

# Uptake of *Leishmania major* by dendritic cells is mediated by Fc $\gamma$ receptors and facilitates acquisition of protective immunity

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**Uptake of *Leishmania major* by dendritic cells (DCs) results in activation and interleukin (IL)–12 release. Infected DCs efficiently stimulate CD4<sup>+</sup> and CD8<sup>+</sup> T cells and vaccinate against leishmaniasis. In contrast, complement receptor 3–dependent phagocytosis of *L. major* by macrophages (M $\Phi$ ) leads exclusively to MHC class II–restricted antigen presentation to primed, but not naive, T cells, and no IL–12 production. Herein, we demonstrate that uptake of *L. major* by DCs required parasite–reactive immunoglobulin (Ig)G and involved Fc $\gamma$ RI and Fc $\gamma$ RIII. In vivo, DC infiltration of *L. major*–infected skin lesions coincided with the appearance of antibodies in sera. Skin of infected B cell–deficient mice and Fc $\gamma$ –/– mice contained fewer parasite–infected DCs in vivo. Infected B cell–deficient mice as well as Fc $\gamma$ –/– mice (all on the C57BL/6 background) showed similarly increased disease susceptibility as assessed by lesion volumes and parasite burdens. The B cell–deficient mice displayed impaired T cell priming and dramatically reduced IFN– $\gamma$  production, and these deficits were normalized by infection with IgG–opsonized parasites. These data demonstrate that DC and M $\Phi$  use different receptors to recognize and ingest *L. major* with different outcomes, and indicate that B cell–derived, parasite–reactive IgG and DC Fc $\gamma$ RI and Fc $\gamma$ RIII are essential for optimal development of protective immunity.**

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Abbreviations used: BMDC, bone marrow–derived DC; CFSE, carboxyl fluorescein succinimidyl ester; CR3, complement receptor 3; IS, immune serum; M $\Phi$ , macrophages; NMS, normal mouse serum; SLA, soluble *Leishmania* antigen.

In cutaneous leishmaniasis affecting mice and man, control of infection is associated with Th1/Tc1–mediated, IFN– $\gamma$ –dependent elimination of intracellular parasites (1, 2). After infection of mice with physiologic low dose inocula of *Leishmania major* parasites, the evolution of skin lesions occurs in three distinct phases (3). In the initial “silent” phase, resident macrophages (M $\Phi$ ) phagocytose *L. major* promastigotes primarily via complement receptor 3 (CR3) (4, 5), which inactivates the infected cells and allows parasite amplification (as intracellular amastigotes) at sites of infection. In the second phase, development of clinically evident lesions occurs coincident with the influx of inflammatory cells, including neutrophils, M $\Phi$ , and eosinophils. Subsequently, immunity is initiated by infiltra-

tion of DCs as well as T and B cells, and lesions resolve (the third phase) (3).

Both M $\Phi$  and DCs, the major APCs in skin, clearly influence the development of cellular immune responses against *Leishmania*. Dermal M $\Phi$  capture organisms at sites of inoculation and, after establishment of protective immunity, they ultimately kill the parasites. However, M $\Phi$  do not actively participate in T cell priming. In all likelihood, DCs take up amastigotes of *L. major* present in lesional skin, become activated, and migrate to draining LN where they present *Leishmania* antigen to naive T cells (6, 7). There are striking differences in the ways that M $\Phi$  and DCs interact with *L. major* parasites in vitro. First, skin DCs preferentially take up *L. major* amastigotes, the obligate intracellular life form of the parasite, rather than promastigotes (transmitted by sand flies), whereas M $\Phi$  efficiently

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phagocytose both life forms (7–9). Second, the phagocytotic capacity of DCs is limited with regard to efficiency and capacity as compared with that of MΦ (7). Third, *L. major*-infected DCs, unlike infected MΦ, release IL-12 and efficiently induce Th1/Tc1 differentiation of naive cells (7, 10–12). Fourth, although both cell types present *Leishmania* antigen via the MHC class II pathway, only DCs prime and restimulate *L. major*-specific CD8<sup>+</sup> T cells (13).

Based on the different behaviors and functional roles of MΦ and DCs in *L. major* infections, we hypothesized that DCs and MΦ might take up *L. major* via different phagocytotic receptors. MΦ ingest *L. major* via CR3-dependent mechanisms (4). Herein, we identified immune IgG and Fcγ receptors (FcγRI and FcγRIII) as critical mediators of *L. major* uptake by DCs in vitro. In vivo, mice infected with IgG-opsonized parasites showed enhanced protective immunity as well as increased numbers of *L. major*-infected lesional DCs. We also determined that B cell- (μMT and J<sub>H</sub>T) and Fcγ-deficient mice had decreased numbers of *L. major*-infected lesional DCs and enhanced lesion progression. In addition, we observed impaired CD4- and CD8-priming in the absence of B cells. Immune IgG production and engagement of DC FcγR are required for timely development of Th1/Tc1-dependent immunity and control of experimental cutaneous leishmaniasis in mice.

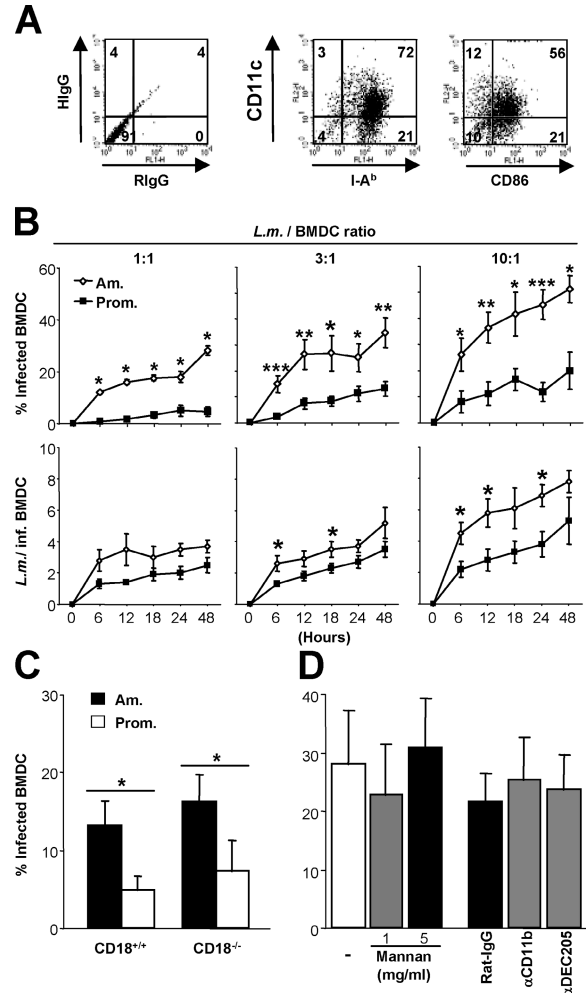
**RESULTS**

**CR3 does not mediate uptake of *L. major* by DCs**

MΦ phagocytosis of *L. major* promastigotes and amastigotes is rapid and efficient (1). In contrast, skin DCs preferentially ingest amastigotes, and this occurs slowly and inefficiently (7). We generated bone marrow-derived DCs (BMDCs) using GM-CSF/IL-4 and confirmed our previous findings obtained with skin DCs. Day 6 immature DCs expressed CD11c, intermediate levels of MHC class II, and low levels of CD86 (Fig. 1 A). BMDCs, like skin DCs, internalized freshly isolated amastigotes in a time- and dose-dependent manner. Normal mouse serum (NMS)-opsonized promastigotes, in contrast, were not readily ingested (27 ± 6 vs. 8 ± 1% infected DCs with a DC/parasite ratio of 1:3 at 18 h; P ≤ 0.05, Fig. 1 B). As expected, DC infection was associated with up-regulation of MHC class I/II and costimulatory markers (reference 7 and unpublished data).

