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Dendritic Cell-Derived IL-12p40 Homodimer Contributes to Susceptibility in Cutaneous Leishmaniasis in BALB/c Mice

Axel P. Nigg,¹ Sabine Zahn,² Dominik Rückerl,¹ Christoph Hölscer,¹ Takayuki Yoshimoto,‡ Jan M. Ehrchen,§ Florian Wölbing,* Mark C. Udey,¶ and Esther von Stebut³*

Protection against Leishmania major in resistant C57BL/6 mice is mediated by Th1 cells, whereas susceptibility in BALB/c mice is the result of Th2 development. IL-12 release by L. major-infected dendritic cells (DC) is critically involved in differentiation of Th1 cells. Previously, we reported that strain differences in the production of DC-derived factors, e.g., IL-1αβ, are in part responsible for disparate disease outcome. In the present study, we analyzed the release of IL-12 from DC in more detail. Stimulated DC from C57BL/6 and BALB/c mice released comparable amounts of IL-12p40 and p70. In the absence of IL-4, BALB/c/DC produced significantly more IL-12p40 than C57BL/6 DC. Detailed analyses by Western blot and ELISA revealed that one-tenth of IL-12p40 detected in DC supernatants was released as the IL-12 antagonist IL-12p40 homodimer (IL-12p80). BALB/c DC released 2-fold more IL-12p80 than C57BL/6 DC both in vitro and in vivo. Local injection of IL-12p80 during the first 3 days after infection resulted in increased lesion volumes for several weeks in both L. major-infected BALB/c or C57BL/6 mice, in higher lesional parasite burdens, and decreased Th1-cytokine production. Finally, IL-12p40-transgenic C57BL/6 mice characterized by overexpression of p40 showed increased levels of serum IL-12p80 and enhanced disease susceptibility. Thus, in addition to IL-1αβ, strain-dependent differences in the release of other DC-derived factors such as IL-12p80 may influence genetically determined disease outcome. *The Journal of Immunology, 2007, 178: 7251–7258.

In murine experimental leishmaniasis, the development of genetically determined Th responses is observed (1). Protective, healing immune responses against Leishmania are dependent on effective IFN-γ release mainly from Th1 CD4⁺ T cells (2), but also from Tc1 CD8⁺ T cells (3). Infected macrophages are subsequently activated by IFN-γ that enables them to kill intracellular organisms. Most mouse strains (including C57BL/6 mice) develop Th1 immune responses associated with limited disease followed by lifelong immunity. In contrast, due to the inability to mount protective Th1 immunity, BALB/c mice experience progressive lesion development, dissemination of parasites, and ultimately succumb to infection. This experimental model has been used extensively to characterize factors determining Th1/Th2 development. Several differences at the level of T cells have been associated with disease outcome: e.g., increased numbers of Leishmania homologue of receptors for activated C-kinase 2-reactive, IL-4-producing CD4⁺ T cells as well as an early IL-4-mediated down-regulation of the IL-12R β2 chain on Th2 cells during the course of infection in BALB/c mice (for a review see Ref. 1).

However, in addition to CD4⁺ T cells, myeloid cells such as macrophages and dendritic cells (DC) have recently been described to influence the outcome of cutaneous leishmaniasis in susceptible BALB/c mice (4, 5). DC contribute to the development of protective immunity against intracellular pathogens (e.g., Leishmania) by initiating Leishmania-specific T cell priming and by directing Th development toward a Th1 phenotype. Particularly, the production of IL-12p70 by DC appears to be a prerequisite for generating optimal Th1 responses and plays a pivotal role in promoting cell-mediated immunity against intracellular pathogens. IL-12p70 is a heterodimeric cytokine composed of a p35 and a p40 subunit. However, beside dimerization with the p35 subunit to IL-12p70, p40 also dimerizes with an IL-12p35-related subunit, p19, to form a novel cytokine IL-23 (6). Additionally, IL-12p40 subunits also form disulfide-linked homodimers among each other (IL-12[p40]₀ or IL-12p80), which exhibit mainly antagonistic functions at the IL-12R (7–9).

