The Role of Interleukin-10 in Susceptibility of BALB/c Mice to Infection with *Leishmania mexicana* and *Leishmania amazonensis*

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The Role of Interleukin-10 in Susceptibility of BALB/c Mice to Infection with Leishmania mexicana and Leishmania amazonensis 1

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Recent studies have demonstrated the critical role of IL-10 in susceptibility to cutaneous and visceral leishmaniasis caused by Leishmania major and Leishmania donovani, respectively. To determine whether IL-10 also plays a similar role in the susceptibility and pathogenesis of cutaneous leishmaniasis caused by the New World species, L. mexicana and L. amazonensis, we analyzed their course of infection in IL-10-deficient BALB/c mice and their wild-type counterparts. Although IL-10-deficient mice infected with either L. mexicana or L. amazonensis failed to control the lesion progression, we did observe consistently lower levels of infection in IL-10−/− mice compared with wild-type BALB/c mice. We also observed increased IFN-γ and NO production and higher levels for IL-12p40 and IL-12Rβ2 mRNA in cells from IL-10−/− mice compared with cells from BALB/c mice. The mRNA levels for IL-4, which increased significantly in both IL-10−/− and BALB/c mice, were comparable. When treated with anti-IL-4 mAb, IL-10−/− mice resolved the infection more effectively and had significantly fewer parasites in their lesions compared with similarly treated BALB/c mice. These findings suggest that IL-10, although not the dominant mediator of susceptibility of BALB/c mice to infection with L. mexicana and L. amazonensis, does play a significant role in regulating the development of a protective Th1-type response. However, effective resolution of infection with these New World parasites requires neutralization of both IL-4 and IL-10. The Journal of Immunology, 2003, 171: 3705–3710.

Infections in mice by Leishmania major have been extensively utilized to examine the parameters influencing the development of protective Th1-type vs nonprotective Th2-type immune responses. Early studies established the concept that the production of IL-4 during initial stages of infection in susceptible BALB/c mice played a dominant role in directing the differentiation of naive CD4+ T cells into Th2-type effector cells (1, 2). However, more recent studies have questioned whether IL-4 production is absolutely essential to the development of nonhealing infections in mice. Thus, although infections with some strains of L. major are controlled in IL-4- or IL-4Rα-deficient mice, infections with other strains are not, suggesting that other IL-4-independent mechanisms can influence the ability of mice to control parasite replication and/or survival (3, 4). With respect to the development of resistance, however, there is general consensus that the activation of an IL-12-dependent Th1 response is required for optimum production of the macrophage-activating cytokine, IFN-γ, and cure of infection (5, 6).

Although L. major has been the most intensively investigated Leishmania species in mice, the immune response to other species has not been neglected, and studies of New World Leishmania such as L. mexicana and L. amazonensis offer interesting contrasts to those with L. major. For example, while L. major, L. amazonensis, and L. mexicana all produce progressive, nonhealing infections in BALB/c mice (7–9), L. major infections, unlike L. mexicana and L. amazonensis infections, heal in the majority of other mouse strains such as C3H, CBA, C57BL/10, and C57BL/6. In contrast, L. mexicana and L. amazonensis infections in these L. major-resistant mouse strains result in the development of nonhealing lesions of varying severity (8, 10, 11). The susceptibility of C57BL/6 mice to infection with L. amazonensis is associated with defective production of IFN-γ rather than the production of high amounts of IL-4 (11). Similarly, control of the closely related L. mexicana in C57BL/6 mice has been shown to be IFN-γ- and STAT4-dependent, although surprisingly independent of IL-12 production (12).

Interestingly, cells from L. amazonensis-infected C3H mice produced low levels of IL-12, but were not induced to heal following treatment with exogenous IL-12 (11). In addition, IL-4-deficient mice on a C57BL/6 background were no more resistant to infection than were wild-type mice suggesting that the failure to resolve infection was independent of the development of a dominant Th2-type response (11). The underlying mechanism responsible for the failure of resistant strains of mice to develop a vigorous Th1-type response following infection with L. amazonensis is unclear, but could involve the production of anti-inflammatory factors such as TGF-β, which has been shown to play a role in the susceptibility of BALB/c mice to this parasite (13). In contrast to C57BL/6 mice, susceptibility of BALB/c mice to L. amazonensis has been shown to be IL-4 mediated. Anti-IL-4 Ab treatment of BALB/c mice before infection with L. major or L. amazonensis reduces the parasite burden and controls the infection (8, 14). Similarly IL-4−/− mice on a BALB/c background have enhanced resistance to L. mexicana, as well as L. amazonensis infection (9, 15).

