INFECTION AND IMMUNITY, June 1997, p. 2448–2450 Vol. 65, No. 6
0019-9567/97/$04.00+0
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Efficient Immunity against *Leishmania major* in the Absence of Interleukin-6

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Received 13 January 1997/Accepted 3 March 1997

Mice genetically deficient in the pleiotropic cytokine interleukin-6 (IL-6) exhibit immunodeficiency against viral, bacterial, and fungal pathogens. When challenged with the protozoan parasite *Leishmania major*, IL-6-deficient mice controlled infection and mounted strong Th1 responses as efficiently as IL-6-sufficient littermates. Thus, the successful activation of parasitized macrophages and class II-restricted T cells does not require a full inflammatory repertoire.

Leishmania major is an intracellular parasite which replicates only in host macrophages (reviewed in reference 15). Control of murine leishmaniasis is dependent on class II-restricted T cells (2, 13). Interleukin-12 (IL-12) is critical for the induction of Th1 effector cells, which secrete gamma interferon (IFN-γ) to activate macrophages to a microbicidal state (10, 20). NO is an essential product of the activated macrophage responsible for intracellular killing (24). In addition to the important signals delivered by such soluble mediators, cognate interaction between Th1 cells and macrophages is essential during infection, as demonstrated by the extreme susceptibility of CD40-deficient mice (11) and CD40L-deficient mice (1) to *L. major*. Natural killer cells contribute to early IFN-γ production, but their in vivo depletion does not significantly affect healing (19). β2-Microglobulin-deficient mice which lack $CD8^+$ T cells also fully control infection (23). Thus, infection with *L. major* provides a useful system to define the requirements for successful cooperation between macrophages and class II-restricted T cells in eradicating intracellular parasitization.

The role of proinflammatory cytokines in cell-mediated immunity in general and in leishmaniasis specifically has not been fully defined. IL-6 is a pleiotropic cytokine which is produced by macrophages, lymphocytes, and many nonhematopoietic cells. It plays a role in B-cell stimulation and in regulation of acute-phase responses and inflammation (6). Although coordinately induced from activated macrophages with IL-1 and tumor necrosis factor alpha (TNF-α), IL-6 suppresses the production of the former two cytokines from macrophages, whereas IL-1 and TNF-α induce further secretion of the proinflammatory triad (6). IL-6-deficient mice develop normally but have reduced inflammatory responses and enhanced susceptibility to infection with vaccinia virus (12), *Listeria monocytogenes* (5, 12), and *Candida albicans* (17). To determine whether these immune defects would adversely alter the outcome of infection with an exclusively intramacrophagic pathogen, we challenged IL-6-deficient mice from a genetically resistant background with virulent *L. major.*

IL-6-deficient mice (12) (Jackson Laboratory, Bar Harbor, Maine) were bred to wild-type mice. Heterozygotic offspring were then backcrossed to parental $IL\text{-}6^{-/-}$ mice to generate litters with $IL\text{-}6^{-/-}$ and $IL\text{-}6^{+/-}$ controls. Resistant-background C57BL/6 $IL\text{-}6^{+/-}$, littermate $IL\text{-}6^{-/-}$, and susceptible BALB/c mice were inoculated with 5×10^5 metacyclic promastigotes of *L. major* (strain WHOM/IR/-/173) in each hind footpad. Infections were monitored with weekly measurements for 6 weeks, using a metric caliper. $IL\text{-}6^{-/-}$ mice controlled infection as well as $IL\text{-}6^{+/-}$ mice, as determined by resolution of footpad swelling (Fig. 1). Both $IL\text{-}6^{+/-}$ and $IL\text{-}6^{-/-}$ mice had a disease course that sharply contrasted with that of BALB/c mice, which developed severe and progressive footpad lesions (Fig. 1). To assess the microbiologic outcome for the mutant animals, quantitative cultures were performed on the feet and spleens of infected mice. Tissues were homogenized in a fixed volume of parasite medium M199 (Fisher Scientific, Pittsburgh, Pa.) supplemented with 20% fetal calf serum and aliquoted to a flat-bottom 96-well plate. Samples were then diluted serially across the plate and stored at 26°C for 2 weeks. The feet and spleens of $IL\text{-}6^{-/-}$ mice had parasite loads equivalent to or lower than those of the matched tissues of $IL\text{-}6^{+/-}$ mice. Susceptible BALB/c mice had parasite burdens greater than 3 orders of magnitude higher than those of both $IL\text{-}6^{-/-}$ and $IL\text{-}6^{+/-}$ mice (Fig. 2). IL-6, therefore, is not required for healing and resolution of infection with *L. major.*