We and others have previously shown that differential production of IL-1αβ by DC provided a partial explanation for disparate disease outcome in Leishmania infections (10–12). The present study was initiated to attempt to identify additional DC-derived cytokines responsible for directing Th differentiation in cutaneous leishmaniasis. Specifically, our previous studies revealed that susceptible BALB/c DC produce more IL-12p40 than resistant C57BL/6 cells, whereas the release of IL-12p70 was comparable in both strains. Because IL-12p40 can be released as monomeric IL-12p40, as inhibitory homodimer, or as bioactive heterodimeric IL-12p70 (7), we analyzed the differential production of IL-12p40-containing cytokines by Leishmania major-infected DC from these susceptible and resistant mouse strains. Interestingly, we found that BALB/c DC released significantly more IL-12p80 than C57BL/6 cells both in vitro and in vivo. We propose that, in

*Abbreviations used in this paper: DC, dendritic cell; Tg, transgenic; BMDC, bone marrow-derived DC; LN, lymph node.
BALB/c mice, the presence of increased levels of inhibitory IL-12p80 during T cell priming might inhibit protective Th1 priming. Genetically determined increased production of IL-12p80 as well as decreased IL-1α levels by infected DC may therefore contribute to progressive disease in susceptible BALB/c mice infected with L. major.

Materials and Methods

Mice

Six- to 8-wk-old BALB/c, C57BL/6, or IL-12p40 transgenic (Tg) mice (C57BL/6 background) (13) were obtained from the specific pathogen-free facility of the Central Animal Facility Production Program of the University of Mainz. All animals were housed in accordance with institutional and federal guidelines. All experiments were undertaken with an approved license from the Animal Care and Use Committee of the Rheinland-Pfalz Region.

Cells and parasites

Bone marrow-derived DC (BMDC) were generated as described previously (14). Bone marrow cells were plated at 2 × 10^6 cells/ml medium (RPMI 1640, 5% FCS) supplemented with 10 ng/ml GM-CSF (PeproTech) ±10 ng/ml IL-4 (PeproTech) in 24-well plates, and medium was partially replaced every 48 h. On day 6, nonadherent immature BMDC were harvested. Purity was determined by assessing the number of CD11c^+ MHC class II^+ cells by flow cytometry (10, 15). Metacyclic promastigotes or amastigotes of L. major clone VI (MHOM/IL/80/Friedlin) were prepared as described previously (12). Isolated parasites were opsonized with 5% BALB/c normal mouse serum and washed before in vitro or in vivo infections.

Quantification of cytokine production by DC in vitro

For stimulation, BMDC were plated at 2 × 10^5 or 2 × 10^6/ml in their basal medium for 18 h and 1LPS (100 ng/ml; Sigma-Aldrich), IFN-γ (1,000 U/ml; Peprotec/Tebu), or parasites (parasite:cell ratio of 3:1 or 10:1) were added as indicated. Cytokine accumulation in 18- or 72-h supernatants was measured using ELISA kits specific for murine IL-12p40, IL-12p70, and TNF-α (BD Pharmingen).

Detection of IL-12p40 homodimer

Aliquots of DC supernatants were stored at −20°C and assayed for the presence of IL-12p80 by Western blot and ELISA. For Western blots, supernatants were mixed with nonreducing loading buffer and resolved on 7% SDS polyacrylamide gels. Aliquots of Blue Pre-Stained Standards (Sigma-Aldrich) were included on each gel. Proteins were transferred to polyvinylidene difluoride-membranes (Millipore) by semidry blotting for 2 h at 4°C, followed by incubation with streptavidin-HRP conjugate (156 ng/ml; Zytomed) diluted in 1% BSA with 0.05% Tween 20 in PBS for 1 h at 4°C, and visualized with the Li-Cor detection system (Li-Cor Biosciences). Presence of IL-12p80 by Western blot and ELISA. For Western blots, infected BMDC from susceptible and resistant mouse strains release detectable amounts of IL-12p40 and IL-12p70 (10, 11). Strain-dependent differences in the production of IL-12p70 that would explain genetic determination of disease outcome were not detected by ELISA. To extend these findings using another source of DC, we initiated studies using BMDC generated in the presence of GM-CSF and IL-4 (Ref. 15 and this study). BMDC were stimulated with L. major promastigotes or amastigotes. Coincubation with promastigotes led to infection of only 4 ± 2% of C57BL/6 DC and 8 ± 1% of BALB/c DC, whereas 28 ± 3% and 26 ± 3% of BMDC were infected when amastigotes were used (parasite:cell ratio 10:1; C57BL/6 and BALB/c, respectively; n ≥ 5). Therefore, as in previous studies using skin-DC (11), no strain-specific difference in the percentage of infected cells or the number of ingested parasites/cell was observed. BMDC derived from genetically resistant C57BL/6 or susceptible BALB/c mice both displayed an activated phenotype after infection with L. major amastigotes, as determined by up-regulation of MHC class II and costimulatory molecules (CD40, CD54 and CD86; data not shown).

