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Interleukin-12 Is Indispensable for Protective Immunity against *Leishmania major*

FRANK MATTNER,† KARIN DI PADOVA, AND GOTTFRIED ALBER*

Department of Infectious Diseases, F. Hoffmann-La Roche AG, Basel, Switzerland

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Interleukin-12 (IL-12)-deficient mice derived from a strain genetically resistant to infection with *Leishmania major* were recently shown to be susceptible toward this parasite, developing a strong Th2 response after injection of a large number of parasites. We further investigated the role of IL-12 in *L. major* infection by studying the responses of mutant mice against smaller numbers of parasites. IL-12-deficient mice infected with only small numbers of parasites showed the progressive lesion development and high parasite burden associated with a polarized Th2 response. Our data show that IL-12 is indispensable for protective immunity against *L. major*. Even at low inocula, no salvage pathway appears to compensate for the lack of IL-12. However, genetically susceptible BALB/c mice infected with small numbers of parasites were able to resolve lesions and restrict the parasite burden to levels which were 10^5-fold lower than those in IL-12-deficient mice. In contrast to mutant mice, BALB/c mice mounted a type 1 response against low inocula of *L. major*. IL-12-deficient BALB/c mice, however, developed a type 2 response. These data emphasize the essential role of IL-12 in resistance against *L. major*. In addition, this study suggests that in the absence of IL-12, susceptibility to *L. major* is determined by the inability to induce a Th1 response rather than the development of a Th2 response. Our results are relevant for potential vaccination strategies that use low inocula of infective microorganisms which fail to induce a protective type 1 response at higher inocula.

It is well established that in mice and humans $CD4^+$ Th clones can be divided into two functional subsets, Th1 and Th2, according to the profile of lymphokines produced. The Th1 subset produces preferentially gamma interferon (IFN-γ), whereas the Th2 subset produces predominantly interleukin-4 (IL-4) (21, 29, 38).

Leishmania major infection of mice is the preferred model for investigating the in vivo relevance of the significance of Th1 and Th2 responses (10, 16, 28, 33). Resistant inbred strains of mice predominantly develop a Th1 response, whereas susceptible strains develop a Th2 response. In fact, both resistant and susceptible mice can be infected persistently with *L. major*. The critical difference is that lesions of resistant mice heal and lesions of susceptible mice do not heal but tend to increase in size and to ulcerate. Therefore, it is more appropriate to distinguish between healer and nonhealer mouse strains (27). IL-12 and IL-4 have previously been shown to be involved in the generation of Th1 and Th2 cells, respectively (12, 32, 34). Susceptible (or nonhealer) mice can be cured by the application of IL-12 during the first week of infection or by treatment with anti-IL-4 monoclonal antibody (MAb) (9, 11, 30, 31, 35). Recently, we demonstrated that mice with homologous disruption of the gene coding for either the p35 or p40 subunit of IL-12 and derived from a genetically resistant strain (healer strain) are susceptible to infection with *L. major* promastigotes (18). Susceptible BALB/c mice injected with a small number of parasites were able to develop stable cell-mediated immunity (3). These mice acquired resistance to a larger, normally pathogenic challenge. The goal of the present study was to compare relative susceptibility and resistance between susceptible BALB/c mice and resistant but IL-12-deficient mice infected with decreasing numbers of parasites. Are IL-12-deficient mice on a healer background able to resist a low-dose infection? Do these mice develop a type 1 response?

* Corresponding author. Present address: University of Leipzig, Zwickauer Str. 53, D-04103 Leipzig, Germany. Phone: (49-341) 97 38 328. Fax: (49-341) 97 38 349. E-mail: alber@rz.uni-leipzig.de.

† Present address: Roche Milano Ricerche, I-20132 Milan, Italy.

MATERIALS AND METHODS

Mice. Female BALB/c, (C57BL/6 × 129/Sv/Ev)–IL-12p40$^{-/-}$, and (C57BL/6 × 129/Sv/Ev)F_2 mice (8 to 10 weeks old) were obtained from BRL Füllinsdorf (Basel, Switzerland). IL-12-deficient mice were originally generated by Jeanne Magram et al. as previously described (17, 18). Chimeric mice derived from targeted 129/Sv embryonic stem cells and heterozygous for a mutation in the IL-12p40 gene were mated to BALB/c mice obtained from the Jackson Laboratory (Bar Harbor, Maine). Progeny from this cross that carried a heterozygous IL-12p40 mutation were backcrossed to the BALB/c strain for a total of five backcrosses. Heterozygous mice were intercrossed in order to obtain mice homozygous for the IL-12p40 mutation. Homozygotes were bred under specific-pathogen-free conditions by BRL Füllinsdorf. Mice used in experiments were 6 to 10 weeks old and were kept in filter-cap cages during experiments.