Phagocytosis of *L. major* by MΦ is CR3 dependent (5). To investigate the role of CR3 and CR4 in *L. major* uptake by DCs, we used CD18<sup>-/-</sup> mice. As expected, DCs generated from CD18<sup>-/-</sup> mice did not express CD11b or CD11c (unpublished data). No differences in the percentages of infected wild type or CD18<sup>-/-</sup> DCs (Fig. 1 C) or the number of parasites/cell was observed after DCs and *L. major* amastigotes were cocultured for 18 h.

We also assessed the involvement of other candidate receptors. Antibodies reactive with CD11b (clone M1/70) (9), CD205 (clone NLDC145) (14), or preincubation with mannan (5) were used at optimal concentrations. This concentration of mannan was able to completely inhibit the uptake of



**Figure 1. *L. major* amastigotes, rather than promastigotes, are preferentially internalized by DCs independent from CR3/CR4.** Bone marrow-derived DCs and amastigotes or promastigotes of *L. major* were cocultured at various ratios at 2 × 10<sup>5</sup> DCs/ml. (A) Before coculture, surface phenotypes of immature DCs were verified by FACS. (B) At the indicated time points, cells were harvested, cytopspun, and the percentage of infected cells was determined (mean ± SEM, n ≥ 3, \*, P ≤ 0.05, \*\*, P ≤ 0.005, \*\*\*, P ≤ 0.002). (C) DCs from CD18<sup>-/-</sup> and wild-type 129 × C57BL/6 controls were cocultured with *L. major* (1:3). (D) C57BL/6 DC were preincubated with 5 mg/ml mannan, 50 μg/ml anti-CD11b, anti-DEC205, or isotype control before amastigotes of *L. major* were added (1:3). (C and D) After 18 h, cells were harvested and cytopspins were analyzed for the percentage of infected DC (mean ± SEM, n ≥ 3, \*, P ≤ 0.05).

*C. albicans* by MΦ (unpublished data) (5). None of the inhibitors tested affected the uptake of *L. major* by DCs (Fig. 1 D). Thus, CR3/CR4 and C-type lectins appear to be dispensable for phagocytosis of *L. major* by DCs.

**Immunoglobulins enhance uptake of *L. major* by DCs**

*L. major* amastigotes are isolated from infected tissues, whereas metacyclic promastigotes are enriched from stationary phase in vitro cultures. Among the most prominent differences between surface characteristics is the large amount of Ig bound

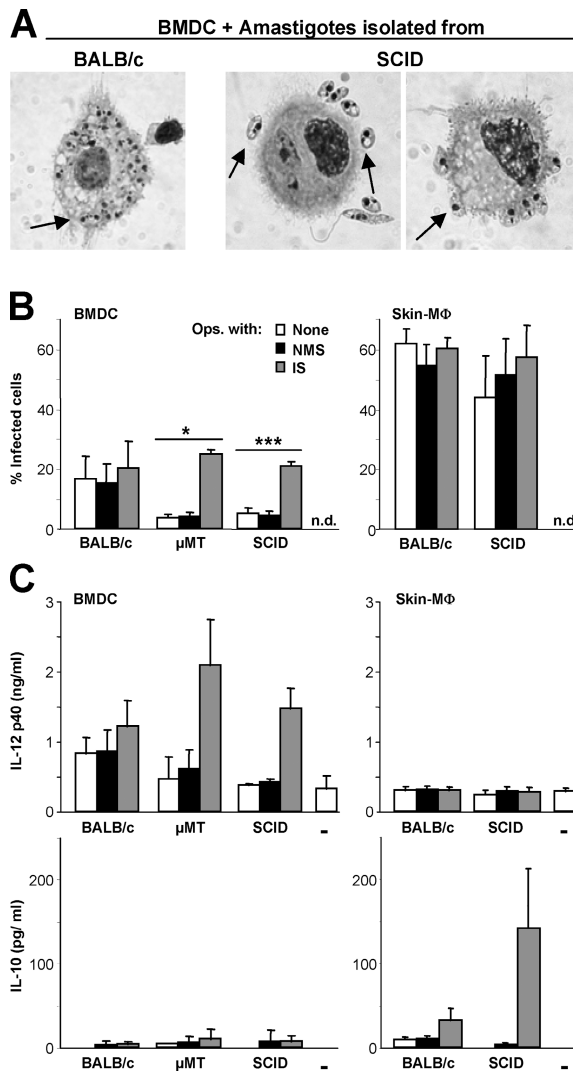
to the surfaces of amastigotes, but not promastigotes. To determine if Ig was involved in parasite uptake, we quantified the ability of amastigotes isolated from B cell-replete, wild-type BALB/c mice,  $\mu$ MT (B cell-deficient) mice and SCID (B cell- and T cell-deficient) mice to parasitize DCs. DCs readily phagocytosed amastigotes from BALB/c mice, but not amastigotes from  $\mu$ MT or SCID mice (Fig. 2, A and B). Opsonization with NMS did not affect uptake. Parasites from B cell-deficient mice efficiently entered DCs only after they had been preincubated with Ig-containing immune serum (IS) from *L. major*-infected BALB/c mice (or C57BL/6 mice; unpublished data). Phagocytosis of amastigotes by M $\Phi$  was not affected by the presence or absence of Ig. Opsonization of amastigotes from B cell-deficient mice with IS (Fig. 2 C) also induced enhanced release of IL-12p40 from DCs, whereas infection of M $\Phi$  did not promote IL-12 production. Ig-mediated uptake of amastigotes did induce IL-10 release from M $\Phi$  (15), whereas little, if any, IL-10 was produced by infected DCs.

To determine if Ig-coated promastigotes could be ingested by DCs, metacyclic *L. major* promastigotes were left untreated or were opsonized with NMS or IS for 10 min at 37°C. After washing, parasites were cocultured with DCs for 18 h. IS-treated promastigotes were efficiently taken up by DCs and induced IL-12 release, whereas untreated or NMS-treated parasites were not ingested (Fig. 3 A). Interestingly, complete transformation of promastigotes into amastigotes was not observed within all DCs, even after 18 h (Fig. 3 B), suggesting that there are differences in the phagosomal compartments of DCs and M $\Phi$  that influence this transition (e.g., differences in pH, content of proteolytic enzymes) (16). Stimulation of antigen-specific, carboxyl fluorescein succinimidyl ester (CFSE)-labeled T cells with parasite-treated DCs revealed that DCs infected with NMS amastigotes or IS promastigotes induced similar expansion of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, whereas DCs treated with NMS promastigotes did not promote T cell proliferation (Fig. 3 C).

### Phagocytosis of *L. major* is IgG dependent

Amastigotes from infected tissue efficiently parasitize DCs. By flow cytometry, we detected both IgG1 and IgG2a/b on the surfaces of amastigotes when analyzed directly after isolation from C57BL/6 mice or after opsonization with NMS (Fig. 3 D). Additional opsonization with NMS or IS led to enhanced binding of IgM to amastigotes. Cell surface Ig was not detected on promastigotes. NMS-opsonized promastigotes exhibited surface-associated IgM, attributable to natural Ig found in the sera of naive mice (17). Because both NMS- and IS-opsonized amastigotes were taken up to similar extents and NMS-opsonized promastigotes were not phagocytosed by DCs (compare with Fig. 3 A), we conclude that IgM is not required for parasite uptake. Interestingly, promastigotes bound similar amounts of IgG1 and IgG2a/b when incubated with sera harvested from infected resistant C57BL/6 mice (Fig. 3 D) or susceptible BALB/c mice (unpublished data).