Infections

Groups of mice were infected intradermally into ear skin with 2 × 10^5 infectious stage metacyclic promastigotes (12, 15). In some experiments, mice were euthanized at wk 3, and DC from draining submandibular LNs were positively selected using anti-CD11c microbeads (Miltenyi Biotec) and plated at 1 × 10^6 cells/200 μl in RPMI 1640/5% FCS. Supernatants were harvested after 18 h and analyzed for the presence of IL-12p40 and IL-12p80 by ELISA as described above.

In other experiments, murine IL-12p70 or IL-12p80 (both R&D Systems) were diluted in serum-free PBS and injected intradermally on days 1–3 postinfection as indicated. Lesion sizes were measured weekly using a caliper and ellipsoid volumes were calculated. Organisms present in lesional tissue were enumerated at the indicated times using limiting dilution assays as described previously (12). For determination of cytokine profiles in infected mice, retroauricular LNs were recovered at wk 3 and wk 5 and single-cell suspensions were prepared. Cells were added to 96-well plates without Ag or with L. major lysate (10^6 cells/200 μl) in complete RPMI 1640 containing 2-ME (5 × 10^-5 M). Culture supernatants were assayed for cytokine production after 48 h using ELISA specific for murine IFN-γ (R&D Systems) and IL-4 (BD Pharmingen).

Statistics

Statistical analysis was performed using the unpaired Student’s t test.

Results

L. major-infected BMDC from susceptible and resistant mouse strains release IL-12p40 containing cytokines

Previous studies have shown that DC are capable of taking up amastigotes or promastigotes (10, 15–17). Infected DC were activated and released detectable amounts of IL-12p40 and IL-12p70 (10, 11). Strain-dependent differences in the production of IL-12p70 that would explain genetic determination of disease outcome were not detected by ELISA. To extend these findings using another source of DC, we initiated studies using BMDC generated in the presence of GM-CSF and IL-4 (Ref. 15 and this study). BMDC were stimulated with L. major promastigotes or amastigotes. Coincubation with promastigotes led to infection of only 4 ± 2% of C57BL/6 DC and 8 ± 1% of BALB/c DC, whereas 28 ± 3% and 26 ± 3% of BMDC were infected when amastigotes were used (parasite:cell ratio 10:1; C57BL/6 and BALB/c, respectively; n ≥ 5). Therefore, as in previous studies using skin-DC (11), no strain-specific difference in the percentage of infected cells or the number of ingested parasites/cell was observed. BMDC derived from genetically resistant C57BL/6 or susceptible BALB/c mice both displayed an activated phenotype after infection with L. major amastigotes, as determined by up-regulation of MHC class II and costimulatory molecules (CD40, CD54 and CD86; data not shown).
Next, release of IL-12p40 and IL-12p70 from stimulated BMDC was determined. As shown previously with skin-DC (11), stimulation with LPS or infection with \textit{L. major} amastigotes led to increased release of IL-12p40 from BMDC compared with unstimulated controls (Fig. 1A). In contrast, the amount of IL-12p70 was \textsim100-fold lower compared with IL-12p40 (Fig. 1B), which is consistent with previous reports using other sources of DC (10, 11, 18). However, the pattern of release of IL-12p70 from BMDC was comparable to IL-12p40. Strain-dependent differences in the release of IL-12p70 from activated BMDC were not evident. As in previous studies, BALB/c DC released more IL-12p40 in response to maximal stimulation with LPS than their C57BL/6-derived counterparts (11). However, these differences did not reach statistical significance.

We also assayed the supernatants of stimulated BMDC for the release of other Th1-inducing cytokines. Release of IL-18 was not found in culture supernatants of \textsim2 \times 10^{5} or \textsim2 \times 10^{6} cells/ml using two commercially available ELISA kits. In parallel to the release of IL-12, TNF-\alpha production of stimulated BMDC was also detected (Fig. 1C). No strain-dependent differences in the amount released were observed. Production of Th2 cytokines (IL-4, IL-10, and TGF-\beta1) by BMDC generated from C57BL/6 or BALB/c mice was also determined (data not shown). All three cytokines were found to be produced by DC at baseline levels, but the amounts were comparably low between the mouse strains analyzed and induction in activated cells was not observed.