Parasites and infection of mice. *L. major* LV 39 (MRHO/SU/59/P strain) was kept virulent by continuous passage in mice. Parasites isolated from skin lesions of infected mice were grown in Iscove's modified Dulbecco's medium (IMDM) (Sigma, Deisenhofen, Germany) containing 5% fetal calf serum (FCS) at room temperature on a solid layer of blood agar as previously described (22).

Mice were infected subcutaneously in one hind footpad with the indicated numbers of *L. major* stationary-phase promastigotes in a final volume of 50 μl. The development of lesions was monitored by measuring footpad swelling weekly with a metric caliper.

A freeze-thaw preparation of *L. major* promastigotes was used as the antigen (F/T Ag) (36). Doses of antigen are expressed in terms of the number of promastigotes from which the antigen was obtained (e.g., 10^6 F/T Ag is the soluble antigen obtained from freezing and thawing 10^6 *L. major* promastigotes).

Quantitative parasite cultures. Viable *L. major* parasites in infected tissues were enumerated by a limiting dilution assay (37). In brief, dilutions of single-cell suspensions of popliteal lymph node cells (LNC) of infected mice were grown on 96-well microtiter plates in medium 199 (GIBCO BRL, Life Technologies, Paisley, Scotland) containing 30% heat-inactivated FCS. The plates were incubated for 10 days at room temperature in a humidified atmosphere, and the wells containing motile parasites were identified with an inverted microscope. The data reported are the geometric means and standard errors of the dilution factors of the last positive wells multiplied by the number of LNC.

Lymphocyte cultures. Popliteal LNC were collected, and erythrocyte-lysed single-cell suspensions were stimulated at 5×10^6 cells per ml with F/T Ag (10^6/ml) as indicated in a final volume of 1 ml at 37°C and in an atmosphere of 7.5% CO_2. Cells were cultured in IMDM supplemented with 5% heat-inactivated FCS, L-glutamine, 2-mercaptoethanol, and HEPES. Culture supernatants were collected after 72 h of stimulation and stored at −20°C until used. To some cultures, MAb directed against the IL-4 receptor was added at 5 μg/ml (Gen-

zyme, Cambridge, Mass.) to prevent IL-4 consumption. The application of anti-IL-4 receptor MAb had no effect on IFN-γ production by cells.

Lymphokine and serum isotype analysis. IFN-γ was measured by enzyme-linked immunosorbent assay (ELISA) with rat immunoglobulin G1 (IgG1) MAbs AN18 and XMG1,2 (4, 26). The detection limit of this assay was 40 pg/ml.

IL-4 was measured by ELISA with rat IgG1 MAb 11B11 (24) and rat MAb BVD6-24G2 (PharMingen, Lugano, Switzerland). The level of sensitivity of IL-4 detection was 200 pg/ml.

The serum levels of *Leishmania*-specific antibodies were determined by ELISA (13). Plates were coated with 10^6 F/T Ag/ml. Bound IgG1 and IgG2a antibodies were detected by isotype-specific, peroxidase-conjugated anti-mouse antisera (The Binding Site, Birmingham, United Kingdom). Immune serum from infected BALB/c mice served as the standard. A 1:100 dilution of this standard was arbitrarily defined as 100 U/ml. The level of sensitivity for the detection of specific IgG1 and IgG2a was 0.5 U/ml.

Total serum IgE was measured by ELISA with rat IgG2a MAb EM95 (1) and a sheep anti-mouse IgE antiserum obtained from The Binding Site. Purified mouse IgE (PharMingen) served as the standard. The level of sensitivity for the detection of total IgE was 10 ng/ml.

