Dendritic Cell (DC)-Based Protection Against an Intracellular Pathogen Is Dependent Upon DC-Derived IL-12 and Can Be Induced by Molecularly Defined Antigens[1]

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Upon loading with microbial Ag and adoptive transfer, dendritic cells (DC) are able to induce immunity to infections. This offers encouragement for the development of DC-based vaccination strategies. However, the mechanisms underlying the adjuvant effect of DC are not fully understood, and there is a need to identify Ag with which to arm DC. In the present study, we analyzed the role of DC-derived IL-12 in the induction of resistance to *Leishmania major*, and we evaluated the protective efficacy of DC loaded with individual *Leishmania* Ag. Using Ag-pulsed Langerhans cells (LC) from IL-12-deficient or wild-type mice for immunization of susceptible animals, we showed that the inability to release IL-12 completely abrogated the capacity of LC to mediate protection against leishmaniasis. This suggests that the availability of donor LC-derived IL-12 is a requirement for the development of protective immunity. In addition, we tested the protective effect of LC loaded with *Leishmania* homolog of receptor for activated C kinase, gp63, promastigote surface Ag, kinetoplastid membrane protein-11, or *Leishmania* homolog of eukaryotic ribosomal elongation and initiation factor 4a. The results show that mice vaccinated with LC that had been pulsed with selected molecularly defined parasite proteins are capable of controlling infection with *L. major*. Moreover, the protective potential of DC pulsed with a given *Leishmania* Ag correlated with the level of their IL-12 expression. Analysis of the cytokine profile of mice after DC-based vaccination revealed that protection was associated with a shift toward a Th1-type response. Together, these findings emphasize the critical role of IL-12 produced by the sensitizing DC and suggest that the development of a DC-based subunit vaccine is feasible. ***The Journal of Immunology,*** **2003, 170: 3171–3179.**

Effective protection from infections with intracellular microorganisms requires the induction of cell-mediated immune responses. To gain effector functions, Th cells and CTL need to be activated by professional APC, in particular dendritic cells (DC).[3] Cells of the DC network monitor tissues for microbial Ag and are highly specialized for presentation of these Ag to quiescent T cells in lymphoid organs. Thus, they are the principal activators of T and B cell immunity in vivo (1, 2). Recent studies revealed an extraordinary plasticity of DC-induced T cell activation which may result in selective priming of Th1- or Th2-mediated immunity (3, 4). Moreover, evidence has been provided that the nature of the pathogen-derived stimulus and the kinetics of DC activation may profoundly influence the capacity of DC to modulate the quality of the developing T cell response (5). The ability of DC to produce IL-12 in response to microbial signals is well-documented and is considered to be critical for the induction of a Th1 response (6, 7).

The pivotal role of DC in priming immune responses has prompted hopes that DC, upon specific loading with Ag, may serve as natural adjuvants. Indeed, several studies have documented the therapeutic potential of DC-based immune interventions in a variety of murine tumor models (8–10) and, more recently, in clinical trials (11–13). However, only a few studies so far have explored the in vivo efficacy of DC-mediated vaccination in infectious disease settings (14). For the elaboration of the parameters that are relevant to the development of anti-infective vaccination strategies based on DC, we chose the model of experimental infection of mice with the protozoan parasite *Leishmania major*. In this model, the immunological mechanisms underlying susceptibility or resistance of mice to parasite infection are well-characterized. A plethora of data demonstrates that protective immunity is associated with the development of Th1 responses, while a Th2 cytokine pattern prevails in mice that succumb to infection (15). Furthermore, DC have been shown to play a decisive role in the initiation, regulation, and maintenance of *Leishmania*-specific T cell responses (16–19). We reported previously that adoptively transferred epidermal Langerhans cells (LC) loaded ex vivo with crude parasite lysate are potent inducers of *L. major*-specific Th1-dominant immune responses that confer protection to genetically susceptible BALB/c mice against challenges with virulent parasites (20). These findings offer encouragement for the development of a DC-based vaccine against leishmaniasis and perhaps other infectious diseases. To this end, it is important to understand the immunizing properties of Ag-pulsed DC mediating protection, and to identify defined *Leishmania* Ag(s) that are suitable for effective DC-based vaccination strategies.

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[3] Abbreviations used in this paper: DC, dendritic cell; LC, Langerhans cell; LACK, *Leishmania* homolog of receptor for activated C kinase; LeIF, *Leishmania* homolog of eukaryotic ribosomal elongation and initiation factor 4a; PSA, promastigote surface Ag; KMP-11, kinetoplastid membrane protein-11; IPTG, isopropyl-β-D-thiogalactopyranoside; i.d., intradermal; LC-LeIF, LeIF-pulsed LC; WT, wild type.

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In the present study, we determined the role of DC-derived IL-12 for the education of protective Th1 cells, and we evaluated the potential of individual *Leishmania* Ag to mediate protection upon delivery by DC. For this purpose, several prominent *Leishmania* Ag were chosen that have already been shown to be strongly immunogenic. The *Leishmania* homolog of receptor for activated C kinase (LACK) is a highly conserved intracellular protein that has been shown to play an important role in the immunopathogenesis of experimental *L. major* infections (21, 22), and vaccination with DNA encoding LACK induces protection against a challenge with parasites (23). *Leishmania* homolog of eukaryotic ribosomal elongation and initiation factor 4a (LeIF) is a *Leishmania* protein that elicits IL-12 production and a Th1 response of human PBMCs (24). The protective *Leishmania* surface protease gp63 (25) has been implicated in infectivity and intracellular survival of the parasites (26). The promastigote surface Ag (PSA) may be involved in cell-cell interactions during the infection process (27). Finally, the protective properties of the kinetoplastid membrane protein-11 (KMP-11) of *Leishmania* have been recently studied in a live vaccine (28). Our data demonstrate that 1) the ability of adoptively transferred DC to protect against leishmaniasis is critically dependent on their potential to release IL-12 and 2) single Ag in a DC-based vaccine are able to mediate high levels of protection. These findings indicate that the development of DC-based subunit vaccines with molecularly defined components is feasible.