An additional cytokine that has been recently shown to play an important role in resistance to leishmaniasis is IL-10. IL-10 suppresses IFN-γ synthesis by inhibiting accessory cell functions and can reduce production of NO by activated macrophages. IL-10 also down-regulates expression of MHC class I and class II molecules as well as costimulatory B7 molecules on macrophages. Of note, a
recent study has shown that IL-10-deficient BALB/c mice can control infection with L. major, suggesting that IL-10 plays a critical role in mediating the susceptibility and pathogenesis of cutaneous leishmaniasis (16). However, C57BL/6 IL-10-deficient mice fail to exhibit increased resistance to L. amazonensis, despite the fact that they developed an enhanced Th1-type response (17). Given the differences in patterns of disease in inbred strains of mice infected with the Old World parasite, L. major, and the New World Leishmania species and given the relative importance of different cytokines in resistance or susceptibility to different Leishmania species, we have explored how a deficiency in IL-10 influences infection with L. mexicana and L. amazonensis in susceptible BALB/c mice. We show that in contrast to effects on L. major, a lack of endogenous IL-10 production has little, if any, effect on lesion development in L. mexicana- and L. amazonensis-infected mice, although IL-10-deficient mice do show an increase in their ability to control parasite numbers. In addition, we demonstrate IL-10-deficient mice, but not wild-type BALB/c mice, treated with Ab to IL-4 can effectively resolve infection with L. mexicana. These results suggest that both IL-4 and IL-10 play significant roles in susceptibility to infection with New World species and that depletion of both cytokines may be required for optimum development of resistance.

Materials and Methods

Parasites and animals

Female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c IL-10−/− mice were originally obtained from Dr. Robert Coffman (DNAX, Palo Alto, CA) and were bred and maintained within the animal resource facility at the University of Pennsylvania. These mice have been fully back-crossed greater than six generations. All mice were 6–10 wk old at the time of infection. L. mexicana (MYNC/BZ/62/M379) and L. amazonensis (IFLA/BR/67/PH8) were maintained in Grace’s insect cell culture medium (Life Technologies, Grand Island, NY) containing 20% FBS, 2 mM l-glutamine, 100 µg streptomycin, and 100 U penicillin G sodium per milliliter.

Infections

Mice were inoculated into one hind footpad with 1 × 106 stationary phase L. mexicana or L. amazonensis promastigotes. Lesion size was measured with Vernier caliper and expressed as the difference in thickness between the infected and the uninfected contralateral footpads. Parasites were enumerated by a limiting dilution assay as previously described (8). In brief, the homogenates of infected lesions were serially diluted in Grace’s insect cell culture medium plus 20% FBS and observed 5–7 days later for growth of promastigotes. Lesion size was measured using the Vernier caliper and expressed as the difference in thickness between the infected and the uninfected contralateral footpads. Parasite numbers are expressed as the negative log10 dilution at which promastigotes growth was observed.

Anti-IL-4 Ab treatment protocol

BALB/c wild-type and BALB/c IL-10−/− mice were treated i.p. with anti-IL-4 Ab (11B11, Harlan, Madison, WI) on day 0 (5 mg/mouse) and then on days 7, 15, and 21 (3 mg/mouse) of infection. Control mice were treated with normal rat IgG.

Cell culture and ELISA

Single-cell suspensions of spleens were cultured at 5 × 106 cells/ml in DMEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 5 × 10−5 M 2-ME in the presence of freeze-thawed leishmanial Ag (5 × 105 parasites Eq/ml) as previously described (8). Supernatants were collected at 72 h and assayed for IFN-γ by ELISA as previously described (18). Recombinant IFN-γ (PeproTech, Rocky Hill, NJ) was used as standard for ELISA.