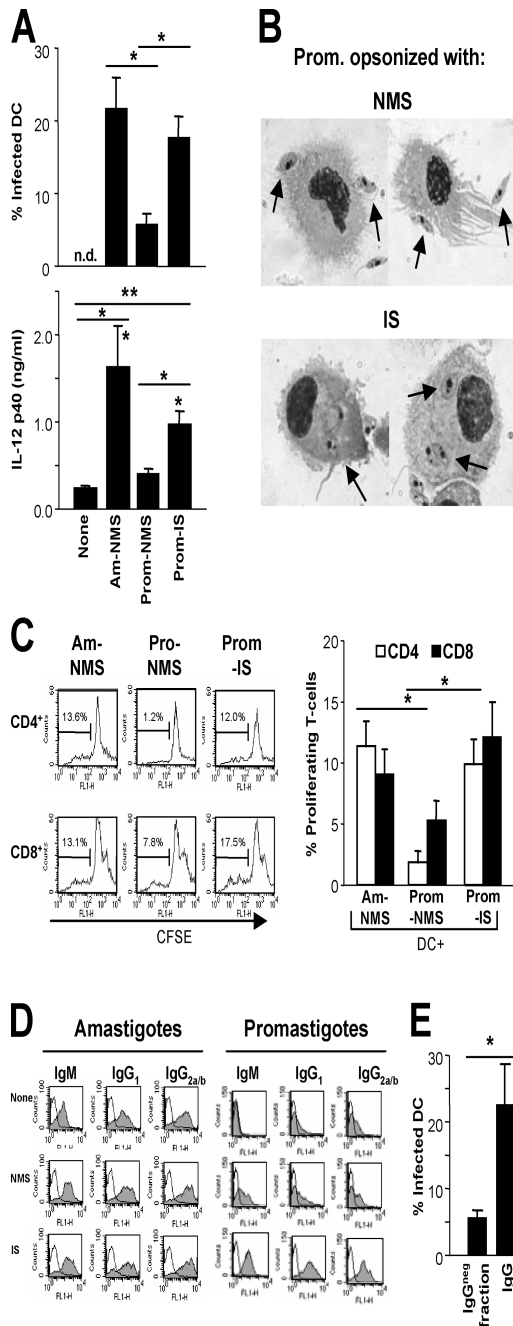
To conclusively implicate immune IgG in DC-parasite uptake, we isolated total IgG from IS using protein G affinity



**Figure 2. Immunoglobulins are required for internalization of**

*L. major* amastigotes by DCs. Amastigotes of *L. major* were prepared from BALB/c mice or mice deficient for B cells ( $\mu$ MT or SCID mice). As indicated, parasites were opsonized for 10 min with 5% of normal mouse serum (NMS) or serum from 6 wk *L. major*-infected BALB/c mice (IS) and added to DCs or skin-M $\Phi$  from C57BL/6 mice ( $2 \times 10^5$  cells/ml) at a parasite/cell ratio of 3:1. (A and B) 18 h later, cells were harvested and cytospun and the percentage of infected cells was determined by light microscopy and expressed as mean  $\pm$  SEM ( $n \geq 4$ , \*,  $P \leq 0.05$ , \*\*\*,  $P \leq 0.002$ ). (C) The coculture supernatants were assayed for the presence of IL-12p40 and IL-10 by ELISA (mean  $\pm$  SEM,  $n \geq 4$ ).

columns and tested the capacity of IgG to trigger phagocytosis. Similar to IS, the IgG fraction mediated uptake of promastigotes, whereas parasites incubated with the IgG-depleted fraction were not phagocytosed (Fig. 3 E). In addition, parasite uptake was associated with IL-12p40 release ( $772 \pm 324$  pg/ml for DCs incubated with IgG promastigotes vs.  $137 \pm 27$  pg/ml for DCs cocultured with promastigotes incubated with the IgG<sup>neg</sup> fraction;  $n \geq 3$ ,  $P = 0.03$ ).



**Figure 3. Infectious stage promastigotes of *L. major* infect DCs in the presence of IgG.** DCs were cocultured with metacyclic promastigotes of *L. major* that had either been left untreated or were opsonized with normal mouse serum (NMS) or serum of 6 wk-infected BALB/c mice (IS) before addition to the cultures. (A and B) Cells were harvested after 18 h and infection rates were determined on cytopins. The IL-12p40 production of DCs was analyzed by ELISA (mean  $\pm$  SEM,  $n = 3$ , \*  $P \leq 0.05$ ; \*\*  $P \leq 0.005$ ). (C) BMDCs infected as indicated were coincubated (1:2) with CFSE-labeled LN cells (at  $10^6$  cells/200  $\mu$ l) obtained from 6 wk-infected C57BL/6 mice. Antigen-specific expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was assessed and the relative number of *Leishmania*-reactive cells (in percents of total CD4<sup>+</sup> or CD8<sup>+</sup> T cells) was determined in DC-stimulated cultures compared with untreated control cultures. One representative FACS result is shown on the left, pooled data is shown on the right (mean  $\pm$  SEM,  $n = 3$ , \*  $P \leq 0.05$ ).

**Both Fc $\gamma$ RI and Fc $\gamma$ RIII mediate uptake of *L. major* by DCs**

IgG1-containing immune complexes bind preferentially to Fc $\gamma$ RIII (and Fc $\gamma$ RII) and IgG2a-containing complexes bind with higher affinity to Fc $\gamma$ RI than to Fc $\gamma$ RIII. Fc $\gamma$ RII typically mediates endocytosis of soluble immune complexes (18). DCs from knockout mice deficient for single Fc $\gamma$ R family members ingested *L. major* as efficiently as DCs from wild-type mice (Fig. 4 A). In addition, blocking antibodies directed against Fc $\gamma$ RII/III (clone 2.4G2) did not have a dramatic effect on *L. major* uptake by wild-type DCs (Fig. 4 B, left). However, significant inhibition of *L. major* phagocytosis by DCs (up to 70%) was observed if DCs from Fc $\gamma$ RI/III- or Fc $\gamma$ -deficient mice were compared with wild-type cells (Fig. 4 B). Uptake of amastigotes and Ig-opsonized promastigotes was impaired to similar extents. Thus, Fc $\gamma$ RI and Fc $\gamma$ RIII each facilitate phagocytosis of *L. major* by DCs, and these receptors can compensate for one another.

**Accumulation of infected DCs in lesions coincides with the appearance of *Leishmania*-specific IgG in sera**

In the setting of physiologic low dose infections, we have shown that increased accumulation of both T cells and DCs at inoculation sites coincides with the onset of lesion involution (3). In addition, infiltration with DCs was delayed as compared with M $\Phi$  recruitment and infection. DCs were identified in lesions beginning 5 wk after inoculation, and their number increased substantially during the healing phase.

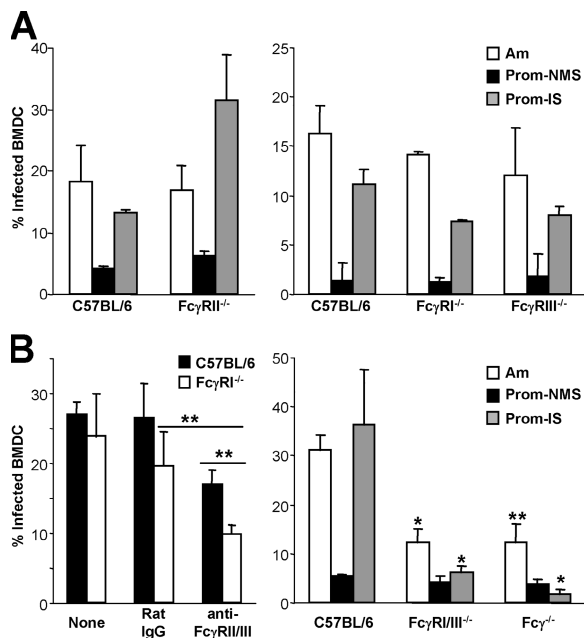
To determine if immune IgG, which dramatically enhances *L. major* infection of DCs in vitro, is present at the time that DCs are recruited to *Leishmania* lesions, we infected C57BL/6 mice with  $10^3$  promastigotes and quantified the number of inflammatory cells in lesional skin as well as the appearance of *Leishmania*-reactive IgG in sera at weekly intervals. Fig. 5 shows that by weeks 5–6 after infection, the numbers of DCs as well as serum parasite-specific IgG levels were increased. This indicates that *Leishmania*-specific IgG is available to opsonize parasites and enhance phagocytosis by DCs at the time that DCs are infected in vivo. Significant accumulation of CD19<sup>+</sup> B cells in lesional skin ( $>10^3$  cells) was not detected within 8 wk after infection.