To ascertain whether IL-6 deficiency had an adverse impact on Th1 differentiation, as has been reported in other systems (17), draining popliteal lymph nodes of $IL\text{-}6^{-/-}$ and $IL\text{-}6^{+/-}$ animals were removed for analysis of cytokine production. Single-cell suspensions were made, and triplicate aliquots of 10^6 cells/well were placed in 96-well plates with Iscove's medium (Fisher Scientific) or medium plus 100 μg of soluble *Leishmania* antigen per ml. After 48 h, supernatants were

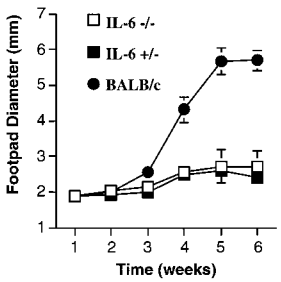

FIG. 1. Resolution of *L. major* infection in $IL\text{-}6^{-/-}$ mice. Mice were inoculated in the hind footpad with *L. major* promastigotes. Symbols represent mean footpad measurements, with standard deviations expressed as *y*-axis error bars. Results shown are representative of two experiments involving four C57BL/6 $IL\text{-}6^{+/-}$, seven C57BL/6 $IL\text{-}6^{-/-}$, and two BALB/c mice.

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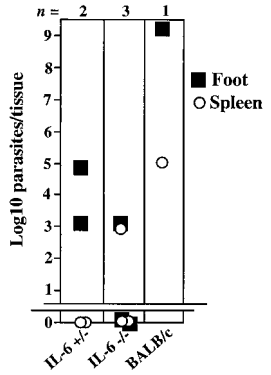

FIG. 2. IL-6$^{-/-}$ mice control parasite growth in tissues. Cohorts of IL-6$^{-/-}$ and IL-6$^{+/-}$ mice together with susceptible BALB/c mice were infected with *L. major*. Parasite burden was assessed by the presence of motile promastigotes as seen through an inverted microscope after 2 weeks. Symbols represent parasite loads from individual animals. Results shown are representative of two separate experiments involving four C57BL/6 IL-6$^{+/-}$, six C57BL/6 IL-6$^{+/-}$, and two BALB/c mice.

collected and analyzed by enzyme-linked immunosorbent assay using commercial antibody pairs (Pharmingen, San Diego, Calif.). Antigen-specific IFN-γ production was equivalently high among individual IL-6$^{+/-}$ and IL-6$^{-/-}$ animals (Fig. 3). BALB/c mice produced less IFN-γ in response to antigen than the healer animals. There was no detectable IFN-γ in any wells without added antigen.

To assess the in vivo pattern of T helper differentiation, we performed competitive quantitative reverse transcription-PCR as described previously (16). Briefly, total RNA was extracted from popliteal lymph nodes taken directly from infected animals by using RNAzol (Cinna/Biotecx, Houston, Tex.) and reverse transcribed by using random hexamer primers. A competitor molecule containing mutated cDNAs approximately 30% larger than wild-type transcripts was used as an internal control for amplification. Concentrations of cDNA were equalized by amplifying the constitutively expressed *HPRT* gene. Adjusted volumes of cDNA were then used in subsequent reactions with primers specific for IFN-γ and IL-4. PCRs were resolved on ethidium-stained agarose gels. The IL-6$^{-/-}$ and IL-6$^{+/-}$ animals all had elevated levels of IFN-γ mRNA and lower levels of IL-4 mRNA compared to susceptible BALB/c