Materials and Methods

Mice

Female BALB/c mice were 6- to 8-wk-old at the onset of the experiments. The mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany) and, during experimentation, were kept under conventional conditions in an isolation facility. IL-12p35$^{-/-}$ mice were generated as described (29) and backcrossed into a BALB/c background (30).

L. major *cultivation and preparation of parasite lysate*

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.

Cloning, recombinant expression, and purification of Leishmania *Ag*

Cloning of the *Leishmania* Ag LACK, gp63, PSA, and KMP-11 into the bacterial expression vector pQE30 (Qiagen, Hilden, Germany) and expression as recombinant proteins in *Escherichia coli* was performed as follows. The 0.95-kb LACK cDNA was excised from the original plasmid pET3a (31), using the restriction enzymes *Eco*RI and *Hin*dIII, subcloned into the pBluescript SK$^-$ vector (Stratagene, Heidelberg, Germany) and finally introduced, in frame, into the polylinker of pQE30. Correct cloning was confirmed by sequence analysis. The 36-kDa full length LACK Ag was expressed in *E. coli* induced for 2 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) as an N-terminal His-tagged fusion protein. Bacteria were harvested from 1-L batch cultures by centrifugation, resuspended in denaturing lysis buffer (8 M urea; 0.1 M sodium phosphate; 0.01 M Tris-HCl, pH 8.0), and sonicated on ice. The lysate was centrifuged to pellet the bacterial debris. The rLACK protein was purified from the clear supernatant by affinity chromatography using the Ni-NTA agarose matrix (Qiagen). Stringent washing steps of the agarose-bound material in the presence of a pH gradient (lysis buffer, pH 8.0 to 5.5) allowed removal of contaminating proteins associated with the His-tagged LACK. Elution of the recombinant protein was performed in lysis buffer, pH 4.5. The fractions with the highest concentration of rLACK were pooled and monitored for purity by SDS-PAGE. In the subsequent immunoblot analysis, a single protein band corresponding to 36 kDa reacted strongly with a LACK-specific Ab (32). To further improve the purity of LACK and to exclude endotoxin contaminations, the recombinant protein was electroeluted from corresponding gel slices after preparative SDS-PAGE of the elution fractions. LPS levels were typically $<$10 U endotoxin/mg protein, as determined by the *Limulus* amebocyte assay (Sigma-Aldrich, Heidelberg, Germany). In a final step, the electroeluted protein was dialyzed against 1$\times$ PBS.

For recombinant expression of gp63, a 2.1-kb fragment encompassing the open reading frame of a member of the C2 subclass of the *Leishmania mexicana* gp63 genes (33), except for the propeptide sequence and the first 64 N-terminal residues of the mature protein, was excised from pSK-4 using *Pst*I and *Hin*dIII. The fragment was subcloned into pBluescript SK$^-$ and finally introduced, in frame, into the polylinker of pQE30. Correct cloning was confirmed by sequence analysis. Induction of recombinant protein expression in *E. coli* in the presence of 1 mM IPTG for 4 h yielded a truncated version of gp63 with a molecular mass of ~48 kDa. Purification from bacterial extract was performed as described above. In the immunoblot analysis, a single band with some minor degradation products strongly reacted with a gp63-specific Ab (33). Endotoxin levels in rgp63 were typically $<$10 U endotoxin/mg protein.

A 1.8-kb PCR-amplified fragment containing the PSA-coding region of *Leishmania infantum* without the signal peptide (34) was introduced, in frame, into the polylinker of pQE30. Sequence analysis confirmed correct cloning. Induction of recombinant protein expression in *E. coli* was performed in the presence of 1 mM IPTG for 4 h and yielded a His-tagged fusion protein of PSA with a molecular mass of ~48 kDa. Purification from bacterial extract by affinity chromatography was performed as described above, but no electroelution step was included in the purification of rPSA because the protein yield after expression in *E. coli* was very low. The endotoxin levels in rPSA were $<$100 U endotoxin/mg protein.

The KMP-11 open reading frame of *L. major* was amplified by PCR using oligonucleotide primers (CB1 and CB2) which have been shown previously to be highly specific for the amplification of KMP-11 from genomic *Leishmania* DNA (35). The amplified 0.3-kb fragment was introduced, in frame, into pQE30. Correct cloning was confirmed by sequence analysis. Induction of expression of the 12-kDa recombinant protein in *E. coli* was performed in the presence of 1 mM IPTG for 4 h. Because rKMP-11 remains soluble to a large extent in the induced *E. coli* cultures, protein purification was performed under native conditions. Bacteria were resuspended in native lysis buffer (0.1 M sodium phosphate, pH 8.0; 0.01 M Tris-HCl, pH 8.0, containing 2 mM PMSF and 20 μg/ml leupeptin), sonicated, and centrifuged to remove bacterial debris. The recombinant protein was purified from the clear supernatant by affinity chromatography using the Ni-NTA agarose matrix (Qiagen). The agarose-bound material was washed several times in the presence of an imidazole gradient (20–100 mM in native lysis buffer) to remove contaminating proteins associated with the His-tagged KMP-11. Elution of rKMP-11 was performed with 250 mM imidazole in native lysis buffer. The immunoblot analysis of the eluted protein yielded a single protein band at 12 kDa which strongly reacted with a KMP-11-specific Ab (36). The recombinant protein was further purified by an electroelution step as described above. The endotoxin level in rKMP-11 was $<$10 U endotoxin/mg protein.

The LeIF Ag from *L. major* comprising amino acid residues 1–226 was produced as previously described (37) and further purified by HPLC. The endotoxin level in rLeIF was $<$10 U endotoxin/mg protein.