**In vivo targeting of DCs with IgG-opsonized promastigotes speeds disease resolution**

Previously, we and others have demonstrated that *L. major*-infected DCs release IL-12 and effectively vaccinate against

(D) Parasites were analyzed for surface binding of Ig subclasses after different opsonization procedures. Amastigotes were isolated from infected C57BL/6 tissue and opsonized. Promastigotes were enriched from stationary phase cultures and opsonized similarly. One out of three independent experiments with similar results is shown. (E) IgG was purified from IS. IgG as well as the IgG<sup>neg</sup> fraction were used for opsonization of promastigotes. Opsonized promastigote preparations were cocultured with DCs for 18 h at a ratio of 3:1 ( $2 \times 10^5$  cells/ml). Cells were harvested and the percentage of infected cells was determined on cytopins (mean  $\pm$  SEM,  $n = 3$ , \*  $P \leq 0.05$ ).

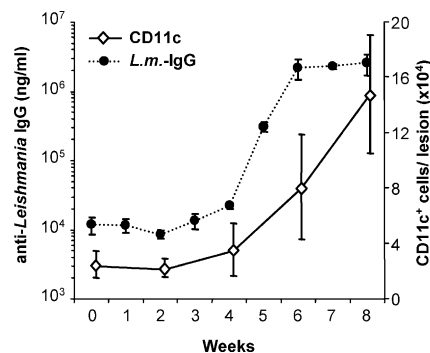




**Figure 4. Fc RI/III mediate uptake of *L. major* parasites by DCs.** Bone marrow–derived DCs from C57BL/6, FcγRII<sup>-/-</sup>, FcγRI<sup>-/-</sup>, FcγRIII<sup>-/-</sup>, Fcγ<sup>-/-</sup>, and FcγRI/III<sup>-/-</sup> were cocultured with *L. major* at a ratio of 1:3 ( $2 \times 10^5$  cells/ml). Parasites (Am = amastigotes, Prom = promastigotes) were opsonized with normal mouse serum (NMS) or serum of 6 wk–infected BALB/c mice (IS) for 10 min before addition to the cultures. As indicated, 50 μg/ml anti-CD16/32 (clone 2.4G2) or isotype control was added to the cells 1 h before the parasites (B). Cells were harvested after 18 h. The percentage of infected cells was determined (mean ± SEM,  $n \geq 3$ , \* $P \leq 0.05$ , \*\* $P \leq 0.005$  compared with isotype treatment [B, left] or wild type control [B, right]).

progressive disease (10–12). Therefore, infection of DCs *in vivo* earlier in the course of infection should accelerate development of Th1 immunity. To test this hypothesis, promastigotes were opsonized either with NMS or IS. After washing, low dose infections using  $10^3$  opsonized parasites were initiated in the ear skin of C57BL/6 mice (Fig. 6). Inflammatory dermal cells from lesional ear skin were studied weekly (Fig. 6, A and B). Interestingly, the numbers of CD11c<sup>+</sup> DCs in IS promastigote–infected skin were significantly higher ( $7.9 \pm 0.9 \times 10^4$ /lesion) than those in NMS promastigote–treated ears ( $2.5 \pm 0.5 \times 10^4$ /lesion in week 1,  $n = 3$ ,  $P \leq 0.005$ ), especially at early time points. At later time points (week 3 and after), this difference was not evident. DCs were enriched by preparative flow sorting and the number of infected DCs was determined (Fig. 6, B and C). At early time points, the percentage of DCs containing intracellular amastigotes was low. However, by week 2, significantly more infected cells were found in ears of mice infected with IS– versus NMS–opsonized parasites ( $9.5 \pm 1.3$  vs.  $3.4 \pm 0.7\%$ ,  $n = 3$ ,  $P \leq 0.002$ ). By week 3, this difference also disappeared.

Lesion development in infected mice was monitored for >3 mo. Interestingly, cutaneous lesions of mice infected with IS–opsonized parasites were significantly smaller and resolved more quickly than those in mice that were infected with par-

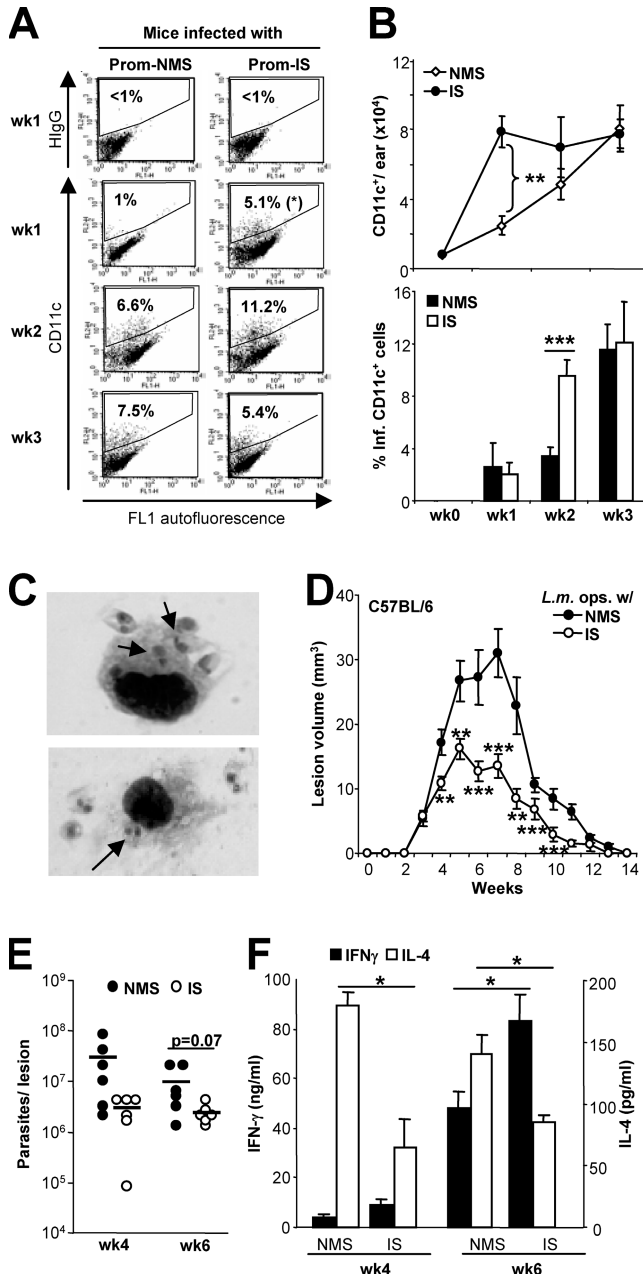


**Figure 5. The appearance of parasite–specific IgG is coincident with DCs recruitment into *Leishmania* lesions.** Accumulation of DCs into skin lesions of *L. major*–infected C57BL/6 mice ( $10^3$  metacyclic promastigotes intradermally, day 0) was determined as described previously. In parallel, the anti–*Leishmania* antibody response was monitored in the serum by ELISA (mean ± SEM,  $n = 5$  mice). One out of two independent experiments with similar results is shown.

asites opsonized with NMS (Fig. 6 D). In addition, lesional parasite loads were decreased in weeks 4 and 6 after infection in mice inoculated with IS–opsonized parasites compared with NMS–treated parasites (Fig. 6 E). Smaller lesion volumes were associated with increased Th1 immunity as measured by antigen–specific restimulation of LN cells at weeks 4 and 6 (Fig. 6 F). The IFN–γ/IL–4 ratio in IgG–parasite infected mice was Th1–predominant (week 6:  $1,068 \pm 250$ ) as compared with mice infected with NMS–opsonized parasites (week 6:  $382 \pm 86$ ,  $n = 6$ ,  $P \leq 0.05$ ). Collectively, these data suggest that enhanced IgG–mediated recruitment and *L. major* infection of DCs *in vivo* leads to enhanced Th1 immunity and more rapid resolution of cutaneous lesions.

