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IL-9 Is a Susceptibility Factor in *Leishmania major* Infection by Promoting Detrimental Th2/Type 2 Responses

Berenice Arendse, Jacques Van Snick, and Frank Brombacher

IL-9 is a cytokine produced by Th2 cells, induced during *Leishmania major* infection. Because the role of IL-9 in leishmaniasis is currently unknown, IL-9-deficient mice were generated by immunization with mouse IL-9 coupled to OVA. This produced strong and long-lasting neutralizing anti-IL-9 Abs in vivo. Anti-IL-9 vaccination showed protective effects, because it enabled *L. major*-infected nonhealer BALB/c mice to better resist to leishmaniasis with doubling the time span until pathological disease progression occurred. Increased resistance was also demonstrated by moderate footpad swelling and histopathology due to reduced parasite burden compared with sham-immunized BALB/c mice. Mechanistically, IL-9 neutralization in BALB/c mice resulted in a reduction of detrimental Th2/type 2 responses with an observed shift toward protective Th1 immune responses. This led to an alteration from alternative to classical macrophage activation with subsequent enhanced killing effector functions, as demonstrated by increased NO production but reduced arginase 1-mediated macrophage responses. Conclusively, the data show that IL-9 is a susceptible factor in leishmaniasis. They further suggest that IL-9 is able to influence Th dichotomy in leishmaniasis by promoting detrimental Th2/type 2 responses in BALB/c mice. The results extend efforts made to generate autoantibodies capable of regulating biological processes, with IL-9 a potential drug target against leishmaniasis. *The Journal of Immunology*, 2005, 174: 2205–2211.
physically linked T cell help to B cells, therefore overcoming B cell tolerance. Using this method, IL-9-depleted BALB/c and C57BL/6 mice were generated, infected with *L. major*, and compared with sham-immunized control BALB/c mice to define a possible role of IL-9 in leishmaniasis by loss of function.

**Materials and Methods**

### Mice and parasites

Mice were kept at the animal facility at the Health Science Faculty, University of Cape Town, under specific pathogen-free conditions. The *L. major* MHOM/IL/81/FEBNI strain was maintained by continuous passage in BALB/c mice as previously described (22). Anesthetized mice were infected s.c. in one hind footpad with 2 × 10⁶ stationary-phase metacyclic *L. major* promastigotes in a final volume of 50 μl of HBSS. Parasites were isolated from skin lesions of infected animals. Parasite burden from homogenized organs was determined by 2-fold limiting dilutions in Schneider’s medium (Sigma-Aldrich). Stationary-phase cultures were also used to prepare frozen-and-thawed (F/T; 1:1000) promastigotes.

### Preparation of IL-9-OVA complexes and immunization protocol

IL-9-OVA complexes were obtained by cross-linking mouse IL-9 and OVA (Sigma-Aldrich) with glutaraldehyde, and purified as previously shown (21). Mice were injected s.c. with 100 μl of 1:1 mixture of 10 μg of IL-9-OVA complexes in PBS and CFA. Two s.c. boosts were performed in IFA at wk 2 and 4. Control mice received an equivalent amount of OVA in Freund’s adjuvant only. Anti-IL-9 titers were measured by the inhibitory activity of the sera on the proliferation of TS1 cells that respond to IL-9. Sera were serially diluted in 96-well plates containing culture medium and incubated in the presence of 2.5 U/ml murine (m)IL-9 for 1 h. TS1 cells were incubated at 37°C, 8% CO₂ for 3 days, and proliferation was measured by hexoseaminidase activity determination (23).

### Cell isolation and CD4⁺ restimulation

CD4⁺ T cells were purified (>90% by FACS) from lymph nodes by positive selection with magnetic mouse CD4 Dynabeads and mouse CD4 DETACHA-BEAD (Dynal; Robbins Scientific) and differentiation performed as described (24). CD4⁺ T cells (2 × 10⁶/ml) were stimulated with anti-CD3 (145-2C-11; BD Pharmingen) or F/T and cytokine concentration from supernatant determined by ELISA 48 h later.

### Macrophage activation

Thioglycolate (3%)-elicited peritoneal exudate cells were harvested at day 6 and further cultured in triplicates at a concentration of 2 × 10⁷/ml in 96-well plates (Nunc) for 4 h. Plastic-adherent macrophages were stimulated with LPS (10 ng/ml; Sigma-Aldrich) and IFN-γ (100 U/ml; BD Pharmingen). After 48 h, the concentration of NO in supernatants was measured by the Griess reaction. Arginase was determined as previously described (25).

### ELISA

Cytokine concentrations were determined by sandwich ELISA. Standards and Abs were purchased from BD Pharmingen and detected using alkaline phosphatase-coupled streptavidin (Southern Biotechnology). Detection limits were as follows: IFN-γ, IL-9, and IL-13, 46 pg/ml; IL-4, 2 pg/ml. Ag-specific Ig ELISA was performed as previously described (22).

### Histology

Tissue samples were fixed in neutral buffered formalin, processed, and 5- to 7-μm sections were stained with H&E.

### Statistics

Data are given as mean ± SD, and the differences were tested using the unpaired two-tailed Student’s *t* test or ANOVA using GraphPad Prism software.