Preparation and Ag pulsing of LC

Epidermal LC suspensions were prepared from mouse ear skin by trypsinization procedures as previously described (38). Briefly, the ventral, thick ear halves were incubated in a solution of 1% trypsin in PBS for 90 min, and the dorsal, thin ear halves were treated with 0.6% trypsin in PBS for 45 min. These preparations contained ~3–5% of LC that constitutively express MHC class II as well as MHC class II-negative keratinocytes, a source of GM-CSF that is essential for LC differentiation. The proportion of LC was determined by fluorescence labeling of MHC class II-positive cells. The preparations were completely devoid of macrophages (38), as documented by the lack of staining with mAb F4/80 (fluorescence labeling). For Ag pulsing, epidermal cell suspensions were cultured for 20 h in Click RPMI 1640 medium (Biochrome, 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 *L. major* lysate (equivalent to 10^7 parasites per 3×10^6 cells per ml) or in the presence of recombinant Ag. For the Ag mixture of rLACK, rgp63, rPSA, and rKMP-11, 70 μg were used for 3×10^6 cells per ml; for rLACK or rLeIF, respectively, 25 μg were used. Control cultures were set up in the absence of parasite Ag.

Treatment of mice

After Ag pulsing, the nonadherent fractions of epidermal cells, containing 30–50% viable LC (as determined by staining for MHC class II expression), were harvested. Cells were washed twice with PBS to remove soluble, nonphagocytosed, parasite Ag, and 2–3 $\times$ 10^5 LC were administered

i.v. into a tail vein of naive BALB/c mice. Control mice were treated with PBS or unpulsed LC. One week later, mice were infected intradermally (i.d.) with 2×10^5 stationary phase promastigotes into the right hind footpad using a syringe with a particularly fine needle (30G). The course of infection was monitored weekly by measuring the increase in footpad size of the infected vs the noninfected footpad using an engineering micrometer. Mice were sacrificed when severely necrotic skin lesions became apparent.

Limiting dilution analysis

For the evaluation of the frequency of *L. major*-infected cells in the lymph nodes draining the infected footpads, single cell suspensions were prepared at the time points indicated. Serial dilutions of lymph node cells ranging from 10^5 cells/ml to 15 cells/ml were seeded into microculture plates (100 μl/well) containing 50 μl of a blood-agar slant to support the growth of parasites. For each dilution, replicates of 20 wells were set-up. After 8 to 10 days of incubation at 28°C in a humidified atmosphere with 5% CO_2, the cultures were scored for the presence of parasites using an inverted microscope. Minimal estimates of the frequency of *L. major*-infected cells were obtained by the minimum χ^2 method from the Poisson distribution relationship between the number of cells and the logarithm of the fraction of negative cultures by using a computer program (39).

Lymphokine assays

Popliteal lymph nodes draining the infected footpads or spleens were removed at different time points before or after infection as indicated. After preparation of single cell suspensions, 2×10^6 cells/ml were cultured in 24-well plates (Sarstedt, Nümbrecht, Germany) in the presence of *L. major* lysate (equivalent to 1 or 3×10^6 parasites) or rLeIF (3 μg) or in the absence of Ag. Supernatants were collected 48 or 72 h later. The levels of IL-2, IFN-γ, IL-10, and IL-4 were determined by sandwich ELISA as described previously (20). Standard curves were generated using recombinant mouse cytokines (R&D Systems, Wiesbaden, Germany). The detection limits were 150 pg/ml for IFN-γ and IL-10, 750 pg/ml for IL-2, and 50 pg/ml for IL-4.

Analysis of IL-12 expression by DC

Epidermal LC suspensions were prepared as described above and resuspended in culture medium. Cultures containing 3×10^5 cells in 1 ml were supplemented with 25 μg/ml of the recombinant *Leishmania* Ag LeIF, LACK, or KMP-11. Bone marrow-derived DC were prepared as described by Lutz et al. (40). Briefly, freshly prepared bone marrow cells were cultured in medium containing 200 U/ml GM-CSF and cultures were fed with GM-CSF at days 3, 6, and 8. On day 10, the cells were collected and 1×10^6 cells/ml were cultured in 24-well plates in medium containing GM-CSF and the recombinant *Leishmania* Ag. Control cultures of LC and bone marrow-derived DC were incubated in the absence of Ag or in the presence of LPS (10 μg/ml). To exclude IL-12 induction by residual LPS contamination in the recombinant protein preparations, equivalent cultures were set up with polymyxin B (50 μg/ml). After 24 h of incubation, cells were separated from culture supernatants by centrifugation. Cells were used for total RNA extraction (RNeasy kit; Qiagen) and 2 μg of RNA were reverse-transcribed (cDNA Omniscript RT kit; Qiagen). The PCR to estimate the relative amounts of the respective mRNA was performed using the following primers: IL-12p35 antisense, 5′-GCC GGC TAT CCA GAC AAT TA-3′; IL-12p35 sense, 5′-CTA CCA AGG CAC AGG GTC AT-3′; IL-12p40 antisense, 5′-ATG GCC ATG TGG GAG CTG GAG-3′; IL-12p40 sense, 5′-TTT GGT GCT TCA CAC TTC AGG-3′. β-actin primers were used to normalize the amount of template RNA (β-actin antisense, 5′-TGT GAT GGT GGG AAT GGG TCAG-3′; β-actin sense, 5′-TTT GAT GTC ACG CAC GAT TTC C-3′). PCR conditions were as follows: 94°C for initial denaturation (2 min), amplification with 35 cycles of 94°C (1 min) for denaturation, 58°C (1 min) for annealing, 72°C (1 min) for elongation, and final extension at 72°C (10 min). The levels of IL-12p70 in the culture supernatants were determined by a sandwich ELISA, using purified rat anti-mouse IL-12p70 mAb (BD PharMingen, Hamburg, Germany) for capture and a biotin-labeled anti-mouse IL-12p40/p70 mAb (BD PharMingen) for detection. The detection threshold was 122 pg/ml.

Statistical analysis

The Student *t* test was used for statistical analyses. Differences were considered significant when $p < 0.05$.