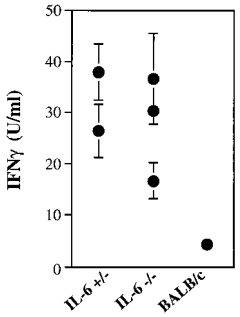

FIG. 3. Lymph node cells from IL-6$^{-/-}$ mice undergo Th1 differentiation during infection with *L. major*. Lymph node cells from infected mice were incubated with and without soluble *Leishmania* antigens, and supernatants were assayed for IFN-γ production. Data points are from antigen-containing wells and represent means of triplicate wells, with standard deviations expressed as *y*-axis error bars. Antigen-free wells had no detectable IFN-γ. Results shown are representative of two separate experiments involving four C57BL/6 IL-6$^{+/-}$, six C57BL/6 IL-6$^{-/-}$, and two BALB/c mice.

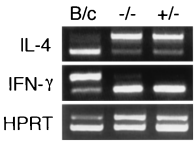

FIG. 4. Lymph nodes from IL-6$^{-/-}$ mice develop Th1 responses in vivo. Competitive quantitative reverse transcription-PCR was performed on RNA from lymph nodes of infected BALB/c, IL-6$^{-/-}$, and IL-6$^{+/-}$ animals. Levels of IFN-γ and IL-4 transcripts (lower bands) are shown in comparison to amplification of the competitor molecule (upper bands). Results are from individual mice and were representative of three C57BL/6 IL-6$^{-/-}$ and two C57BL/6 IL-6$^{+/-}$ mice. B/c, BALB/c.

mice (Fig. 4). IL-6, therefore, is not required for strong Th1 responses against *L. major.*

IL-6-deficient mice have enhanced susceptibility to infection with vaccinia virus (12), *Listeria monocytogenes* (5, 12), and *C. albicans* (17). Control of these infections is associated with effector functions ascribed to cell-mediated immunity. Although infection with *L. major* represents another well-characterized example of the cell-mediated control of intracellular parasitization (15), we could find no impairment in the resistance of IL-6$^{-/-}$ mice to parasite challenge. It is possible that IL-6 plays a negative role in the control of *L. major*. This would be consistent with the ability of IL-6 to inhibit antileishmanial macrophage activation in vitro (9). It may be easier, however, to implicate IL-6 as a negative regulator of antileishmanial immunity if BALB/c IL-6$^{-/-}$ mice display enhanced resistance to *L. major.*

Neutrophils have been shown to play an important role in control of *C. albicans* (17) and *Listeria monocytogenes* (3, 4). IL-6-deficient mice infected with either *Candida* or *Listeria* have reduced peripheral blood neutrophilia (5, 17). Furthermore, administration of recombinant IL-6 to *Candida*-infected and *Listeria*-infected mice restored neutrophilia and increased resistance to disease (5, 17). Thus, inefficient neutrophilia resulting from lack of IL-6 may be of major consequence in candidiasis or listeriosis but inconsequential to the resolution of leishmaniasis owing to the limited intramammalian habitat of the parasite.

Assessment of other proinflammatory mediators has also revealed differential requirements among various models of cell-mediated immunity. TNF receptor 1 (p55)$^{-/-}$ mice have enhanced susceptibility to *Listeria monocytogenes* (14, 18) and *Mycobacterium tuberculosis* (7), suggesting an important contribution of TNF-α in the control of intracellular infection. TNF-α also synergizes with IL-12 to promote IFN-γ production from NK cells (8, 21). TNF receptor 1 (p55)$^{-/-}$ mice, however, exhibit full resistance to *L. major* (22), suggesting, again, that the requirements for eradication of an exclusively intramacrophagic organism may be less stringent than for microbes with more complex life cycles. It may be of interest to challenge IL-1-deficient mice with *L. major* in order to fully exclude a contribution by the proinflammatory triad in control of this organism.

D.R.B. was supported by the University of Chicago Medical Scientist Training Program and Immunology Training Grant AI-07090. S.L.R. was supported by the Burroughs Wellcome Fund and NIH grant AI-01309.

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Editor: R. E. McCallum