functions in vivo.

Our observations have significance for the understanding of Th polarization by DC in vivo and the development of immune intervention strategies. In particular, they show that DC are guided by the conditions of their activation to drive the immune response to a pathogen in vivo. The protocol for in vitro generation of DC inducing highly effective antileishmanial activity in the host, as described in this study, can be readily adapted to other models of infectious diseases and may help to exploit DC for anti-infective therapies.

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