**Effect of IL-4 on IL-12 release from BMDC**

IL-4 is commonly used for the generation of murine BMDC. However, IL-4 has been shown to regulate the production of IL-12p40 and IL-12p70 by DC (19–23). To test the effect of IL-4 on IL-12p40 containing cytokine production, DC were generated from C57BL/6 or BALB/c mice in the presence or absence of this cytokine. Cells were subsequently stimulated with either LPS or \textit{L. major} amastigotes (plus or minus IL-4 during stimulation; Fig. 1, D–F). Interestingly, although BALB/c DC stimulated in the presence of IL-4 produced marginally more IL-12p40 than C57BL/6 DC (a difference that never reached statistical significance), in the absence of IL-4 BALB/c DC produced more IL-12p40 than C57BL/6 DC (Fig. 1D). This effect was most dramatic in LPS-treated, maximally stimulated cells, but was also detectable in \textit{L. major}-promoting activated DC (Fig. 1F). A similar effect was observed with regard to the release of Th1-promoting TNF-\alpha (data not shown).

Interestingly, confirming previous findings (19, 21), the effect of IL-4 on IL-12p70 production was reversed. In the absence of IL-4, the DC produced significantly less bioactive IL-12p70. With regard to IL-12p70, IL-4-activated DC from both mouse strains produced equivalent amounts of this cytokine (Fig. 1E).

To confirm that DC generated with or without IL-4 have otherwise similar phenotypes and allostimulatory properties, we determined their surface Ag profiles and performed proliferation assays with alloreactive T cells (data not shown). The expression levels of CD11c, MHC class II, and costimulatory molecules were comparable on DC generated in the presence or absence of IL-4. In addition, their ability to activate alloreactive T cells in proliferation assays was similar.

**DC from susceptible BALB/c mice release more antagonistic IL-12p80 than C57BL/6 DC**

IL-12p40 is released as a component of bioactive IL-12p70 (in association with p35) or IL-23 (together with p19), as inactive IL-12p40 monomer, or as antagonistic IL-12p80 (8). We therefore analyzed whether DC stimulated with LPS or infected with \textit{L. major} also released IL-12p80. DC were generated in the presence or absence of IL-4, and stimulated with LPS for 18 h at \textsim2 \times 10^{5} cells/ml. The culture supernatants were analyzed by Western blot for the presence of IL-12p40 reactivity under nonreducing conditions. In Fig. 2A, reactivity at \sim80 kDa reflecting IL-12p80 production was detected. IL-12p70 production was not detected. Interestingly, under all conditions studied, supernatants of BALB/c DC contained more IL-12p80 than C57BL/6 cultures. From four independent experiments performed, semiquantitative analyses of band densities revealed that BALB/c DC cultures contained \textsim1.5- to 2-fold more IL-12p80 compared with supernatants of C57BL/6 cells (Fig. 2A). In contrast to a previous report (19), no dramatic effect of IL-4 on the amount of homodimer produced from the DC was observed.

To quantitate the amount of IL-12p80 synthesis, we developed an ELISA specific for IL-12p80 (Fig. 2B). To accomplish this process, we used the same monoclonal anti-IL-12p40 Ab recognizing a single epitope on the p40 monomer (clone 17.8) both for capturing as well as detecting the protein. Using this approach, we determined that only rIL-12p80 was detectable by this ELISA, whereas IL-12p70 or IL-23—both containing one IL-12p40 subunit—were not measurable. Small amounts of immunoreactivity were detected at high concentrations of the commercial IL-12p40 standard, suggesting that the IL-12p40 preparation contains small amounts of contaminating IL-12p80. The IL-12p80 ELISA was highly reproducible and the detection limit was 31.25 pg/ml (similar to other commercially available ELISA).

We next measured IL-12p80 release from activated DC by ELISA. DC were generated in the absence or presence of IL-4,
harvested, and stimulated for an additional 18 h with LPS/IFN-γ/H9253 or amastigotes of L. major (2 × 10^5 cells/ml). Significant IL-12p80 release by both BALB/c and C57BL/6 DC was detected after stimulation (Fig. 2C). BALB/c DC produced more IL-12p80 than C57BL/6 DC after stimulation with either LPS/IFN-γ or L. major. This difference was statistically significant in the absence of IL-4, confirming previous findings regarding the role of IL-4 in IL-12p80 generation (19). Interestingly, approximately one-tenth of total IL-12p40 appeared to be released from DC in the form of the inhibitory IL-12p80, an amount ~10-fold greater than stimulatory IL-12p70. In a prior study, it was postulated that up to 40% of p40 protein in the serum is present as IL-12p40 homodimer (24).