NO production assay

NO production from the culture supernatants harvested at 72 h was assessed using the Griess reagent (19).

RNase protection assay

Total RNA was isolated from popliteal draining lymph nodes using RNA STAT-60 (Tel-Test, Friendswood, TX) as directed by the manufacturer. mRNA was quantified by RNase protection assay using a Riboquant kit (BD Pharmingen, San Diego, CA) as directed. A custom probe from BD Pharmingen was prepared using [32P]UTP and hybridized to 15 µg of each sample RNA. The protected probe was purified and resolved on 5% denaturing polyacrylamide gel using Ultra Pure Sequagel reagents (National Diagnostics, Atlanta, GA). Dried gels were exposed to a phosphor-imaging screen and protected fragments were visualized using a phosphor imager GS-525 Molecular Imager System (Bio-Rad, Richmond, CA). Cytokine mRNA levels were normalized to level L32 and the results are expressed as the increase in message level in experimental mice and in control mice.

Statistical analysis

Statistically significant differences between groups were determined using the unpaired Student t test. Significance was assumed if p < 0.05.

Results

A deficiency in endogenous IL-10 has little effect on lesion development, but does limit parasite numbers in mice infected with L. mexicana or L. amazonensis

To determine whether IL-10 also plays a role in susceptibility to New World cutaneous leishmaniasis, we inoculated BALB/c IL-10−/− mice with 1 × 106 stationary-phase promastigotes of L. mexicana or L. amazonensis into one hind footpad and compared the course of infections with that in similarly infected BALB/c mice. As can be seen in Fig. 1A at week 13 postinfection, both wild-type and IL-10−/− BALB/c mice inoculated with L. mexicana parasites failed to control lesion progression and pathogenesis of infection. When lesion parasite numbers were enumerated

![FIGURE 1. BALB/c IL-10−/− mice inoculated with L. mexicana parasites failed to control lesion progression, but had reduced parasite numbers in BALB/c mice infected with L. mexicana. BALB/c IL-10−/− and wild-type mice were inoculated into one hind footpad with 1 × 106 stationary phase L. mexicana promastigotes and the course of infection was followed for 13 wk. A, Lesion size was measured with a Vernier caliper and expressed as the difference in thickness between the infected and the uninfected contralateral footpads. B, Parasites were enumerated by a limiting dilution assay. The results are presented as the negative log10 dilution at which promastigote growth was observed. Error bars represent the mean ± SE of four to five mice per group and values are representative of results of three separate experiments.](http://www.jimmunol.org/Downloadedfrom)
at week 13 of infection, however, we did observe that IL-10−/− BALB/c mice had −3 log10 fewer parasites in their cutaneous lesions as well as in their draining lymph nodes and spleens compared with wild-type mice (Fig. 1B). However, the parasite burden was found to be similar in these groups of mice at week 5 of infection (log10 3.85 and log10 3.9 lesion parasites in IL-10−/− and wild-type mice, respectively). We observed a similar pattern of infection in BALB/c IL-10−/− mice infected with L. amazonensis. Lesion size did not differ significantly between wild-type and IL-10−/− mice during infection however we did observe a statistically significant difference by week 13 of infection (Fig. 2A). The parasite numbers in lesions, lymph nodes, and spleens were significantly lower in IL-10−/− compared with wild-type mice (Fig. 2B). The deficiency in IL-10 production appeared to have a more dramatic effect on levels of infection in L. amazonensis-infected mice as evidenced by 5 log10 fewer lesion parasites and an absence of detectable parasites in the spleen at week 15. However, analysis of parasite numbers at interim time points (data not shown) demonstrated that the levels of lesion infection increased continuously throughout the course of disease. Thus there was no indication that the reduced numbers of parasites in IL-10−/− mice reflected their ability to resolve infection.