Results

The availability of donor DC-derived IL-12 is required for the induction of protective immunity

DC have been shown to release IL-12 in response to activation by microbial Ag (7, 18, 19, 41, 42). Furthermore, our previous findings demonstrated that DC pulsing with *L. major* lysate induces IL-12 expression (20). These observations suggest that production of the Th1-polarizing cytokine IL-12 by the Ag-bearing DC may be an important parameter for the initiation and development of a protective T cell immune response in mice vaccinated with Ag-loaded DC. To test this hypothesis, we compared the potential of adoptively transferred LC obtained from IL-12-deficient mice with that of LC from wild-type (WT) mice to mediate protection against a challenge with *L. major* parasites. Freshly prepared LC from WT BALB/c mice or from IL-12p35$^{-/-}$ BALB/c mice were loaded ex vivo with parasite lysate and were injected i.v. into WT BALB/c recipients. One week later, mice were infected i.d. with *L. major* parasites and the course of infection was monitored by measuring the lesion size at weekly intervals. The results show that *L. major* Ag-loaded LC from IL-12p35-deficient mice failed to induce protection, as opposed to equivalently pulsed LC from WT mice (Fig. 1). Mice that had been treated with Ag-pulsed LC from IL-12-deficient mice exhibited progressive disease with large lesions that were comparable in size to those of the control animals. By 7 wk of infection, the skin lesions were strongly necrotic and the mice had to be sacrificed, whereas there was no indication of skin necrosis and no disease progression in mice immunized with LC from WT mice. Determination of the frequency of parasitized cells in the lymph nodes draining the lesional skin revealed that the ability of mice vaccinated with WT LC to control the disease was associated with an approximately five times lower parasite burden when compared with the nonimmunized control group or the group treated with LC from IL-12-deficient mice (Table I). These results show that the protection induced by *Leishmania*-pulsed DC depends upon the ability of the donor DC to produce IL-12.

DC pulsed with molecularly defined parasite Ag are able to confer significant levels of protection against L. major *infection*

For the development of a DC-based subunit vaccine against an infectious disease, the choice of microbial Ag(s) for DC loading is

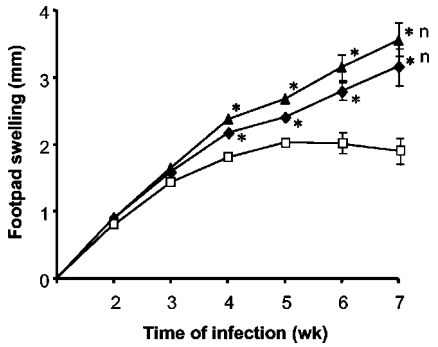

FIGURE 1. DC-derived IL-12 is required for the control of *L. major* infection. BALB/c mice were immunized i.v. with 2–3 $\times$ 10^5 *L. major* lysate-pulsed LC derived from WT mice (□) or IL-12p35-deficient mice (▲). Control animals were treated with PBS (♦). One week later, animals were infected i.d. with *L. major* promastigotes into the right hind footpad. The differences in the thickness of the infected footpad compared with the noninfected contralateral footpad were determined weekly. The results show the mean ± SD of five mice per group and are representative of two independent experiments. *, Values that were significantly different ($p <$ 0.01) from those of the protected animals (mice treated with lysate-pulsed LC derived from WT mice); "n" denotes severe footpad necrosis.

Table I. *Frequency of parasitized lymph node cells from mice vaccinated with* L. major *Ag-pulsed LC from WT mice or from IL-12-deficient mice*

Mice Treated With	Reciprocal of Frequency of Infected Cells[a]
PBS	105 (94–118)[b]
Ag-pulsed LC, WT	522 (445–631)
Ag-pulsed LC, IL-12$^{-/-}$	125 (112–141)

[a] Eight weeks after infection, single cell suspensions were prepared from lymph nodes draining the site of infection. Lymph node cells were seeded into blood agar-containing microculture plates by using limiting dilution techniques. After 8 days of culture, plates were scored for the growth of parasites. The data represent the mean values from groups of five mice.

[b] Confidence limits, 95%.

going to have a profound influence on the efficacy of vaccination. The Ag preparation should be molecularly defined and it should be possible to manufacture it in a reproducible manner. Therefore, we chose several well-characterized *Leishmania* proteins that have already been documented to be highly immunogenic. In a first set of experiments, a mixture of the *Leishmania* proteins LACK, gp63, PSA, and KMP-11 was used for pulsing of LC in vitro and subsequent immunization of mice. The Ag were expressed as recombinant proteins in *E. coli* and purified under vigorous conditions to exclude bacterial contaminants. The preparations of LACK, gp63, and KMP-11 were virtually free of endotoxin (<10 U endotoxin/mg protein), and PSA contained <100 U/mg, as determined by the *Limulus* amebocyte assay. One week after i.v. injection of Ag-loaded LC, the animals were challenged by i.d. inoculation of virulent *L. major* parasites. Vaccination with LC that had been pulsed with the mixture of recombinant *Leishmania* Ag or with parasite lysate greatly affected disease manifestation. The skin lesions were significantly smaller than those of mice that had been treated with PBS (Fig. 2*A*). The size of lesions of vaccinated mice did not increase after 5 wk of infection and there were no signs of skin necrosis. In contrast, the control animals exhibited progressive lesions with severe tissue necrosis by 7 wk postinfection. These results suggest that a DC-based subunit vaccine with a combination of the *Leishmania* proteins LACK, gp63, PSA, and KMP-11 is able to induce high levels of protection against experimental cutaneous leishmaniasis. Subsequently, we tested the immunizing properties of a single component of the Ag mixture. LC were loaded with rLACK alone and used for vaccination according to the same protocol as described above. As shown in Fig. 2*B*, mice treated with LACK-pulsed LC exhibited smaller footpad lesions than the control animals treated with PBS or unpulsed LC, although lesion sizes of mice vaccinated with LACK-pulsed LC tended to be larger than those of the mice immunized with whole parasite lysate-pulsed LC or with LC pulsed with the mixture of recombinant *Leishmania* Ag.