Finally, we attempted to quantitate the production of IL-12p80 by DC from C57BL/6 and BALB/c mice in a more physiologic setting. Production of IL-12p40 in draining LN cells restimulated with soluble Leishmania Ag usually ranges between 200 and 400 pg/ml if cells were plated at 10^6/ml and IL-12p70 and IL-12p80 are below the detection limit. Thus, we enriched for LN DC in an attempt to increase the sensitivity of our approach. Mice were infected by injection of 2 × 10^5 metacyclic promastigotes of L. major into ear skin. Three weeks later, draining LN CD11c+ cells were enriched using microbeads (Miltenyi Biotec) and plated in medium at 1 × 10^6/ml for 18 h, and supernatants were assayed for the presence of IL-12p40 containing cytokines by ELISA. As determined previously, production of total IL-12p40 was comparable between DC from the two mouse strains analyzed (BALB/c, 10.2 ± 2.0 vs C57BL/6, 12.5 ± 2.3 ng/ml; n = 11). In contrast, release of inhibitory IL-12p80 by BALB/c DC in vivo was ~2-fold increased as compared with C57BL/6 DC (BALB/c, 162 ± 49 vs C57BL/6, 76 ± 30 pg/ml; n = 11).

**IL-12p40 homodimer blocks IL-12p70 signaling**

In prior studies it was demonstrated that IL-12p80 preferentially binds IL-12Rβ1, but not IL-12Rβ2 (25–29). Although naive T
cells from BALB/c mice initially respond to IL-12, T cells from L. major-infected animals lose their IL-12 responsiveness due to down-modulation of the IL-12Rβ2 chain (30–32). The IL-12β2 down-regulation of BALB/c T cells was in part dependent on IL-4. However, because BALB/c mice that express a transgene for IL-12β2 are still susceptible to infection, the role of IL-12β2 for disease outcome is unclear to date (33).

To confirm that IL-12p80 inhibits IL-12R-mediated signaling in T cells, we investigated the role of IL-12p40 homodimer on the phosphorylation of STAT-4 and IFN-γ release by IL-12-stimulated cells (Fig. 3). Fig. 3A shows a representative Western blot in which phospho-STAT-4 levels were visualized after stimulation of splenocytes with IL-12p70 (1 ng/ml) and/or IL-12p80 (1 ng/ml). Phospho-STAT-4 was readily detectable in IL-12p70-stimulated cultures (Fig. 3A, lane 3), whereas addition of IL-12p80 abrogated this effect (Fig. 3A, lane 4). Next, BALB/c CD4+ T cells were subjected to Th1-inducing conditions (IL-12/anti-IL-4) and IL-12p80 was added in different concentrations (Fig. 3B). Resulting IFN-γ levels were determined in supernatants of anti-CD3-restimulated cells. As shown in Fig. 3B, IL-12p80 inhibited the IL-12p70-induced IFN-γ release in a concentration-dependent fashion. Thus, our data confirm that BALB/c T cells respond to IL-12p80 by down-modulating IL-12p70-induced events.

**IL-12p80 augments Th2 immunity in vivo**

To determine whether IL-12p80 has the ability to regulate Th development in cutaneous leishmaniasis, susceptible BALB/c and resistant C57BL/6 mice were infected with 2 × 10⁵ infectious stage promastigotes of L. major into ear skin and treated locally with recombinant cytokine during T cell priming. In previous reports demonstrating an effect of IL-12p80 in disease settings, recombinant cytokine was applied in doses ranging from 1 to 100 μg/mouse i.p. (34, 35). In addition, DC-supernatants generally contain ~100- to 1,000-fold more IL-12p40 than IL-12p70. Therefore, we chose to apply 2 μg of IL-12p80/mouse on days 1, 2, and 3 post-infection—a treatment schedule that has proven to be efficacious for IL-1α to protect BALB/c mice from progressive disease (12). For comparison, IL-12p70 was administered in a similar fashion (50 ng/mouse) (12).

In BALB/c (Fig. 4A) as well as C57BL/6 mice (Fig. 4B), transient treatment with IL-12p80 resulted in significantly increased lesion volumes that persisted for several weeks. In addition, increased leishmanial parasite loads as well as impaired Th1 and enhanced Th2 development were observed in BALB/c mice as determined 3 wk postinfection (Fig. 4, C and D). Interestingly, prolonged treatment with IL-12p80 for an additional 3 wk did not further promote disease progression (data not shown).