Partial control of parasite burdens in BALB/c IL-10−/− mice is associated with increased IFN-γ and NO production
Because susceptibility and pathogenesis of BALB/c mice to cutaneous leishmaniasis is associated with low levels of IFN-γ and NO production, we assessed the production of these products at various times of the infection. Mice infected with either L. mexicana or L. amazonensis were sacrificed at weeks 3, 5, and 13–15 postinfection, and cells from lymph nodes draining to lesion sites and/or spleens were cultured in vitro for 72 h following stimulation with freeze-thawed promastigotes. The supernatants of the in vitro cultures were analyzed for NO or IFN-γ. Cells from IL-10-deficient mice infected with L. mexicana produced significantly higher levels of IFN-γ (Fig. 3A) and NO (Fig. 3B) compared with those in wild-type mice. A similar increase in levels of IFN-γ and NO production was noted in L. amazonensis-infected IL-10−/− mice throughout infection (Fig. 4). This increased pattern of IFN-γ and NO production in IL-10-deficient mice is consistent with the known functions of IL-10.

We extended our analysis of the immune response by assessing levels of mRNA expression for cytokine and cytokine receptors. Because both L. mexicana- and L. amazonensis-infected mice showed a similar pattern of infection, we used only L. mexicana infections for these studies. Total mRNA was extracted from lymph nodes draining to lesion sites, and mRNA expression levels of IL-12p40, IL-12Rβ2, and IL-4 were compared in IL-10−/− and wild-type mice. As can be seen in Fig. 5, IL-10−/− mice expressed increased mRNA levels of IL-12p40 and IL-12Rβ2 at weeks 5 and 13 of infection compared with those expressed by wild-type mice, whereas the levels for IL-4 message were comparable. The production of IL-4 protein by Ag-stimulated lymph node cells was also similar in both groups of mice (data not shown). Thus, it appears that a deficiency in endogenous IL-10 production results in an increase in the production of Th1-type cytokines (IFN-γ and IL-12) and effector molecules (NO) as well as the β2 subunit of the IL-12 receptor, but does not block the production of the type 2 cytokine, IL-4.

FIGURE 2. Deficiency in IL-10 production has a more dramatic effect on levels of infection in L. amazonensis-infected mice. BALB/c IL-10−/− and wild-type mice were inoculated into one hind footpad with 1 × 106 stationary phase L. amazonensis promastigotes and the course of infection was followed for 15 wk. A, Lesion size was measured and parasites were enumerated (6) as described in Fig. 1. Statistically significant differences between mouse groups of p < 0.05 are indicated. Error bars represent the mean ± SE of six to eight mice per group.

FIGURE 3. IFN-γ and NO production at week 13 of L. mexicana infection. Splenocytes from BALB/c IL-10−/− and wild-type mice were stimulated in vitro in the presence of freeze-thawed Ag (5 × 106 parasites Eq/ml) and cell supernatants were harvested at 72 h and assayed for IFN-γ and NO2. A, IFN-γ was measured by ELISA. B, NO2 was measured using Griess reagent. Error bars represent the mean ± SE of four to five mice per group. The results shown are representative of two experiments.
Course of L. mexicana infection in anti-IL-4-treated IL-10+/−/− mice

A previous study had shown that treatment with anti-IL-4 enables BALB/c mice to control infection with L. amazonensis. However, despite the small lesion size and substantial reduction in parasite numbers, significant numbers of parasites (>10^5 footpad) were recovered from anti-IL-4-treated BALB/c mice suggesting the existence of additional factors mediating susceptibility to L. amazonensis infection (8). Similarly, substantial numbers of parasites were obtained from L. mexicana-infected BALB/c IL-4+/−/− mice (15). Further, BALB/c IL-4+/−/− mice, when also deficient in IL-13 gene, were found to express a significant amount of IL-10 during the infection, although the level was reduced in IL-4+/−/−IL-13+/−/− mice compared with wild-type BALB/c mice (15). Based on our observations on IL-4 mRNA levels in IL-10+/−/− mice, we predicted that in the absence of IL-10, treatment with anti-IL-4 mAb might promote effective resolution of infection. To examine the effect of anti-IL-4 mAb treatment during L. mexicana infection in IL-10+/−/−, we inoculated IL-10+/−/− mice with L. mexicana and treated them with anti-IL-4 mAb from day 0 weekly up to and including week 3 of infection and compared the course of lesion development with those in similarly treated wild-type mice. As seen in Fig. 6A, although treatment with anti-IL-4 Ab enabled both IL-10+/−/− and wild-type mice to control the infection as evidenced by reduced lesion size and reduced parasites burden in their lesions and lymphoid organs (Fig. 6B), only IL-10-deficient mice totally resolved their lesions and reduced parasite numbers to negligible levels. Interestingly, although only anti-IL-4-treated IL-10+/−/− mice completely resolved infection, their draining lymph node cells did not produce elevated amounts of IFN-γ compared with cells from nontreated IL-10+/−/− mice and anti-IL-4-treated wild-type mice, neither of which healed (Fig. 6C).