RESULTS

IL-12-deficient mice but not BALB/c mice are unable to resist infection with small numbers of parasites. Mice with homozygous disruption of the gene encoding IL-12p40 on the resistant C57BL/6 × 129/Sv/Ev background, congenic control mice, and genetically susceptible BALB/c mice were infected with various numbers of *L. major* promastigotes (Fig. 1) subcutaneously in one hind footpad, and the development of lesions was monitored once weekly. Figure 1A shows that BALB/c mice infected with relatively large numbers of parasites developed large lesions. In contrast, BALB/c mice infected with only 100 parasites were (like C57BL/6 × 129 mice) resistant to infection, since only small lesions developed (with a delay compared to development in C57BL/6 × 129 mice) and subsequently resolved. It has previously been shown that IL-12-deficient mice are susceptible to infection with large numbers of promastigotes (18). Figure 1B presents confirmatory data; in addition, it shows that genetically resistant IL-12-deficient mice did not resist infection with *L. major* when they were infected with only a small number of parasites (100 promastigotes per footpad). The lesions of BALB/c mice started to ulcerate earlier than did the lesions found in IL-12-deficient mice on a healer background. At later stages of infection, parasites were disseminated into visceral organs in both strains of mice.

Figure 2 presents the enumeration results for parasites at 168 days postinfection in LNC draining the site of infection in BALB/c mice, genetically resistant IL-12-deficient mice, and resistant wild-type C57BL/6 × 129 control mice. BALB/c mice infected with only 100 parasites showed a parasite load comparable to that of the resistant C57BL/6 × 129 strain (infected with 10^6 parasites). In contrast, independent of the number of injected promastigotes, IL-12-deficient mice were found to have a 10^5-fold increase in the number of parasites compared to that in low-dose-infected BALB/c mice. Taken together, these results confirm the crucial role of IL-12 in controlling *L. major* infection even at extremely low infection doses.

Low-dose infection induces a type 2 response in genetically resistant IL-12-deficient mice, whereas genetically susceptible BALB/c mice develop a type 1 response. Susceptibility and resistance to *L. major* infection have previously been shown to be related to the expansion of Th2 and Th1 cells, respectively (16, 28). In order to determine the nature of the immune response, type 1 or type 2, we measured lymphokine production by draining LNC after specific stimulation in vitro with *Leishmania* antigen in BALB/c, IL-12-deficient mice, and wild-type control mice 168 days after infection with different numbers of promastigotes. Figure 3 shows that as expected, resistant C57BL/6 × 129 mice developed a clear type 1 response, with relatively high amounts of IFN-γ and no detectable IL-4. BALB/c mice showed an interesting pattern of IFN-γ production; the smaller the number of injected parasites, the higher the levels of IFN-γ. BALB/c mice infected with 100 promastigotes showed a large excess of IFN-γ over IL-4 production. With decreasing numbers of injected promastigotes, the immune response shifted more and more to type 1 with significantly enhanced IFN-γ production (3.2-fold increase between the value for mice injected with 10^2 parasites and that for mice injected with 10^4 parasites [$P = 0.05$]), whereas IL-4 levels appeared to be almost unchanged (0.6-fold decrease between the value for mice injected with 10^2 parasites and that for mice injected with 10^4 parasites [$P = 0.35$]). In contrast, IL-12-deficient mice did not show any development of a type 1 response and IL-4 levels were always higher than IFN-γ levels, independent of the number of injected parasites. Interestingly, IL-4 levels produced by splenocytes of BALB/c mice and IL-12-deficient mice were similar and almost dose independent in the range of 10^2 to 10^4 promastigotes.

We next investigated the nature of the humoral response. It is generally accepted that a type 1 response is accompanied by the production of preferentially IgG2a antibodies, whereas a type 2 response is characterized by IgG1 and IgE (6, 8, 13). Figure 4 shows the results of serum IgE analysis of BALB/c mice, IL-12-deficient mice, and wild-type control mice. BALB/c mice infected with the smallest number of promastigotes were found to have very low serum IgE levels, even lower than those of resistant C57BL/6 × 129 mice. On the contrary, all groups of IL-12-deficient mice showed enormous amounts of total serum IgE, independent of the inoculum size used for infection, indicating a strong type 2 response. In addition, upon infection with 100 to 10,000 parasites, all groups of IL-12-deficient mice developed minimal specific IgG2a responses (2.5 relative units), whereas the amount of IgG2a produced by BALB/c mice was similar to that produced by resistant C57BL/6 × 129 mice (42 and 19 relative units, respectively).

Experiments to compare the immune response of high-dose-infected, wild-type BALB/c mice with that of IL-12-deficient BALB/c mice consistently showed a considerable reduction in IFN-γ, a reduction in *Leishmania*-specific IgG2a, and dramatic production of IgE in mutant mice (Fig. 5). This indicates a role for IL-12 in IFN-γ-dependent isotype regulation during *L. major* infection in susceptible BALB/c mice. Moreover, this shows that there are Th1-dependent mechanisms in BALB/c mice even at high doses of parasites.