Treatment with bioactive IL-12p70 during T cell priming protected BALB/c mice from progressive disease, but did not promote healing. This result was in accordance with previous findings demonstrating that sustained IL-12p70 expression is required to mediate full protection in susceptible mice (36). In contrast, C57BL/6 mice treated with IL-12p70 did not develop significant lesions (Fig. 4B). However, similar to BALB/c mice, treatment of C57BL/6 mice with IL-12p80 resulted in enhanced lesion volumes and delayed healing for several weeks.

**IL-12p40 Tg mice showed enhanced disease susceptibility due to increased levels of IL-12p80**

Previously, we generated Tg mice that overexpress IL-12p40 under the control of a liver-specific promoter (13). Serum levels of IL-12p40Tg mice were high and IL-12p40 circulates as monomer and homodimer. In addition, previous studies demonstrated that Ag-driven cytokine responses in these mice were skewed toward a Th2 phenotype.

We additionally investigated the relevance of IL-12p80 in L. major infections using IL-12p40Tg mice on a resistant C57BL/6 genetic background. Wild-type C57BL/6 or IL-12p40Tg mice were infected with 2 × 10⁵ metacyclic promastigotes of L. major. On day 1, 2, and 3, mice were treated locally with 2 μg of IL-12p80, 50 ng of IL-12p70, or 20 μl of PBS. A and B. Lesion development was monitored weekly over the course of 2 mo in three dimensions and lesion volumes were calculated as ellipsoid (mean ± SEM; *, p < 0.05, **, p < 0.005, and ***, p < 0.002 compared with PBS-treated control mice). C. Lesional parasite loads of different BALB/c treatment groups were compared with controls at wk 3 postinfection using limiting dilution assays (pooled data from three independent experiments are shown, n = 8 mice/group; *, p < 0.05). D, LN cells were harvested from infected BALB/c mice and plated at 10⁶ cells/200μl. Cells were stimulated with soluble Leishmania Ag (SLA) for 48 h, and Ag-specific cytokine release was determined using ELISA specific for murine IFN-γ and IL-4 (mean ± SEM). For each mouse, the ratio between IFN-γ and IL-4 was calculated and statistical significance was determined (*, p < 0.05 and ***, p < 0.002; n ≥ 12).

FIGURE 4. Administration of inhibitory IL-12p80 leads to increased lesion development and inhibition of sufficient Th1 development. Groups of n = 5 BALB/c (A, C, and D) or C57BL/6 (B) mice were infected with 2 × 10⁵ metacyclic promastigotes of L. major. On day 1, 2, and 3, mice were treated locally with 2 μg of IL-12p80, 50 ng of IL-12p70, or 20 μl of PBS. A and B. Lesion development was monitored weekly over the course of 2 mo in three dimensions and lesion volumes were calculated as ellipsoid (mean ± SEM; *, p < 0.05, **, p < 0.005, and ***, p < 0.002 compared with PBS-treated control mice). C. Lesional parasite loads of different BALB/c treatment groups were compared with controls at wk 3 postinfection using limiting dilution assays (pooled data from three independent experiments are shown, n = 8 mice/group; *, p < 0.05). D, LN cells were harvested from infected BALB/c mice and plated at 10⁶ cells/200 μl. Cells were stimulated with soluble Leishmania Ag (SLA) for 48 h, and Ag-specific cytokine release was determined using ELISA specific for murine IFN-γ and IL-4 (mean ± SEM). For each mouse, the ratio between IFN-γ and IL-4 was calculated and statistical significance was determined (*, p < 0.05 and ***, p < 0.002; n ≥ 12).
mice were significantly decreased in both male and female mice. Interestingly, IFN-γ production was almost absent in LN cultures of female IL-12p40Tg mice. In contrast, IL-4 release was increased in IL-12p40Tg mice as compared with C57BL/6 controls.

Finally, we quantitated IL-12p40 levels in the serum of IL-12p40Tg mice as compared with wild-type controls (Fig. 6B). As expected, the amount of total IL-12p40 was increased ~100- to 150-fold in naive mice Tg for IL-12p40. IL-12p40 levels in the serum of naive C57BL/6 were almost absent. No increase of serum IL-12p40 was detected in wild-type mice infected with L. major over the course of several weeks (data not shown), suggesting that IL-12p40 is not a cytokine abundant in serum under physiological conditions. We next determined the proportion of IL-12p40 present in the serum of mice in the form of the inhibitory IL-12p80. The levels of IL-12p80 were ~20- to 40-fold lower as compared with total IL-12p40. Interestingly, the amount of IL-12p80 detected in the serum of naive female IL-12p40Tg mice was significantly higher as compared with IL-12p80 in male IL-12p40Tg animals or controls. The levels of IL-12p80 in serum of C57BL/6 controls were very low. As in wild-type mice, the levels of IL-12p40 or IL-12p80 were not additionally increased upon infection with L. major.