Discussion

IL-10 has been shown to inhibit accessory and effector cell functions that are required for the induction and expression of cell-mediated immunity required to control many intracellular pathogens such as Toxoplasma gondii, Leishmania sp., and Trypanosoma cruzi (20–23). In humans, the severity of visceral leishmaniasis has been most closely associated with increased levels of IL-10 (24, 21). IL-10 production is also correlated with lesion progression in patients with cutaneous leishmaniasis (25). A
number of recent studies have also demonstrated the critical role of IL-10 in mediating susceptibility and pathogenesis to both cutaneous and visceral leishmaniasis. IL-10 transgenic mice are highly susceptible to progressive L. major disease despite producing IFN-γ (26). BALB/c mice deficient in IL-10 control L. major infection and harbor reduced parasite numbers (16). IL-10-deficient C57BL/6 mice or C57BL/6 mice treated with anti-IL-10 receptor Abs achieve sterile cure to L. major infection when inoculated intradermally into the ear dermis (27). In addition, we have shown that IL-10-deficient mice exhibit a dramatic increase in resistance to infection with L. donovani (28). Treatment with anti-IL-10 receptor Ab has also been shown to promote increased resistance to L. donovani (29). In the present study, we examined whether a deficiency in IL-10 production has a similar effect on infections with L. mexicana and L. amazonensis. Our results show that although IL-10 clearly plays a role in susceptibility to infection with New World species of Leishmania, a deficiency in IL-10, alone, is insufficient to promote healing of infection with either L. mexicana or L. amazonensis. Although we consistently observed lower parasite numbers in IL-10-deficient BALB/c mice compared with wild-type mice, the absence of IL-10 had little effect on the pathogenesis of infection and did not modify the continued expansion of lesions in mice inoculated with either parasite. With respect to infection with L. mexicana, numbers of parasites in cutaneous lesions were similar in both wild-type and IL-10-deficient mice through week 5 of infection, but increased by only 100- to 200-fold in IL-10-deficient mice compared with 1,000,000-fold in wild-type mice between weeks 5 and 13 of infection. The fact that parasite numbers within lesions continue to increase throughout infection may account for the observation that lesion growth was not significantly reduced in IL-10-deficient mice. IL-10 is also a potent anti-inflammatory cytokine and its absence could promote an increase in multiple cytokines and/or chemokines involved in cell recruitment to parasitized lesions. In contrast to infection with L. mexicana, we did note a more dramatic reduction in parasite numbers in IL-10-deficient mice infected with L. amazonensis, although as with L. mexicana, the lack of endogenous IL-10 did not markedly alter lesion progression and the overall pattern of nonhealing disease. Whether L. mexicana and L. amazonensis have differing capacities to regulate the development of immunity or differ in their susceptibility to killing by activated macrophages is currently unclear.