### B cell–deficient mice show enhanced lesion progression associated with decreased numbers of infected DCs and impaired CD4– and CD8–priming

Because our data suggested that IgG mediates parasite uptake by DCs, we characterized *L. major* infections in B cell–deficient μMT mice (19). Herein, wild–type C57BL/6 or μMT mice were infected with physiologically relevant doses of *L. major* ( $10^3$  promastigotes). Compared with wild–type mice, μMT mice showed significantly enhanced lesion progression from week 6 after infection (Fig. 7 A). Lesion involution was delayed by ~4 wk in μMT compared with control mice. Furthermore, the skin of μMT mice contained greater numbers of parasites reaching a peak load of  $4 \pm 2 \times 10^5$  parasites/ear at week 6 as compared with  $3 \pm 2 \times 10^4$  parasites/ear in wild types ( $P \leq 0.05$ ) (Fig. 7 B). The IFN–γ/IL–4 ratios of μMT LN cell cultures stimulated with soluble *Leishmania* antigen (SLA) were also skewed toward a Th2 profile as compared with C57BL/6 cells. In weeks 6 and 8 after infection, μMT LN cells released significantly less IFN–γ and more IL–4 compared with C57BL/6 mice (e.g.,  $40.1 \pm 12.6$  in μMT compared with  $100.7 \pm 19.2$  ng IFN–γ/ml in C57BL/6 mice in week 6,  $n \geq 9$ ,  $P \leq 0.05$ ; Fig. 7 C).



**Figure 6. Increased recruitment and infection of DCs after injection of IgG-opsonized promastigotes of *L. major* leads to enhanced protection in vivo.** C57BL/6 mice were infected intradermally into ear skin with  $10^3$  metacyclic promastigotes that have been opsonized with normal mouse serum (NMS) or serum from 6 wk-infected BALB/c mice (IS) and washed. Inflammatory ear cells were isolated and analyzed for the presence of CD11c<sup>+</sup> DCs at different time points by flow cytometry (A). The number of DCs per ear was calculated (mean  $\pm$  SEM,  $n = 3$ , \*\*,  $P \leq 0.005$ ; B). CD11c<sup>+</sup> DCs were purified using flow sorting and cytospun. The percentage of infected DCs was determined by light microscopy (mean  $\pm$  SEM,  $n = 3$ , \*\*\*,  $P \leq 0.002$ ; B and C). (D) Lesion development was monitored over the course of >3 mo. Lesion volumes are shown as mean  $\pm$  SEM (\*\*,  $P \leq 0.005$  and \*\*\*,  $P \leq 0.002$ ,  $n \geq 10$  mice). (E) Parasite loads of infected mice were determined at the indicated time points using limiting dilution assays. Each data point represents the number of organisms from one ear and the bars indicate arithmetic means. One repre-

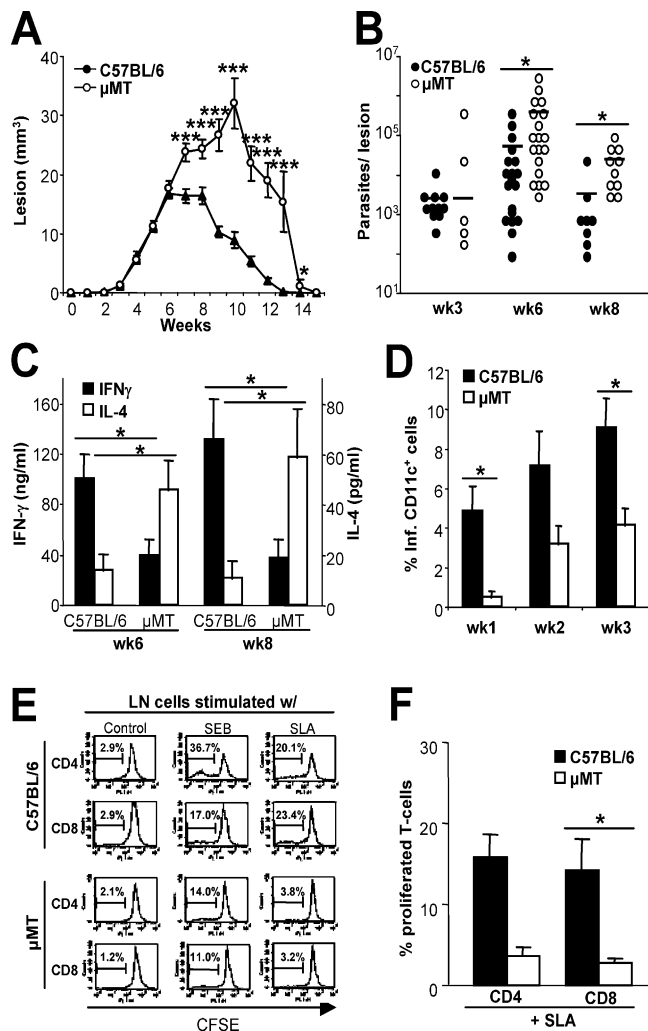
We also isolated inflammatory cells from infected ears of  $\mu$ MT and wild-type mice. No significant difference in the number of CD11c<sup>+</sup> DCs that accumulated in the lesions of  $\mu$ MT mice as compared with C57BL/6 ears was found (unpublished data). However, lesions of  $\mu$ MT mice contained significantly fewer *L. major*-infected DCs at several time points (Fig. 7 D). Finally, we sought to determine if there was a correlation between numbers of infected DCs and the ability to prime CD4 and CD8 T cells in situ. LN cells of infected C57BL/6 or  $\mu$ MT mice were isolated 6 wk after infection and labeled with CFSE. Antigen-specific expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was assessed 5 d after restimulation of LN cells with SLA.  $\mu$ MT LN cells exhibited decreased SLA-specific CD4 expansion as compared with C57BL/6 cells ( $3.7 \pm 1\%$  vs.  $15.7 \pm 3\%$ ; Fig. 7, E and F). Interestingly, the number of *Leishmania*-reactive CD8<sup>+</sup> T cells was also greatly reduced in the absence of B cells (SLA:  $2.9 \pm 0.5\%$  compared with  $14.1 \pm 3.9\%$  in C57BL/6 mice,  $n = 5$ ,  $P \leq 0.05$ ). In summary, enhanced lesion progression in the  $\mu$ MT mice was associated with decreased numbers of infected DCs and defective T cell priming.

**Infection of  $\mu$ MT mice with IgG-opsonized parasites normalizes lesion development**

To investigate whether the deficiency in B cells or the lack of antibody contributed to the phenotype of  $\mu$ MT mice, we infected  $\mu$ MT mice with  $10^3$  NMS- or IS-opsonized promastigotes. In this setting,  $\mu$ MT mice infected with *L. major* developed lesions in the presence of immune IgG that were significantly smaller than those caused by NMS-opsonized parasites (Fig. 8 A). In parallel, decreased lesion volumes in IgG-opsonized parasite-infected  $\mu$ MT mice correlated with significantly smaller parasite burdens in week 6 (Fig. 8 B). In IS parasite-infected  $\mu$ MT mice, the IFN $\gamma$ /IL-4 ratio was shifted from a Th2-predominant ( $828 \pm 94$ ) to a Th1 immune response ( $3,680 \pm 1,515$ ,  $n = 4$ , week 6). Thus, the lack of host IgG is responsible for disease outcome in  $\mu$ MT mice. The skin of  $\mu$ MT mice infected with NMS-opsonized or IS-opsonized promastigotes was analyzed for the presence of infected CD11c<sup>+</sup> DC (Fig. 8 C). As shown before, infection of maximally 5% of DCs was found in  $\mu$ MT mice infected with NMS-treated parasites. Interestingly, inoculation of IgG-containing parasites led to dramatically increased numbers of infected DCs in the early course of infection (Fig. 8 C), even higher than those found in wild types (compare with Fig. 7 D).