FIGURE 5. IL-12p40 Tg mice show enhanced susceptibility in infections with L. major. Groups of ~5 IL-12p40 Tg or control C57BL/6 mice were infected with 2 × 10⁵ metacyclic promastigotes of L. major. A, Lesion volumes were assessed weekly over the course of ~4 mo and expressed as mean ± SEM (pooled data from four independent experiments are shown; *, p < 0.05 and ***, p < 0.002 as compared with sex-matched controls; n = 7). B, Parasite loads of lesions and spleens were determined in wk 3 and 5 using limiting dilution assays, and bars indicate arithmetic means. Pooled data of three experiments are shown (*, p < 0.05; n ≥ 3).

FIGURE 6. Infected IL-12p40 Tg mice display impaired Th1 immunity which correlates with increased levels of serum IL-12p40 homodimer. A, LN cells of infected IL-12p40Tg or control C57BL/6 mice were harvested in wk 3 and 5, and Ag-specific cytokine release was determined after 48 h using ELISA specific for murine IFN-γ and IL-4 (mean ± SEM; *, p < 0.05 and ***, p < 0.002; n ≥ 5). B, Levels of IL-12p40 and IL-12p80 were determined in serum of naive IL-12p40Tg or C57BL/6 mice by ELISA (mean ± SEM; *, p < 0.05 and ***, p < 0.002; n ≥ 9 mice).
Discussion
DC influence Th cell priming and differentiation in vivo by producing an array of cytokines including IL-12p70. Using the system of resistance and susceptibility in cutaneous leishmaniasis, we and others have previously identified DC-derived IL-1αβ to be an important regulator of Th1 immune responses in addition to IL-12p70 (12, 37, 38). In the present study, we investigated strain-specific differences in the release of IL-12 in more detail. We found that DC derived from susceptible BALB/c mice produced significantly more inhibitory IL-12p80 than DC from resistant C57BL/6 mice. In addition, treatment with IL-12p80 resulted in decreased Th1 development and more progressive lesions after infection. Therefore, BALB/c DC may inhibit IL-12p70 signaling in T cells by overproducing IL-12p80 that blocks the IL-12R and thus contributes to genetic determination of disease outcome.

Previous studies using Langerhans cell-like fetal skin-derived DC or freshly isolated DC have shown that DC take up L. major amastigotes, are activated and release detectable amounts of IL-12p40 and IL-12p70 (10, 11, 39). In this study, BMDC were used to confirm these initial findings. Again, no strain-specific differences in the percentages of infected cells or the number of ingested parasites per cell were detected. In addition, BMDC derived from C57BL/6 or BALB/c mice were both activated after infection with L. major amastigotes, and strain-dependent differences in the release of IL-12p40 or IL-12p70 from activated BMDC, as detected by ELISA, were not obvious.

Several groups have previously demonstrated potent effects of IL-4 on the production of IL-12p70 from DC (19–23). In an attempt to determine the amount of IL-4 present in L. major-infected skin during DC activation, we harvested ear skin of infected BALB/c or C57BL/6 mice at several time points postinfection (data not shown). Using either Ab-based detection methods (ELISA, cytometric bead array) or the RT-PCR technique, we found no or only minute amounts of IL-4 a few days after parasite inoculation (<25 pg/ear). Biedermann et al. (21) suggested that BALB/c mice have a deficiency in local IL-4 production early on after infection resulting in inefficient DC activation. Administration of sufficient amounts of IL-4 during DC activation (within the first 8 h postinfection) in BALB/c mice resulted in efficient Th1 priming and resistance against Leishmania infection (21). We therefore investigated how IL-4 influences the release of IL-12p40 subunits in our system of L. major-infected or LPS-stimulated DC. We confirmed that DC stimulated in the presence of IL-4 produce more IL-12p70 (presumably by up-regulating IL-12p35 expression) compared with control treatment (19, 21). In the context of Leishmania infections, IL-4R expression was increased on infected LC from susceptible BALB/c mice, but not on those from resistant mice (20). In these studies, IL-4 treatment of infected BALB/c DC strongly decreased LPS-induced IL-12p40 responses, but IL-12p70 production was not determined. In this study, BALB/c DC produced more IL-12p40 than cells derived from C57BL/6 mice in the absence of IL-4. Under these conditions, the difference in IL-12p40 production that was observed in previous studies (11) became statistically significant. In vivo, increased IL-12p40 production was found in infected BALB/c mice (as compared with C57BL/6 mice) especially very early on after infection with 2 × 106 L. major promastigotes (40). When compared with C57BL/6 mice, ~2- to 6-fold more IL-12p40 was detected in LN cell cultures of BALB/c mice on day 1 and 7 postinfection.