The intriguing properties of the *Leishmania* protein LeIF, which has been shown to be a potent stimulator of the innate immune system (43) and to elicit IL-12 production and Th1-type responses in PBMC from leishmaniasis patients (24), prompted us to explore the efficacy of this Ag in inducing protective immunity to *L. major* infection upon delivery by DC. For this purpose, the N-terminal half of LeIF (aa 1–226), which is known to contain the IL-12/IFN-γ-inducing regions of the molecule (37), was expressed as recombinant protein in *E. coli* and purified by affinity chromatography using the Ni-NTA matrix. LC were loaded with rLeIF in vitro and were subsequently used for immunization of susceptible BALB/c mice. Following infection with *L. major* parasites a week later, the development of skin lesions was monitored. Vaccination with LeIF-pulsed LC (LC-LeIF) had a major impact on the course of the

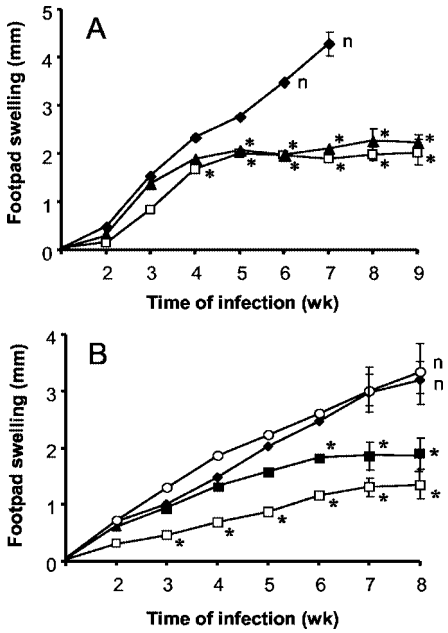

FIGURE 2. LC loaded with the *Leishmania* Ag LACK, gp63, PSA, and KMP-11 mediate high levels of protection against a challenge with *L. major*. BALB/c mice were immunized i.v. with 2–3 × 10^5 LC that had been pulsed ex vivo with a mixture of rLACK, rgp63, rPSA, and rKMP-11 (▲) (*A*) or with rLACK alone (■) (*B*), or with *L. major* lysate (□). Control mice were treated with unpulsed LC (○) or with PBS (◆). One week later, animals were infected i.d. with stationary phase *L. major* promastigotes into the right hind footpad. The increase in size of the infected compared with the noninfected footpad was measured weekly. Data represent the mean ± SD of five mice per group. One of two independent experiments with similar results is shown. *, Values that were significantly different ($p < 0.01$) from those of the nonprotected control animals (PBS-treated mice); "n" denotes severe footpad necrosis.

disease. Mice that had been immunized with this DC-based subunit vaccine developed significantly smaller lesions as compared with control mice and were able to restrain the infection (Fig. 3*A*). In two of three independent experiments, the protection levels induced by LC-LeIF were similar to those observed after immunization with LC pulsed with whole parasite lysate. Comparison of the parasite loads showed that the frequency of parasitized cells in the lymph nodes draining the site of infection was reduced 10-fold in mice immunized with LC-LeIF as compared with unprotected control mice treated with LC alone (Table II). Mice vaccinated with LACK-pulsed LC, resulting in less efficient protection (see above, Fig. 2*B*), showed only an ~3-fold reduction in the frequency of *L. major*-infected lymph node cells. In one experiment, the observation period for the groups protected by treatment with rLeIF- or *L. major* lysate-pulsed LC was extended to 16 wk postinfection. At that time, the skin lesions of three of five mice of the LC-LeIF-treated group and four of five animals immunized with lysate-pulsed LC had completely healed, whereas all of the unprotected control mice had to be sacrificed at 8 wk of infection because of severe tissue necrosis (Fig. 3*B*). Together, these data indicate that a DC-based vaccine delivering the single Ag LeIF is effective in protecting susceptible animals against cutaneous leishmaniasis.

DC pulsed with Leishmania *LeIF express high levels of IL-12*

The distinct capability of LeIF-pulsed DC to mediate protection against leishmaniasis correlated with a high level of IL-12 expression. Upon exposure of DC from skin or bone marrow to the recombinant *Leishmania* Ag LeIF, LACK, or KMP-11, LeIF was the

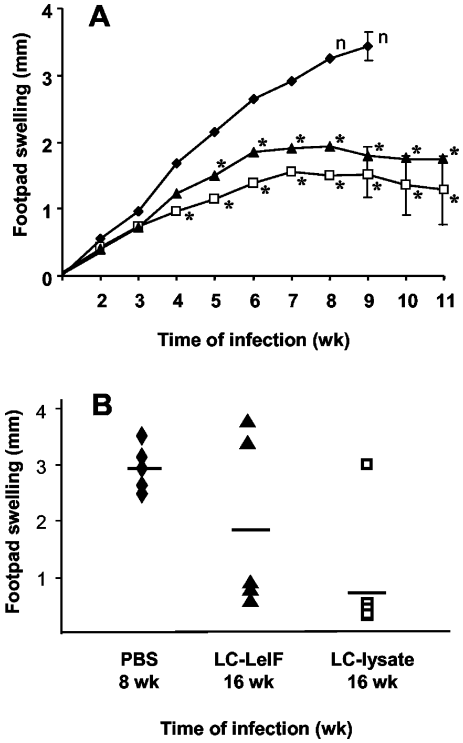

FIGURE 3. Vaccination with LeIF-pulsed LC protects mice from *L. major* infection. BALB/c mice were immunized i.v. with 2–3 × 10^5 LC loaded ex vivo with *L. major* lysate (□) or rLeIF (▲). Control mice were treated with PBS (♦). One week later, animals were infected i.d. with stationary phase *L. major* promastigotes into the right hind footpad. The increase in size of the infected compared with the noninfected footpad was measured thereafter. Effect on (*A*) the course of footpad swelling and (*B*) the footpad swelling of individual mice after 8 wk (PBS-treated controls) or 16 wk (mice treated with *L. major* lysate-pulsed LC, LC-lysate, or LeIF-pulsed LC, LC-LeIF) of infection. *A*, The symbols and bars represent the mean ± SD of five mice per group. One of three independent experiments with similar results is shown. ∗, Values that were significantly different from those of the nonprotected PBS-treated control mice at the same time points ($p < 0.01$); "n" denotes severe footpad necrosis. *B*, In one experiment, the observation period for the protected groups was extended to 16 wk postinfection, while the PBS-treated control mice had to be sacrificed at 8 wk because of severe footpad necrosis. Bars represent the mean.

most potent inducer of IL-12, whereas the other Ag triggered a much weaker or no response (Fig. 4). When highly pure DC from the bone marrow were stimulated with the different *Leishmania* Ag, only LeIF induced the formation of bioactive IL-12p70 molecules (Fig. 4*B*) and the level of LeIF-stimulated IL-12p70 production was similar to that observed after treatment of DC with bacterial LPS as control. These results suggest that leishmanial proteins with a high protective potential in the DC vaccine regimen, such as LeIF, activate DC to express high levels of IL-12.