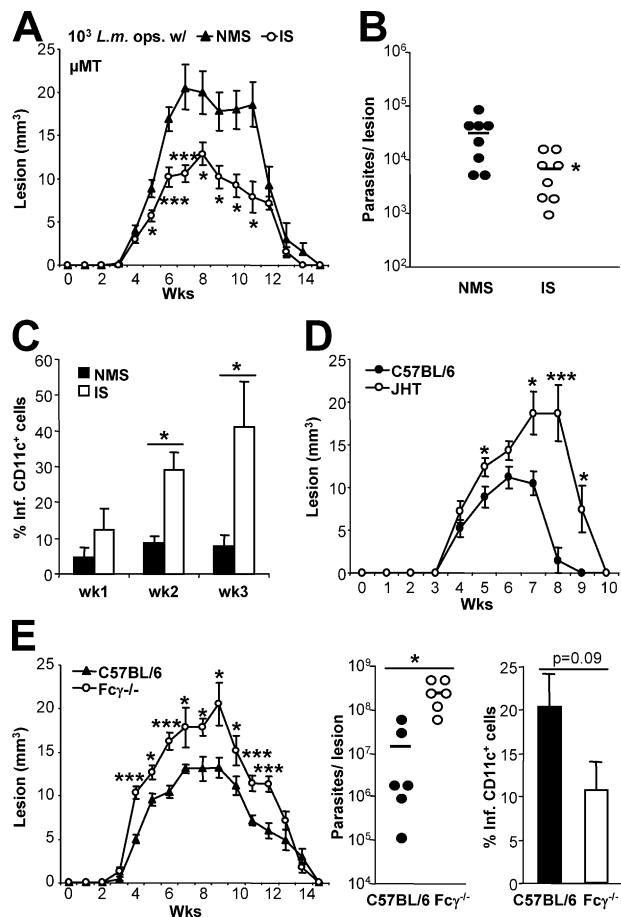
The  $\mu$ MT mice were previously shown to contain Ig in the sera, at least when mice were of BALB/c genetic background (20, 21). The presence of soluble Ig is due to low-level leakiness of the locus (21). To confirm critical

representative out of two independent experiments is shown. (F) Cytokine levels were determined in LN cultures stimulated in the presence of soluble *Leishmania* antigen by ELISA (mean  $\pm$  SEM, \*,  $P \leq 0.05$ ,  $n \geq 10$ ).



**Figure 7. Increased lesion progression in  $\mu$ MT mice due to decreased numbers of *L. major*-infected lesional DC and impaired T cell priming.** Groups of  $\geq 5$  C57BL/6 or B cell-deficient  $\mu$ MT mice were infected into ear skin with  $10^3$  metacyclic promastigotes. (A) Lesion development was monitored over the course of  $>3$  mo (mean  $\pm$  SEM, \*,  $P \leq 0.05$ , \*\*,  $P \leq 0.005$ , and \*\*\*,  $P \leq 0.002$ ,  $n \geq 3$ ). (B) Lesional parasite loads were determined; bars indicate arithmetic means. Pooled data of two to three experiments are shown. (C) LN cells were harvested and antigen-specific cytokine release was determined after 48 h using ELISA specific for murine IFN- $\gamma$  and IL-4 (mean  $\pm$  SEM,  $n \geq 5$ , \*,  $P \leq 0.05$ ). (D) Inflammatory ear cells were isolated and CD11c $^+$  DCs were purified using flow sorting. The percentage of infected DCs was determined on cytopins (mean  $\pm$  SEM,  $n = 3$ , \*,  $P \leq 0.05$ ). (E and F) Antigen-specific proliferation of CD4 $^+$  and CD8 $^+$  T cells was determined in week 6 after infections. LN cells were labeled with CFSE and subcultured in the presence of soluble *Leishmania* antigen (SLA). T cells were pre-gated using staining for CD4 or CD8. For each mouse, the relative number of *Leishmania*-reactive cells (in percentage of total CD4 $^+$  or CD8 $^+$  T cells) was calculated in antigen-stimulated compared with untreated control cultures (mean  $\pm$  SEM,  $n = 2$ , \*,  $P \leq 0.05$ ).

experiments in a truly B cell-deficient mouse strain, we infected C57BL/6 J<sub>H</sub>T mice characterized by deletion of the Ig heavy chain (22). As shown in Fig. 8 D, increased lesion



**Figure 8. Normalization of cutaneous leishmaniasis in B cell-deficient  $\mu$ MT infected with IgG-opsonized promastigotes of *L. major*.** Groups of  $\geq 5$   $\mu$ MT mice were infected into ear skin with  $10^3$  NMS- or IS-opsonized metacyclic promastigotes. (A) Lesion volumes are presented as mean  $\pm$  SEM ( $n = 3$ , \*,  $P \leq 0.05$ , and \*\*\*,  $P \leq 0.002$ ). (B) Parasite loads of infected ears were determined in week 6 after infection using limiting dilution assays. Each data point represents the number of organisms from one ear and bars indicate arithmetic means. One representative out of two independent experiments is shown. (C) Lesional CD11c $^+$  DCs were purified using flow sorting at the indicated time points and the percentage of infected DCs was determined on cytopins (mean  $\pm$  SEM,  $n = 2$ , \*,  $P \leq 0.05$ ). (D) B cell-deficient J<sub>H</sub>T mice were infected with  $10^3$  metacyclic promastigotes and developing lesions monitored every week (mean  $\pm$  SEM, \*,  $P \leq 0.05$ , and \*\*\*,  $P \leq 0.002$ ,  $n \geq 10$  mice/group). (E) Groups of  $\geq 5$  C57BL/6 Fc $\gamma$  $^{-/-}$  mice were infected with  $10^3$  metacyclic promastigotes. Lesion volumes were assessed for  $>3$  mo and are presented as mean  $\pm$  SEM (\*,  $P \leq 0.05$  and \*\*\*,  $P \leq 0.002$ ,  $n = 2$ ). Parasite loads were determined in week 4 by limiting dilution assay. One representative out of two independent experiments is shown (\*,  $P \leq 0.05$ ). The number of infected lesional DCs was determined on cytopins in week 4 ( $n = 2$ ).

development was observed in J<sub>H</sub>T mice over the course of 4 wk identical to the course of infections in  $\mu$ MT mice. This is in contrast with the findings of Miles et al., who reported that J<sub>H</sub> mice on a BALB/c background were less susceptible to infection than their controls (23).

### Enhanced lesion progression and decreased numbers of infected DCs in vivo in mice lacking Fc receptors

Our data suggested that Fc $\gamma$ R-mediated uptake of *L. major* parasites by DCs mediates protection. Thus, we infected Fc $\gamma$  chain-deficient mice lacking all three known activating FcR with physiologically low dose inocula of *L. major* (Fig. 8 E). Lesions were monitored for >3 mo. Fc $\gamma^{-/-}$  C57BL/6 mice developed more progressive lesions between weeks 4 and 9 as compared with wild-type controls. Maximum lesion sizes in Fc $\gamma^{-/-}$  mice were detected in week 9, reaching  $21 \pm 2 \text{ mm}^3$  (C57BL/6:  $13 \pm 1 \text{ mm}^3$ ,  $n = 14$ ,  $P \leq 0.008$ ). Increased lesion volumes were paralleled by significantly higher parasite burdens as determined in week 4 after infection (Fig. 8 E). Similar to the course of disease in B cell-deficient mice, lesion involution in Fc $\gamma^{-/-}$  mice was normal and all mice ultimately healed their infection. This data suggests that FcR-mediated antibody effects are not an absolute requirement for healing.

Finally, we assessed the number of parasite-containing CD11c<sup>+</sup> DC in lesions of Fc $\gamma^{-/-}$  mice infected for 4 wk with low doses of *L. major* (Fig. 8 E). Ear skin of FcR-deficient mice harbored fewer parasite-infected DC ( $10.5 \pm 2.3\%$ ) as compared with wild-type DCs ( $20.2 \pm 3.8\%$ ,  $n = 4$ ,  $P = 0.09$ ). This finding confirmed our in vitro data obtained with BMDCs generated from Fc $\gamma^{-/-}$  mice that demonstrated inhibited parasite uptake in cocultures with *L. major* (Fig. 4 B).