Bioactive IL-12p70 is a heterodimeric cytokine composed of IL-12p40 and IL-12p35 (7). Production of IL-12p40 and IL-12p35 have been shown to be regulated independently. IL-12p35 is secreted only in association with IL-12p40 as part of the IL-12p70 heterodimer. IL-12p40, however, is released as part of the bioactive heterodimer, but also as inactive monomeric IL-12p40 and IL-12p80. Both IL-12p40 and IL-12p80 are secreted in excess of IL-12p70, but IL-12p80 has been shown to act as potent modulator of IL-12p70 signaling at its receptor. In several studies, IL-12p80 was shown to have inhibitory properties (Refs. 13, 25, 41, 42 and present study), whereas we have also demonstrated an agonistic function in IL-12p35/p40-deficient mice (8). In cutaneous leishmaniasis, the role of IL-12p80 has not been previously studied. We now confirmed that a substantial amount of IL-12p40 was released by DC in the form of the inhibitory IL-12p40 homodimer (19). In addition, BALB/c DC produced more IL-12p80 than C57BL/6 DC.

As compared with prior studies (19–23), the effect of IL-4 on IL-12p80 release in our study was not strong. Even though we were able to reproduce the up-regulating effect of IL-4 on IL-12p70 release, the influence of IL-4 on down-modulating IL-12p80 protein synthesis from BMDC in vitro was less striking. Thus, IL-4 may not be the only cytokine/factor responsible for the induction/regulation of IL-12p80 production.

In vivo, administration of IL-12p80 led to protection from IL-12-dependent shock (42). We therefore analyzed the role of IL-12p80 during T cell priming in cutaneous leishmaniasis in vivo. Both susceptible BALB/c and resistant C57BL/6 mice demonstrated increased susceptibility in the presence of IL-12p80 during T cell priming and this effect lasted for >6 wk, although IL-12p80 was only applied during days 1–3 postinfection. The cytokine profile in BALB/c LN cultures was altered toward a Th2 phenotype, whereas IL-12p70 treatment induced a shift toward Th1 cell differentiation as expected. Interestingly, prolonged treatment with IL-12p80 did not additionally worsen disease outcome, suggesting that physiological IL-12p80 acts early after infection.

IL-12p40Tg mice, producing increased amounts of IL-12p80, displayed reduced Th1 responses and increased susceptibility toward infection with e.g., Plasmodium berghei (13). In this study, we demonstrate that IL-12p40Tg mice on a genetically resistant C57BL/6 background are also more susceptible to infection with L. major. The IL-12p40Tg mice displayed increased lesion volumes, higher leisolated parasite loads, and enhanced spreading of the parasite to visceral organs. In addition, the Tg mice showed IFN-γ/IL-4 ratios that were altered toward Th2. Interestingly, the level of susceptibility (in female vs male IL-12p40Tg mice) strongly correlated with the amount of homodimer present in the serum. The fact that female IL-12p40Tg mice displayed higher levels of IL-12p80 despite similar levels of IL-12p40 as compared with male IL-12p40Tg is unexplained and requires further examination.

In summary, our data indicate that in BALB/c mice, DC may contribute to disease susceptibility not only by producing less IL-1αβ, but also by releasing more inhibitory IL-12p80 after infection with L. major amastigotes than their C57BL/6 counterparts. In comparison with C57BL/6 cells, BALB/c DC display an imbalance of factors regulating Th development. BALB/c DC produce lesser amounts of cytokines (e.g., IL-1) that favor the development of Th1 immunity and increased amounts of others (e.g., IL-12p80) that promote Th2 cell differentiation. In addition, we also demonstrated an important role of IL-12p80 for the efficient induction of protective Th1-predominant immunity in vivo. These data provide additional support for the concept that genetic differences that contribute to susceptibility to cutaneous leishmaniasis in mice are expressed at the level of DC as well as in T cells.

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