Protection mediated by a DC-based subunit vaccine correlates with changes in the cytokine production

The findings described above show that mice immunized with DC that had been pulsed with molecularly defined *Leishmania* Ag, or combinations of Ag, are able to control the development of skin lesions and have a significantly reduced parasite burden. Next we determined whether the protection mediated by DC-based vaccination is associated with alterations in the cytokine profile. Animals were sacrificed after 11 wk of infection and single cell suspensions were prepared from the popliteal lymph nodes draining the lesions. The lymph nodes from nonprotected control mice were

Table II. *Frequency of parasitized lymph node cells from mice vaccinated with LC-lysate, LC-LeIF, or LC-LACK*[a]

Mice Treated With	Reciprocal of Frequency of Infected Cells[b]
LC only	172 (150–203)[c]
LC-LeIF	1965 (1548–2746)
LC-LACK	600 (476–827)
LC-lysate	3051 (2251–4835)

[a] LC-lysate, *L. major* lysate-pulsed LC, LC-LeIF, *L. major* LeIF-pulsed LC; LC-LACK, *L. major* LACK-pulsed LC.
[b] Lymph nodes were collected at 6 wk after infection. For culture conditions, see footnote of Table I. Data show the results of one of two independent experiments with similar results.
[c] Confidence limits, 95%.

~2- to 3-fold larger than those from protected animals (data not shown). Cells were cultured in the absence or presence of *Leishmania* Ag for subsequent determination of the cytokine production by ELISA. The levels of IFN-γ production in response to *L. major* Ag were found to be 2- to 4-fold higher in mice protected from

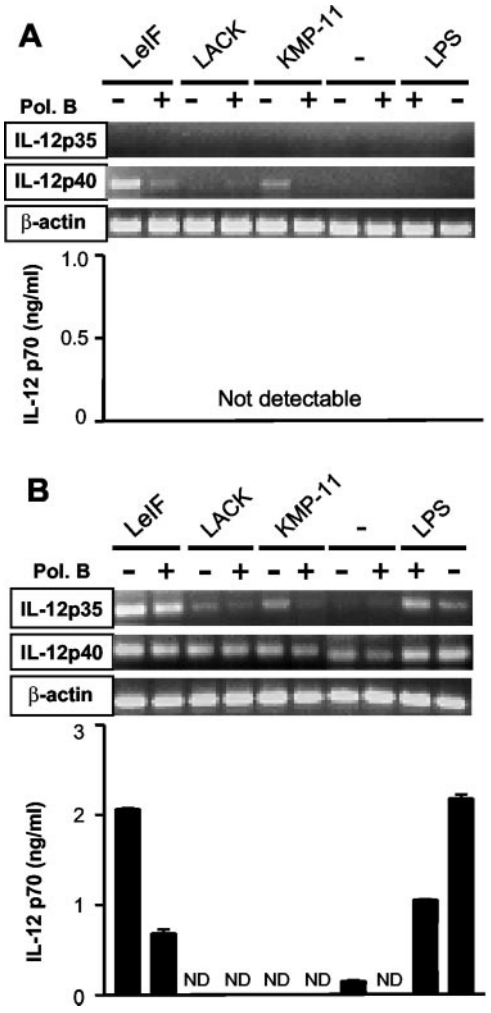

FIGURE 4. IL-12 levels in LC (*A*) and bone marrow-derived DC (*B*) after treatment with recombinant *Leishmania* Ag. Controls were untreated or stimulated with bacterial LPS. After incubation in the presence or absence of stimuli for 24 h, the cells were assayed for mRNA of IL-12p35, IL-12p40, or β-actin by RT-PCR. Culture supernatants were assayed for IL-12p70 production by ELISA. To reveal IL-12 induction by residual LPS contamination in the recombinant protein preparations, DC cultures were set up in the absence or presence of polymyxin B (Pol. B). ND, not detectable.

infection by immunization with rLeIF- or *L. major* lysate-pulsed LC when compared with PBS-treated mice with uncontrolled lesion development (Fig. 5). In contrast, IL-4 production was 2- to 3-fold higher in the animals with progressive disease. Significant differences in the production of the anti-inflammatory cytokine IL-10 were also observed. The amounts of IL-10 were 3- to 4-fold increased in PBS-treated control mice. In the unprotected control group, in vitro restimulation with *L. major* Ag did not further enhance the production of IL-4 and IL-10, presumably due to the presence of large amounts of endogenous parasite Ag in these lymph node cell cultures. In conclusion, these data suggest that DC-based vaccination redirects the Th2 response normally developing in susceptible BALB/c mice toward a Th1-type cytokine profile that is associated with protection.

Vaccination with Ag-pulsed LC induces a primary T cell response

The cytokine milieu that is present at the initiation of infection has been shown to instruct the developing immune response and to be critical for the outcome of the disease (22, 44). To determine whether the protective treatment with Ag-loaded DC mediates the primary stimulation of cytokine-producing cells, we analyzed the cytokine profile in the lymph nodes and spleens of immunized mice before the challenge with parasites. Mice were immunized i.v. with LC-LeIF or with LC pulsed with whole parasite lysate and were sacrificed 1 wk later. Single cell suspensions of popliteal lymph nodes and spleens were prepared and restimulated with parasite lysate or rLeIF for the subsequent assessment of cytokines released into the culture supernatants (Fig. 6). The elevated levels of IL-2 produced by spleen cells from LC-LeIF- and LC-lysate-treated animals indicated that the vaccination protocol induces significant T cell activation. Most notably, splenocytes from these mice produced elevated amounts of IFN-γ in response to parasite Ag, while only marginal levels of IL-4 were observed in the cultures with cells from LC-LeIF-vaccinated mice and, as expected, the PBS control group. Interestingly, splenocytes derived from animals that had been treated with whole lysate-pulsed LC produced detectable amounts of IL-4. This finding indicates that, in contrast to the molecularly defined LeIF Ag, components present in the crude parasite extract may also prime IL-4-producing T cells.