Throughout the course of infection, cells from IL-10−/− mice produced more NO compared with cells from BALB/c mice suggesting that IL-10 increase susceptibility to L. mexicana or L. amazonensis infection by inhibiting effector cell function required for parasite killing. We also noted an increased IFN-γ production and higher expression levels for IL-12p40 and IL-12Rβ2 mRNA in IL-10−/− BALB/c mice compared with those in wild-type mice. These observations were similar to those recently reported for L. amazonensis-infected IL-10-deficient C57BL/6 mice (17). Although C57BL/6 mice deficient in IL-10 exhibited a modest increase in resistance as evidenced by reduced numbers of lesion parasites, they failed to resolve infection and maintained a pattern of nonhealing chronic lesions similar to those in wild-type C57BL/6 mice. The failure to heal in IL-10-deficient C57BL/6 mice occurred despite the development of a more vigorous Th1-type response and in the absence of a detectable IL-4 production. In contrast to C57BL/6 mice, previous studies have shown that BALB/c mice infected with L. mexicana or L. amazonensis exhibit a significant increase in IL-4 production (8, 9). We also detected an increase in IL-4 mRNA levels in lymph nodes of IL-10−/− mice with respect to infection with L. amazonensis. Although we consistently observed lower parasite numbers in IL-10-deficient mice infected with L. mexicana, we did not observe a significant increase in resistance as evidenced by reduced numbers of lesion parasites, they failed to resolve infection and maintained a pattern of nonhealing chronic lesions similar to those in wild-type C57BL/6 mice. The failure to heal in IL-10-deficient C57BL/6 mice occurred despite the development of a more vigorous Th1-type response and in the absence of a detectable IL-4 production. In contrast to C57BL/6 mice, previous studies have shown that BALB/c mice infected with L. mexicana or L. amazonensis exhibit a significant increase in IL-4 production (8, 9). We also detected an increase in IL-4 mRNA levels in lymph nodes of IL-10−/− mice infected with L. mexicana. Interestingly, however, levels of IL-4 mRNA were not reduced in infected IL-10-deficient mice, despite the fact that these mice concurrently exhibited a heightened Th1 response as evidenced by increased production of IFN-γ and increased mRNA levels for IL-12p40 and IL-12Rβ2. This observation is similar to that in a recent study showing unimpaired IL-4 production by cells from L. mexicana-infected BALB/c mice lacking IL-4Rα compared with infected wild-type mice, despite the fact that the IL-4R-deficient mice controlled infection and their cells produced increased amounts of IFN-γ (15). The ability of
IL-4Rα-deficient, but not IL-4-deficient, BALB/c mice to totally control the infection may be attributable to IL-13 in part compensating for IL-4 in IL-4-deficient mice (15). Interestingly with regard to the current study, the disease enhancing activity of IL-13 may be through its ability to up-regulate IL-10 production (30–32) since mRNA transcripts for IL-10 are significantly suppressed L. mexicana infection in IL-13-deficient B6/129 mice (15). Confirmation that the IL-4 production in IL-10-deficient mice plays a significant role in the inability of these mice to control infection comes from our observation that lesions resolved in IL-10-deficient mice treated with anti-IL-4 mAb and that healed mice had few parasites in their lesions compared with nontreated IL-10-deficient mice or wild-type BALB/c mice treated with anti-IL-4 Ab. A recent study reported similar observations with L. major infection and implicated both IL-4R signaling and IL-10 to contribute to susceptibility, and found that BALB/c mice deficient in both IL-4Rα and IL-10 are more resistant to L. major infection, regardless of parasite strain. However, in relative terms, IL-10 appears to exert a more profound effect on L. major parasite growth and the development of disease (33).

In summary, we have shown that IL-10-deficient BALB/c mice exhibit increased resistance to infection with L. mexicana and L. amazonensis, although in contrast to similar mice infected with L. major, they are ultimately unable to limit either lesion growth or parasite number. Enhanced resistance in IL-10–/– mice is correlated with increased production of IFN-γ and NO, although heightened production of these factors does not effectively down-regulate the development of a concurrent Th2-type response as evidenced by continued production of IL-4. The biological significance of continued IL-4 production in IL-10-deficient mice was demonstrated by their ability to resolve infection following in vivo neutralization of IL-4, even though cells from these mice produced comparable levels of IFN-γ to those in nonhealing IL-10-deficient mice or wild-type mice treated with anti-IL-4 Ab. Because a recent study has implicated IL-13 in susceptibility to infection with L. mexicana and because IL-13 is known to suppress NO production by macrophages as well as promote IL-10 production, it is possible IL-13 plays a role in the inability of IL-10–/– or anti-IL-4-treated wild-type mice to heal. Thus, depletion of IL-4 plus IL-10 or IL-4 plus IL-13 may have the same generalized effect on the outcome of infection. Why a deficiency in endogenous IL-10 production seems to be less critical to the susceptibility of BALB/c mice to infection with L. mexicana and L. amazonensis compared with L. major is currently unclear. However, our results do support previous observations suggesting that fundamental differences may exist in the response to infection with Old World and New World Leishmania species.

References