### DISCUSSION

Microbe-binding receptors orchestrate events that occur subsequent to phagocytosis by transducing specific cellular signals (24). The main receptor for uptake of *Leishmania* promastigotes by M $\Phi$  is CR3 (4, 5). In the initial stages of cutaneous leishmaniasis, most parasites are taken up by M $\Phi$ . CR3-mediated phagocytosis of *Leishmania* by M $\Phi$  leads to selective inhibition of IL-12 release (5, 25–27). Production of IL-12 in leishmaniasis is delayed (3), and we and others have suggested that DCs, rather than M $\Phi$ , are the primary source of this Th1-promoting cytokine. It has also been demonstrated that infected DCs are activated and effectively present *L. major* antigen to both naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells in vitro and vaccinate against leishmaniasis in vivo (7, 10, 12, 13).

Although M $\Phi$  and DCs are ontogenically related, their roles in initiation and propagation of immune responses against *L. major* are distinctly different. Uptake of *L. major* by DCs differed significantly from that by M $\Phi$  with regard to kinetics as well as efficiency. Therefore, we speculated that phagocytosis of parasites by DCs might be promoted by receptors other than CR3. However, a previous report suggested that uptake of *L. major* amastigotes by Langerhans cells/DCs was mediated via CR3 (9). In the present study, using both blocking antibodies as well as cells deficient for CR3 and CR4 (from CD18<sup>-/-</sup> mice), we were not able to detect CR3-mediated uptake of *L. major* by DCs. Recently, C-type lectins (DC-SIGN, DEC-205, and Dectin-1) have also been implicated in the uptake of various pathogens by DCs (14, 28–31).

We were unable to implicate mannan-binding C-type lectins in phagocytosis of *L. major* by murine DCs.

In this study, we demonstrate that *L. major* parasites are predominantly phagocytosed by DCs via Fc $\gamma$ RI and Fc $\gamma$ RIII. In line with several studies, Fc $\gamma$ R ligation was associated with DC activation and IL-12 release (32–34). We have previously shown that DCs can cross-present *Leishmania* antigen to CD8<sup>+</sup> T cells (13), whereas CR3-mediated phagocytosis by M $\Phi$  leads exclusively to MHC class II-restricted antigen presentation. These results bear some similarity to experiments evaluating the role of Fc $\gamma$ R in antitumor immunity. In Fc $\gamma^{-/-}$  mice, effective cross-presentation of tumor antigens by DCs was also dependent on Fc $\gamma$ R-dependent activation (35). In addition, signaling through Fc $\gamma$ RI/III facilitated efficient restimulation of tumor-reactive T cells (36). Thus, cross-presentation of both tumor-derived and *L. major*-associated antigens by DCs requires Fc $\gamma$ R, and is presumably dependent on production of specific antibody as well.

In M $\Phi$ , ingestion of amastigotes, in contrast with CR3-phagocytosed promastigotes, appears to occur through both the Fc $\gamma$ R and CR3 (15, 37). In our work and consistent with prior findings, IgG did not play an important role in the uptake of amastigotes from SCID versus BALB/c mice by inflammatory skin M $\Phi$  (38). Our results also confirm the finding that IgG-mediated phagocytosis of *L. major* by M $\Phi$  leads to strong release of IL-10, and no IL-12 synthesis (15), which might promote parasite survival (39). Thus, Fc $\gamma$ R-mediated uptake by M $\Phi$  and DCs has opposing roles in initiating immune responses in cutaneous leishmaniasis.

The role of B cell-derived IgG in cutaneous leishmaniasis in vivo is not fully understood yet. Polyclonal activation of human B cells leads to the production of large amounts of parasite-specific and nonspecific Ab, particularly IgM and IgG (40). Also, amastigotes released into lesional tissue from infected and lysed M $\Phi$  appear to be coated with antiparasite antibodies (41). In this study, we show that *Leishmania*-specific IgG was present in sera at the time of DC accumulation in lesions. Consistent with prior findings, intradermal infection with IgG-opsonized parasites led to enhanced early recruitment of CD11c<sup>+</sup> DCs into the lesions (38), most likely by IgG-triggered chemokine release from M $\Phi$  (42, 43). Administration of IgG-opsonized parasites also led to enhanced infection of DC, augmented T cell priming, and limited disease as compared with inoculation of IgG-free parasites.

Prior data and our experiments suggest that IgG-mediated effects differ significantly, dependent on the genetic background of the mice. B cell-deficient J<sub>H</sub> BALB/c mice showed improved disease outcome after infection with supraphysiologic doses of *L. pifanoi* and coinjection of anti-*Leishmania* IgG reversed their phenotype (44). Administration of IgG at or near the time of parasite inoculation worsened disease outcome in BALB/c mice (40, 45, 46). This is consistent with studies demonstrating that Fc $\gamma$ R ligation on infected M $\Phi$  induced IL-10 release, which in turn prevented parasite elimination and promoted disease progression (15, 23). Final



proof was provided by the demonstration that anti-*Leishmania* IgG reconstitution of J<sub>H</sub> BALB/c mice correlated with increased IL-10 production and blocking of IL-10R prevented antibody-mediated disease exacerbation (23).

Mice on a *Leishmania*-resistant background lacking functional B cells (e.g.,  $\mu$ MT C57BL/6 mice) did not exhibit a phenotype with regard to lesion development after high dose infection with *L. major* (19, 37, 47, 48). However, DeKrey et al. reported that C57BL/6  $\mu$ MT mice infected with high-dose inocula of *L. major* showed reduced IFN- $\gamma$  production after pathogen challenge (48). In our experiment, using physiologically relevant low dose inocula,  $\mu$ MT as well as J<sub>H</sub>T C57BL/6 mice consistently exhibited enhanced lesion progression and delayed lesion involution, higher parasite loads, and cytokine profiles consistent with a Th2-predominant immune response as compared with C57BL/6 mice. In accordance with our in vitro data, significantly fewer infected DCs were found in lesions of  $\mu$ MT mice. In addition, we determined that in the absence of *Leishmania* IgG-mediated infection of DCs, decreased numbers of *Leishmania*-reactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells developed. The defects observed in  $\mu$ MT mice were reversed by using IgG-opsonized parasites for infection indicating that the deficiency in Ig is responsible for worsened disease outcome in B cell-deficient mice.

As expected from the in vitro results obtained with BM-DCs generated from Fc $\gamma$ -deficient mice, we also found decreased numbers of infected DC in Fc $\gamma$ <sup>-/-</sup> mice paralleled by increased lesion volumes over the course of several weeks and higher parasite burdens. In contrast, in prior studies, improved disease outcome of Fc $\gamma$ <sup>-/-</sup> mice was observed using infections with *L. pifanoi* or *L. major* (44, 49). However, the mice used were on a BALB/c background and, thus, are not comparable to those used for this study. Data generated with *Leishmania*-resistant mice might be more physiologically relevant in a clinical setting because the course of disease in, for example, C57BL/6 mice more closely mimics *L. major* infections of humans.

In summary, we propose that the two predominant APCs in skin, M $\Phi$  and DCs, are sequentially engaged via different pathogen recognition receptors as cutaneous leishmaniasis evolves. Although in the initial “silent” phase, *L. major* promastigotes are primarily phagocytosed by resident M $\Phi$  via CR3, Fc $\gamma$ R and DCs become critically important in established infections. IgG-mediated uptake of *L. major* by DCs leads to IL-12 production and priming of Th1/Tc1 cells, both of which are required for efficient parasite killing by lesional M $\Phi$ . In contrast, Fc $\gamma$ R-mediated uptake of amastigotes by M $\Phi$  induces counterregulatory IL-10 production. This may facilitate activation of regulatory T cells, which, in turn, promotes parasite persistence and maintenance of T cell memory (39, 50). The balance between CR3 and Fc $\gamma$ R-triggered anti- and proinflammatory mechanisms involving M $\Phi$  and DCs is critical for disease outcome. The unexpected identification of immune IgG production as a prerequisite for efficient cross-priming of *Leishmania*-specific Th1/Tc1 cells is intriguing. In future experiments it will be important to as-

sess the T cell dependence of *Leishmania*-reactive antibody production, and to identify the APCs that are involved in B cell and, if relevant, Th priming.