Restimulation with rLeIF had the strongest impact on IFN-γ production by spleen cells from both LC-LeIF- and LC-lysate-vaccinated mice, thus suggesting the priming of LeIF-reactive T cells in the spleen. In agreement with our previous finding that i.v.-injected LC migrate to the spleen, but not to the lymph nodes (20), the cytokine expression by lymph node cells was found to be negligible (data not shown). These results indicate a DC vaccine-induced Th1 polarization of the *Leishmania*-specific T cell response and suggest that the spleen is the site of primary T cell activation after i.v. administration of Ag-pulsed DC.

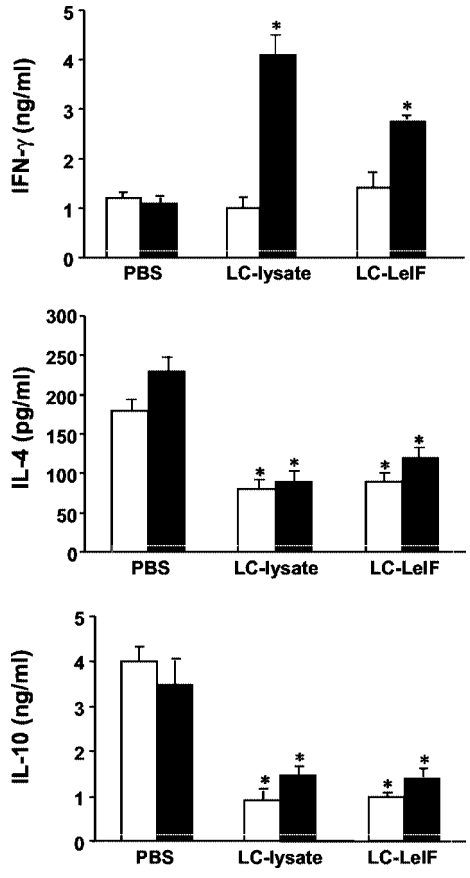

FIGURE 5. Cytokine expression patterns in the lymph nodes draining the infected footpads correlate with the course of *L. major* infection. BALB/c mice were immunized i.v. with 2–3 $\times$ 10^5 *L. major* lysate-pulsed LC (LC-lysate), rLeIF-pulsed LC (LC-LeIF), or PBS. After 7 days, mice were infected with stationary phase promastigotes. After 11 wk of infection, mice were sacrificed for preparation of single cell suspensions of the lymph nodes draining the infected footpads. Cells were cultured in the presence (■) or absence (□) of *Leishmania* lysate for 48 h. Cytokines in the culture supernatants were determined by ELISA. Data represent the mean $\pm$ SD of five mice per group. One of two independent experiments with similar results is shown. *, Bars showing values that were significantly different ($p < 0.01$) from those of the control animals (PBS-treated mice).

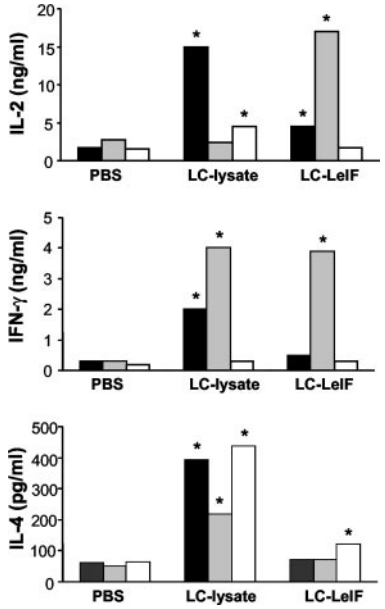

FIGURE 6. A single i.v. injection of Ag-loaded LC induces a primary immune response in the spleen. BALB/c mice were immunized i.v. with 2–3 $\times$ 10^5 *L. major* lysate-pulsed LC (LC-lysate) or rLeIF-pulsed LC (LC-LeIF). PBS-injected animals were used as controls. After 7 days post-vaccination, mice were sacrificed and single cell suspensions of the spleens were cultured in the absence of Ag (□) or in the presence of *Leishmania* lysate (■) or rLeIF (▨) for 48 h. Cytokines in the culture supernatants were determined by ELISA. Results of one of two independent experiments with similar results show the mean values of five mice per group. *, Bars showing values that were significantly different ($p < 0.05$) from those of the control animals (PBS-treated mice).

Discussion

Specific targeting of candidate vaccine Ag to DC, the most potent APC, may be a promising strategy for the design of improved vaccines against intracellular pathogens. We and others have previously shown that this approach is feasible, i.e., immunization of mice with DC that had been loaded with microbial Ag confers protection against infection with various pathogens (14, 20, 45–48). However, some key questions regarding the immunological mechanisms and requirements of DC-mediated vaccination remain to be answered. In the present study, using the murine model of leishmaniasis, we have shown that DC-derived IL-12 plays a decisive role in the priming of protective immune responses and that recombinant Ag, applied as a mixture or as a single Ag, can be used for the development of a molecularly defined DC-based subunit vaccine.