DISCUSSION

Our data show that IL-12 is crucial in protective immunity against even low doses of *L. major*. A comparison of genetically resistant IL-12-deficient mutant mice with genetically susceptible wild-type BALB/c mice demonstrated an enormous difference in parasite burden after infection with only 100 promastigotes. An extremely high parasite burden in IL-12-deficient mice and a 10^5-fold-lower parasite burden in BALB/c mice were associated with type 2 and type 1 T-cell responses, respectively. These results, together with previously published data (3), demonstrate that BALB/c mice (commonly known for their genetically determined susceptibility against *L. major*) are capable of mounting a protective immune response to a low-dose infection. Our results corroborate the findings of Doherty and Coffman (5), who treated low-dose-infected BALB/c mice with anti-IL-12 antibodies. This treatment was able to abrogate resistance by low-dose infection, emphasizing the critical role of IL-12. In addition, Bretscher et al. (2) and Menon and Bretscher (19) recently reported that low-dose infection of

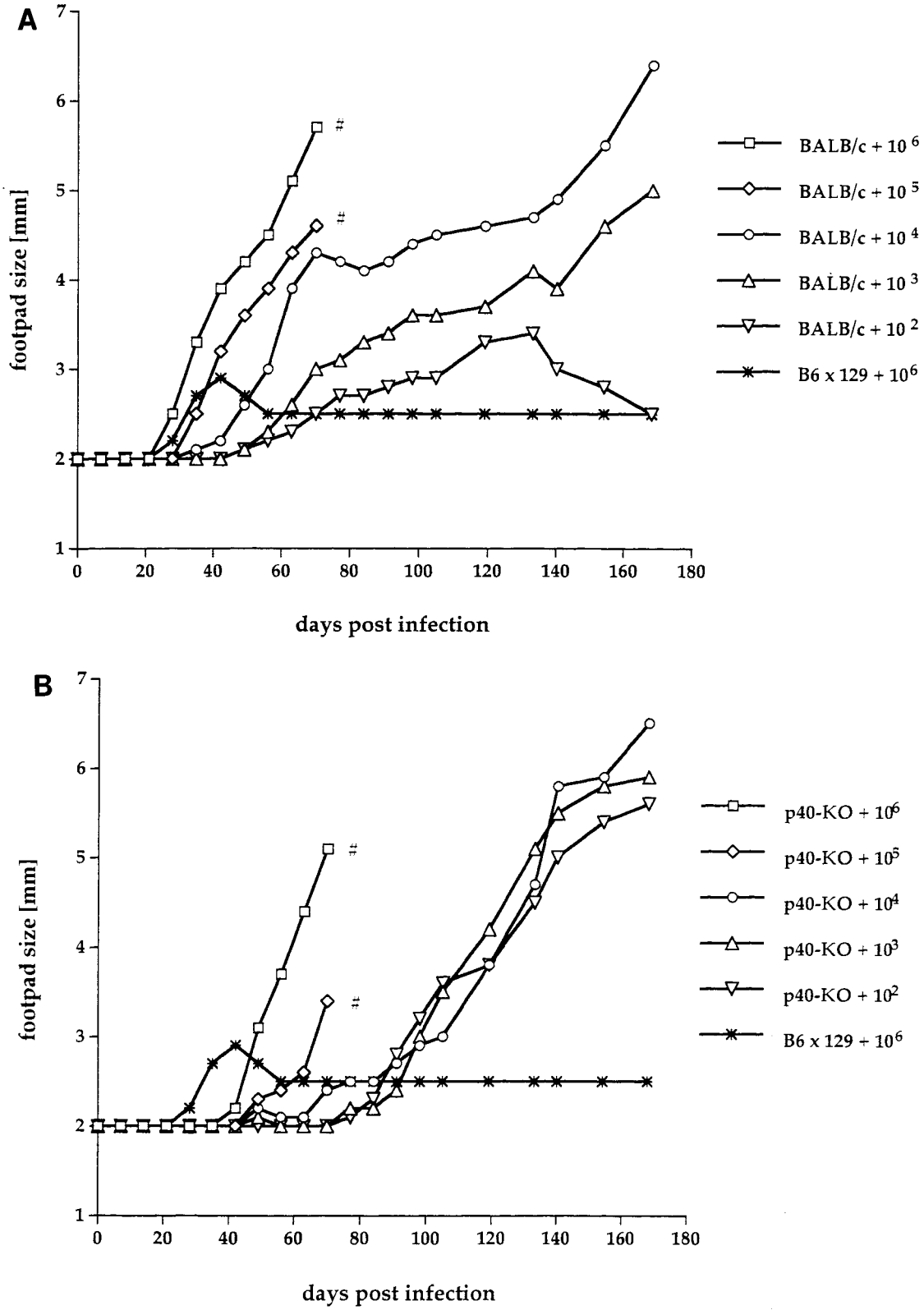