## MATERIALS AND METHODS

**Animals.** 6–8-wk-old BALB/c and C57BL/6 mice were purchased from the Central Animal Facility of the University of Mainz. CD18<sup>-/-</sup> mice (51) on a mixed C57BL/6J and 129/SV background were provided by K. Scharfetter-Kochanek (Department of Dermatology, University of Ulm, Ulm, Germany). Fc $\gamma$ R2<sup>-/-</sup> mice (52) were obtained from H. Mossmann (Max Planck-Institut für Immunbiologie, Freiburg, Germany). Mice deficient for Fc $\gamma$ R3 (53) and Fc $\gamma$ R1 (54) as well as Fc $\gamma$ R1/3 double deficient mice (all C57BL/6 background) were provided by S. Verbeek. C57BL/6 Fc $\gamma$ <sup>-/-</sup> were obtained from T. Saito (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) (55) or from Taconic. B cell-deficient mice (C57BL/6 SCID,  $\mu$ MT, J<sub>H</sub>T) were gifts from M. Neurath, K. Steinbrink, and A. Waisman (all from University of Mainz, Mainz, Germany). All animals were housed in accordance with institutional and federal guidelines. All experiments were undertaken with approved license from the Animal Care and Use Committee of the Region Rheinland-Pfalz.

**Cells.** Inflammatory skin-derived M $\Phi$  (M $\Phi$ ) were elicited by subcutaneous injection of polyacrylamide beads and enriched to homogeneity (7). BMDCs were generated in GM-CSF- and IL-4-containing media (56) and harvested on day 6 of cell culture. The characteristics of the cell populations were assessed by flow cytometry using relevant surface markers. The following antibodies were used: anti-I-A<sup>b,d</sup>/I-E<sup>d</sup> (2G9), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD40 (3/23), anti-CD54 (3E2), anti-CD80 (1G10), anti-CD86 (GL1) (all from BD Biosciences/Becton Dickinson), anti-F4/80 (Serotec), and respective isotype control mAb.

**Parasites.** Metacyclic promastigotes or amastigotes of *L. major* clone VI (MHOM/IL/80/Friedlin) were prepared as described previously (25, 57). Amastigotes were prepared from infected footpads of BALB/c or C57BL/6 mice, or mice genetically deficient in B cells ( $\mu$ MT, SCID) to obtain parasites devoid of Ig. Isolated parasites were opsonized with 5% NMS or serum from 6 wk-infected BALB/c or C57BL/6 mice (immune serum, IS) for 10 min (37°C) and washed before in vitro or in vivo infections. Parasites were stained for surface-associated Ig using isotype-specific secondary antibodies reactive with mouse Ig: anti-IgM (Serotec), anti-IgG1 (A85-1), and anti-IgG2a/b (R2-40, all from BD Biosciences). After staining, parasites were washed with PBS/2% BSA, fixed, and analyzed by flow cytometry. Anti-*Leishmania* IgG was prepared from pooled sera of  $\geq$ 5–6-wk *L. major*-infected BALB/c mice using protein G columns (Pierce Chemical Co.) following the manufacturer's protocol. Sera were stored at -20°C before IgG purification. Purified IgG was stored at 4°C (0.8 mg/ml) in PBS before use.

**Phagocytosis and inhibition studies.** Isolated cells were subcultured in medium (RPMI 1640/5% FCS) at 2  $\times$  10<sup>5</sup>/ml and parasites were added at the parasite/cell ratios indicated. In some experiments, cells were preincubated for 60 min with mannan (Sigma-Aldrich, 1 and 5 mg/ml), anti-CD11b, anti-CD16/32, anti-CD205, or control rat IgG (all at 50  $\mu$ g/ml, all from BD Biosciences). Cells were harvested after several hours and cytopins were prepared. DiffQuick-stained cells were analyzed for the presence of intra- and extracellular parasites. At least 200 cells were counted per sample. Supernatants from parasite/cell cocultures were collected and assayed for the presence of IL-12p40 or IL-10 by ELISA (BD Biosciences).

**Assessment of B cell and DC infiltration and function in vivo.** Groups of  $\geq$ 5 C57BL/6 mice were infected intradermally in ear skin with 1,000 *L. major* promastigotes. At several time points, ears were harvested and the number of B cells and DCs that had accumulated at the site of infection was determined (3). In brief, ears were incubated with 2 mg/ml Liberase (Boehringer Ingelheim). After 2 h, cells were dissociated mechanically and counted and the frequency of CD19<sup>+</sup> and CD11c<sup>+</sup> cells was assessed using

flow cytometry. In addition, serum from infected mice was obtained at several time points and stored at  $-20^{\circ}\text{C}$ .

*Leishmania*-specific IgG in serum was quantified by ELISA. Flat-bottom 96-well plates (Nunc) were coated overnight with 0.5 mg/100  $\mu\text{l}$  of soluble freeze-thaw *Leishmania* lysate (SLA), blocked for 1 h with PBS/2% BSA/0.05% Tween 20, and incubated for 2 h with dilutions of sera or reference standard anti-*Leishmania* IgG (prepared from pooled sera of immune infected mice). Subsequently, biotinylated goat anti-mouse IgG (Caltag) was added (125 ng/ml) for 2 h at  $20^{\circ}\text{C}$ . ELISA plates were developed using commercially available ELISA kit components (BD Biosciences) and reaction products were quantified spectrophotometrically.

**In vivo infections using IgG-opsonized parasites and B cell- or Fc $\gamma$ -deficient mice.** C57BL/6,  $\mu\text{MT}$ ,  $\text{J}_\text{H}\text{T}$ , or  $\text{Fc}\gamma^{-/-}$  mice were infected intradermally with  $10^3$  metacyclic *L. major* promastigotes. In some experiments, parasites were opsonized for 10 min with either NMS or IS and washed. Lesion development was assessed weekly in three dimensions using a caliper, and lesional volumes are reported (in  $\text{mm}^3$ ) as ellipsoids  $[(a/2 \times b/2 \times c/2) \times 4/3\pi]$ . Organisms present in lesional tissue were enumerated using limiting dilution assays (57). For measurement of cytokine production,  $10^6$  retroviral LN cells/200  $\mu\text{l}$  were added to 96-well plates in the presence of SLA (25  $\mu\text{g}/\text{ml}$ ). Antigen-specific IFN- $\gamma$  and IL-4 production was determined after 48 h using ELISA (R&D Systems).

At several time points, ears were harvested and inflammatory cells isolated using Liberase and mechanical disruption (3). The cells were counted and the frequencies of  $\text{CD11c}^+$  DC were determined using flow cytometry.  $\text{CD11c}^+$  cells were enriched to  $>98\%$  purity using a high speed cell sorter (FACS Vantage SE System, Becton Dickinson) and cytopins were analyzed by light microscopy to estimate the number of infected DCs/ear.

The frequency of daughter cells of proliferating antigen-reactive compared with nonproliferating LN T cells was estimated using flow cytometry (58–60). 6 wk after infection, LN cells were harvested and  $5 \times 10^6$  cells/ml were labeled with 1  $\mu\text{M}$  CFSE (Invitrogen). LN cells were subsequently plated at  $10^6/200 \mu\text{l}$  media in a 96-well U-bottom plate and left untreated or stimulated with SEB (10  $\mu\text{g}/\text{ml}$ ; Sigma-Aldrich), or SLA (61). After 5 d, proliferation was determined using flow cytometry. T cells were selected for analysis using mAbs against CD4 (L3T4, RM4-5), CD8 (Ly2, 53-6.7), or isotype control mAb (all from BD Biosciences). For each mouse, the percentage of *Leishmania*-reactive cells compared with nonproliferating cells was calculated.

**Statistics.** Statistical analysis was performed using the unpaired Student's *t* test.

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