Several studies have identified IL-12 as a key component in the development of the early immune response after *Leishmania* infection (29, 44). DC have been shown to mediate the priming of *Leishmania*-specific T cells (49) and to produce high levels of IL-12 after exposure to parasite Ag (18, 19), thus promoting the development of protective Th1 cells. To test the role of IL-12 production by the priming DC in the initiation of antileishmanial immunity after DC-mediated vaccination, we compared the protective efficacy of Ag-loaded LC from IL-12-deficient mice with that of LC obtained from WT mice. The results revealed that the depletion of the IL-12p35 subunit in the priming LC completely abrogated their potential to induce protection against experimental cutaneous leishmaniasis. All mice that had been immunized with Ag-pulsed LC from IL-12p35$^{-/-}$ mice developed progressive lesions with tissue necrosis, similar to the course of disease in control animals. In contrast, mice treated with Ag-pulsed LC from WT mice were able to control *L. major* infection. Because cells from IL-12p35$^{-/-}$ mice are still capable of secreting the IL-12p40 subunit (29), the possibility exists that an antagonist effect caused by the formation of p40 homodimers (50) may mediate a reduction of Th1 responses (51). However, because both WT and IL-12p35$^{-/-}$ mice are able to produce IL-12p40 in similar amounts (29) but differ in their capability of forming IL-12p70, it can be concluded that p70, but not p40, is responsible for mediating protection and that the possible presence of p40 homodimers or, alternatively, p40/p19 heterodimers (IL-23) is unable to substitute for the functions of the IL-12p70 molecule. Thus, our results suggest a key role of donor LC-derived IL-12p70 in the priming of a protective antileishmanial immune response. This finding is consistent with the previous observation that disruption of either the IL-12p40 or the IL-12p35 gene locus in the genetically resistant mouse strain 129/Sv/Ev results in susceptibility to infection with *L. major* (29).

The results of this study emphasize the notion that the cytokine milieu present at the initiation of the immune response to infection plays an important role in the subsequent development of either protective or disease-promoting Th cell responses. The complete failure of LC from IL-12-deficient mice to mediate protection against leishmaniasis demonstrates that the potential to secrete IL-12 is a critical parameter for the capacity of the priming DC to trigger immunity. Interestingly, a recent report by MacDonald and Pearce (52) indicated that donor DC-derived IL-12 is necessary for the induction of an optimal Th1 response to *Propionibacterium acnes* but is not an absolute requirement for Th1 development. Our findings in the leishmaniasis model extend this observation by showing that IL-12 production by the immunizing DC, however, is vital for the induction of host resistance and control of infectious disease. In this context, it is important to note that our previous studies (20) have documented that, upon. i.v. administration of Ag-loaded LC, the number of cells settling in the spleen and presumably initiating the immune response leading to resistance is very small (~2000 LC). Remarkably, the amount of IL-12 released by these cells appears to be sufficient to overcome the deleterious effects of the early IL-4 burst that is associated with *L. major* infection of susceptible BALB/c mice (22).

In addition to demonstrating the critical role of IL-12 produced by the sensitizing DC, we showed that recombinant Ag can be used for DC pulsing and induction of protective immunity. The effect of DC-based vaccination with molecularly defined microbial Ag has not, to our knowledge, been evaluated before. This is an important aspect because the reproducibility of the Ag preparation for DC loading is going to have a profound influence on the efficacy of vaccination. Crude parasite extracts may vary in their quality and, moreover, may also contain Ag that induce deleterious rather than host-protective immune responses, leading to exacerbated pathology. Our data provide evidence that a DC-based subunit vaccine can be highly immunogenic. Upon adoptive transfer into naive recipients, LC loaded with a mixture of the recombinant *Leishmania* Ag LACK, KMP-11, gp63, and PSA were shown to mediate significant protection against a challenge with *L. major* parasites. The significance of LC as the vaccine carrier is emphasized by the previous observation that gp63, LACK, and KMP-11 are unable to induce protective responses when administered as proteins without adjuvant (23, 28, 31). Most importantly, we identified LeIF as a single leishmanial Ag that, upon loading into LC, protected mice from uncontrolled parasite infection. LeIF has been revealed to be a unique candidate vaccine Ag because it bridges the innate immune system with adaptive responses. It is able to induce IFN-γ production by NK cells (43) and stimulates macrophages and DC to produce IL-12 and IL-18, thus favoring the expansion of LeIF-specific Th1 cells (37, 41). On the basis of these observations, LeIF has been proposed to serve as an adjuvant for the induction of Th1 responses. Therefore, loading of DC with LeIF may not only provide a potent trigger for DC maturation and activation but may also deliver peptide epitopes for the effective priming of LeIF-reactive Th1 cells by DC. In fact, we observed a strongly increased expression of IL-12 by DC that had been pulsed with LeIF as compared with unpulsed DC and DC pulsed with *Leishmania* Ag that were less efficient in DC-based vaccination against leishmaniasis. This finding indicates that the potential of a leishmanial Ag to induce DC-mediated protection may closely correlate with its capacity to stimulate IL-12 expression by DC.

Notably, a single i.v. administration of LeIF-bearing LC was able to efficiently prime LeIF-reactive Th1 cells in the spleen, as reflected by a pronounced up-regulation of the IFN-γ level and a virtual lack of IL-4 production (Fig. 6), and thus modulated the cytokine microenvironment in the lymphoid organ targeted by i.v. immunization with LC. These results suggest that LeIF-pulsed LC settling in the T cell compartments of the spleen trigger the immune responses that lead to the development of a protective Th1-type cytokine profile which can later on, after infection of the mice with *L. major* parasites, be detected also in the lymph nodes draining the lesion (Fig. 5).

In conclusion, our results show that 1) the ability of the Ag-delivering DC to produce IL-12 is critical for their potential to mediate protection against leishmaniasis and 2) DC charged with molecularly defined Ag are capable of inducing protective immunity, suggesting that the development of a DC-based subunit vaccine holds promise of success. Future studies will have to address the question of whether the efficacy of DC as vectors for vaccination may be further enhanced by improved strategies for targeting microbial Ag to DC. Such strategies may include transfection with RNA or transduction with cDNA encoding prominent Ag. Genetic

engineering of DC may also comprise the transduction with genes encoding chemokines or cytokines to improve the migratory and/or T cell-activating functions of DC (53). Another challenge is the elaboration of protocols for therapeutic immune interventions with DC because large-scale immunoprophylaxis with ex vivo-generated DC is not feasible. This strategy is currently being tested in cancer patients but, in infectious disease situations, information on the therapeutic potential of DC is still missing. Our data provide the basis for addressing these important issues.

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