FIG. 1. *L. major* infection in BALB/c (A), (C57BL/6 × 129/Sv/Ev)–IL-12p40$^{-/-}$ (p40-KO; B), and C57BL/6 × 129 (B6 × 129; A and B) mice. Groups of five mice were infected in the left hind footpad with the indicated numbers of *L. major* promastigotes. The course of disease was monitored weekly with a metric caliper to quantitate the swelling at the site of inoculation. Infected mice included BALB/c, p40-KO, and resistant wild-type B6 × 129 mice. BALB/c mice infected with 10^5 and 10^6 promastigotes were sacrificed because they had developed large ulcerating lesions after 70 days (#). The standard deviation was always <10%.

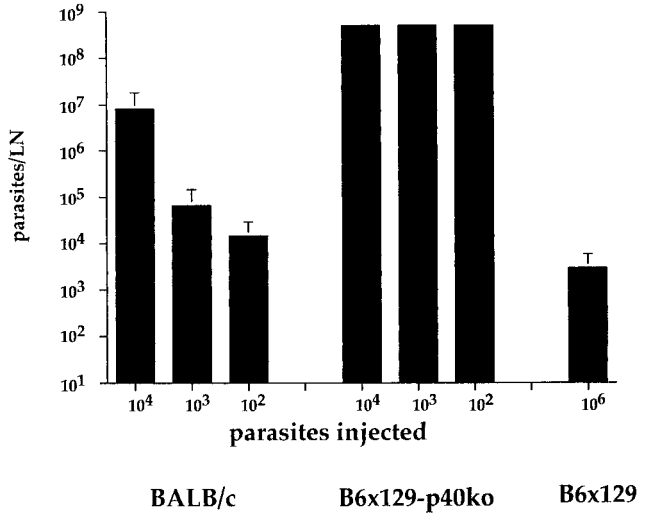

FIG. 2. Parasite load in lymphoid tissues (LN) of mice infected with *L. major*. Data are the numbers of viable parasites, as determined by limiting dilution assay as described in Materials and Methods. (C57BL/6 × 129/Sv/Ev)–IL-12p40$^{-/-}$, B6×129-p40Ko; C57BL/6 × 129, B6×129.

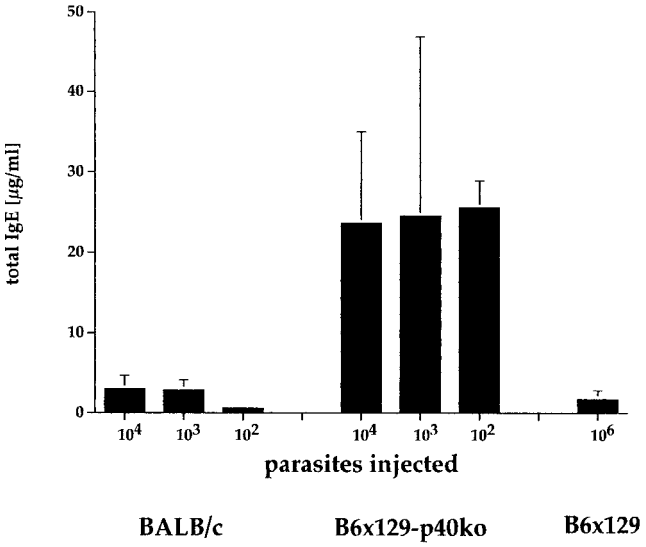

FIG. 4. Total IgE analysis of sera taken from *L. major*-infected mice at 168 days postinfection. Mice (five per group; as identified in the legend to Fig. 2) were sacrificed on day 168, and blood samples were taken from the heart. Total serum IgE was measured by ELISA.

BALB/c mice led to increased IFN-γ/IL-4 ratios. Our data extend this observation to IL-12, which appears to be the principal regulator of IFN-γ production in *L. major* infection.

In our study, genetically resistant IL-12 knockout mice showed an absolute dependence on IL-12 for protective immunity to *L. major*, even at the lowest inoculum. This brought up the following critical question: is the major function of IL-12 in *L. major* infection suppression of a Th2 response or induction of a Th1 response? In other words, is it the Th2 response or the inability to mount a Th1 response which renders mice susceptible? At low inocula, otherwise susceptible BALB/c mice were able to develop a protective Th1 response, as judged by increased IFN-γ production and considerable IL-4 levels. On the other hand, IL-12 knockout mice showed similar IL-4 levels but almost no IFN-γ production, which led to a 100,000-fold increase in parasite burden compared to that of low-dose-infected BALB/c mice. Clearly, this points to IL-12 as the principal regulator of protective immunity to *L. major*. Indeed, IL-12-deficient BALB/c mice showed a significantly reduced IFN-γ response associated with reduced specific IgG2a levels and enhanced IgE levels after *L. major* infection. Therefore, the conclusion from our study is that the inability to induce a Th1 response rather than the development of a Th2 response determines susceptibility to *L. major*.

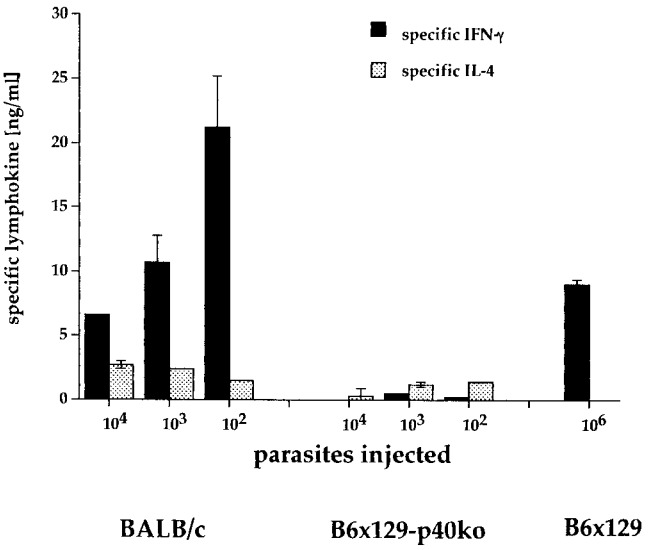

FIG. 3. Net IFN-γ and IL-4 production in vitro by specifically stimulated LNC from *L. major*-infected mice at 168 days postinfection. Suspensions of LNC of designated mice (as identified in the legend to Fig. 2) were cultured for 72 h in the presence of F/T Ag equivalent to 10^6 promastigotes/ml. Supernatants were assayed for IL-4 and IFN-γ by ELISA. Cells cultured in medium alone yielded cytokine levels which were <10% of those produced in response to antigen. Spontaneous cytokine production was subtracted from specifically stimulated levels.

This raises the question of the role of IL-4 in susceptibility to *L. major*. A recent report about *Leishmania*-infected BALB/c mice with a targeted disruption of the *IL-4* gene surprisingly presented evidence for continued susceptibility in the absence of IL-4 production (23). This contrasts with another recent study of *Leishmania*-infected IL-4-deficient BALB/c mice which were created by using embryonic stem cells from 129/Sv mice and then backcrossing with BALB/c mice (14). These mice were found to be resistant against *L. major* infection. Whatever the reasons for these conflicting results, these studies, together with our data, indicate that IL-4 may not be the only essential factor for determining susceptibility.

For a decisive judgment on susceptibility or resistance, however, it is important to follow the course of infection long enough. Our study demonstrates that after low-dose infection, it takes more than 2 months for the nonhealing phenotype observed in IL-12-deficient mice to develop. In the first 2 months, these mice would have been scored as resistant. In an other experimental system examining high-dose infection of BALB/c mice, the treatment of BALB/c mice with anti-IL-4 MAb was found to be protective (30). It is conceivable that IL-4 becomes operative when IL-12-mediated T-cell activation is insufficient. This could include IL-12 responsiveness, which has previously been shown in vitro to be rapidly lost upon antigen-induced stimulation of T cells from BALB/c mice but

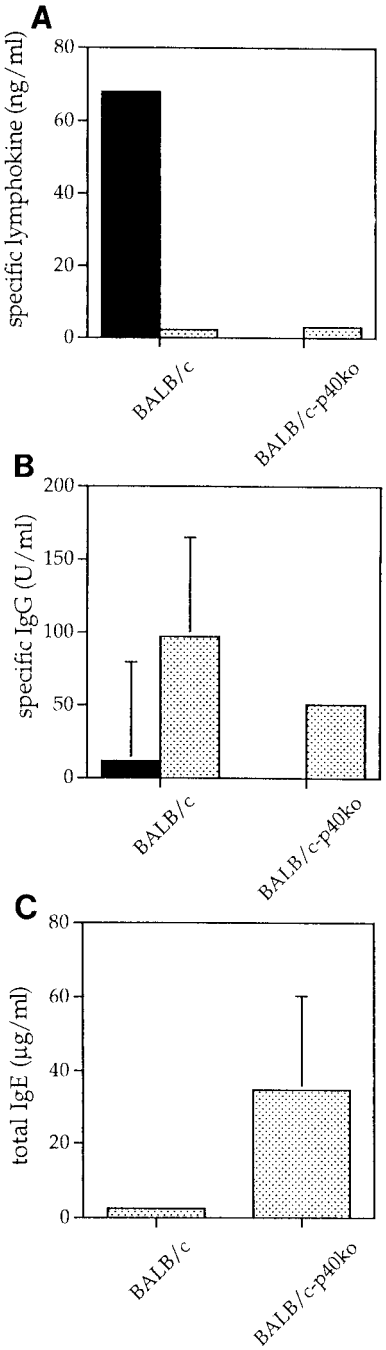

FIG. 5. (A) Net IFN-γ (black bars) and IL-4 (stippled bars) production in vitro by specifically stimulated LNC from mice infected with 3×10^6 *L. major* promastigotes for 58 days. Suspensions of LNC from the mouse strains indicated were cultured for 72 h in the presence of F/T Ag equivalent to 3×10^6 promastigotes/ml. Supernatants were assayed for IL-4 and IFN-γ by ELISA. The standard deviation was always <5%. Cells cultured in medium alone yielded cytokine levels which were <10% of those produced in response to antigen. Spontaneous cytokine production was subtracted from specifically stimulated levels. (B and C) Analysis of specific IgG1 (stippled bars) and IgG2a (black bars) (B) and total IgE (C) levels in sera taken from mice infected with 3×10^6 *L. major* promastigotes for 58 days. Mice (five per group) were sacrificed on day 58, and blood samples were taken from the heart. The levels of specific IgG1 and IgG2a and total serum IgE were measured by ELISA.

not from B10.D2 mice (7). Whether this phenomenon depends on the antigen dose is not known. Alternatively, very early IL-4 production by $CD4^+$ T cells from BALB/c mice but not from C57BL/6 mice may be a relevant regulatory mechanism (15). Interestingly, this early IL-4 peak has been described for a high-dose challenge with *L. major*, which leads to the commonly known genetic susceptibility of BALB/c mice. In addition, the dose-dependent regulation of resistance and susceptibility in BALB/c mice associated with type 1 and type 2 T-cell responses may reflect dose-dependent activation of different antigen-presenting cell types, e.g., dendritic cells versus macrophages, both of which can be infected with *L. major* (20).

L. major infection of BALB/c mice can serve as an important in vivo model for dose-dependent regulation of T-cell responses. Understanding the underlying cytokine-dependent regulation of type 1 versus type 2 responses potentially allows for more rational vaccine approaches. The results from our study as well as from studies by Bretscher et al. (2, 3), Doherty and Coffman (5), and Menon and Bretscher (19) might provide a basis for vaccination strategies that use low inocula of infective microorganisms which tend to induce a nonprotective type 2 response at higher inocula.

Recently, a new cytokine, IGIF (for IFN-γ-inducing factor; more recently termed IL-18), that induces IFN-γ production by T cells was cloned (25). IGIF was described as being even more potent for IFN-γ induction than is IL-12. There was speculation about IGIF involvement in the development of Th1 cells. From the data presented, it is unlikely that IGIF (IL-18) plays a role in *L. major* infection of mice, since IL-12-deficient mice did not show any Th1 differentiation, even after infection with very low numbers of parasites (100 promastigotes). Our data strongly suggest that IL-12 is the crucial cytokine for protective type 1 T-cell responses against *L. major* and that no salvage pathway exists to compensate for the lack of IL-12.

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