### Results

#### Effective production of anti-IL-9 autoantibodies

IL-9-deficient mice were generated by immunization with mIL-9, chemically complexed to OVA. For this purpose, BALB/c and C57BL/6 mice were immunized with three injections of IL-9 cross-linked to OVA. Two weeks after the last injection, the anti-IL-9 response was evaluated by measuring inhibitory activities of the sera in a bioassay by using an IL-9-dependent T cell line TS1. As shown in a representative experiment for BALB/c (Fig. 1a) and C57BL/6 (b), sera were found to strongly inhibit IL-9-induced proliferation, independent of the mouse strain used. Half-maximal inhibition of 2.5 U/ml mIL-9 was obtained at mean serum dilutions of 3 and 7 × 10⁻⁴ in BALB/c and C57BL/6 mice, respectively (shown in Fig. 1c). The vaccination was 1 specific for IL-9, because proliferation of TS1 by IL-4 was not influenced from sera, and 2) long lasting, with only slight reductions in the observed inhibition titers after a year of vaccination (data not shown), as reported before (21).

Anti-IL-9 vaccination increases resistance in *L. major*-infected BALB/c mice

To determine a possible role of IL-9 in leishmaniasis, IL-9-OVA-immunized or control OVA-immunized BALB/c or C57BL/6 mice were infected with 2 × 10⁶ virulent *L. major* (MHOM/IL/81/FEBNI strain) and com-

### Figure 1

**Induction of IL-9 specific autoantibodies.** a and b, Eight mice per group of BALB/c mice (a) or C57BL/6 mice (b) were immunized with IL-9 complexed to OVA in CFA (IL-9-OVA) or with OVA alone to produce anti-IL-9 autoantibodies. Sera was collected 2 wk after the last immunization and tested in serial dilutions for IL-9 inhibition in an IL-9-dependent TS1 cell proliferation assay. c, The serum titer able to inhibit 50% of TS1 cell proliferation with 2.5 U/ml mIL-9 is shown from individual mice, including the mean (horizontal line). Shown is a representative of three independent experiments.
FEBNI) metacyclic promastigotes into one hind footpad. As expected, control BALB/c mice developed massive footpad swelling (Fig. 2a) with ulceration and necrosis (indicated by the asterisks) from wk 3 onwards. Mice had to be killed at wk 8 postinfection due to disease progression, with necrosis and ulceration in all infected control BALB/c mice. In contrast, IL-9-OVA-immunized BALB/c mice stabilized footpad swelling on a moderate level within the first 4 wk postinfection. Parasite burden in the draining popliteal lymph node (Fig. 2b) and in the infected footpad (c) was significantly lower in IL-9-OVA-immunized BALB/c mice compared with infected control BALB/c mice at 8 wk postinfection and confirmed at wk 5 and 9 in independent experiments. Histopathology developed from wk 3 onwards in the control OVA-immunized BALB/c mice with severe bone destruction in the footpad at wk 8 (data not shown). IL-9-OVA-immunized BALB/c mice more than doubled their time span until ulceration and necrosis developed, which started from wk 10 onward with termination of the experiment at wk 17 postinfection with similar disease progression as observed in the control group 9 wk earlier. In contrast, the healer strain C57BL/6 showed a predominant L. major-specific Th1-polarized response with high IFN-γ and very low IL-4 production (Fig. 3) without an Ag-specific effect of IL-9 OVA vaccination. These results, first, suggest an influence on Th cell differentiation by IL-9 and, second, confirm that Th2 cells are major IL-9 producers during leishmaniasis. This resulted in slightly increased type 1 Ab responses but significantly impaired Ab-specific type 2 responses in comparison to control BALB/c mice (Fig. 4). IL-9-OVA or sham-immunized C57BL/6 mice showed no significant differences in their Ab responses. Together, these results show evidence for a shift toward Th1/type 1 responses by neutralizing endogenous IL-9 in BALB/c mice.

Increased NO killing effector macrophage activity

Macrophages are the major cellular host for L. major where amastigotes propagate in the phagolysosome. NO is the crucial killing effector molecule against leishmaniasis, produced by IFN-γ-stimulated and iNOS-induced classical macrophages. To determine the influence of IL-9-OVA immunization on L. major-specific killing effector functions, macrophages were isolated from thioglycollate-elicited peritoneal exudate cells of infected mice and restimulated with IFN-γ/LPS to determine their killing effector function. Macrophages from 5-wk-infected mice showed no differences (data not shown). Macrophages from 8-wk-infected IL-9-OVA-immunized BALB/c mice showed small but significant increased induced NO synthase (iNOS)-catalyzed NO production compared with cells from sham-immunized BALB/c (Fig. 5a). This was verified by showing a striking reduction in urea production in the earlier (Fig. 5b), which is a side product of arginase 1 activity. This differential outcome can be explained by competition between iNOS and arginase 1 for the common substrate L-arginine. IL-9-OVA immunization had no effect on the NO production of C57BL/6 mice.

IL-9 acts downstream from IL-4Ra-mediated functions

To determine whether anti-IL-9 vaccination is dependent on IL-4 or IL-13-mediated functions, L. major infection studies in IL-9-OVA-immunized BALB/c IL-4Ra-deficient mice were performed. These mice are IL-4 and IL-13 unresponsive, because the IL-4Ra chain is a crucial component of the IL-4 and IL-13 receptor (19). Anti-IL-9 vaccination was similarly effective in IL-4Ra-deficient mice with a half-maximal inhibition of 2.5 U/ml mIL-9, obtained at mean serum dilution of 5 × 10^4 (data not shown). Anti-IL-9 or sham-immunized control BALB/c IL-4Ra-deficient mice (eight mice per group) were infected with 2 × 10^6 virulent L. major (MHOM/IL/81/FEBNI) metacyclic promastigotes into one hind footpad, and the swelling of the footpad was monitored. As previously shown using a different L. major strain, IL-4Ra-deficient BALB/c mice were resistant, with slightly increased footpad swelling compared with C57BL/6 (Fig. 6a). Although IL-9-OVA immunization had the described protective effect in BALB/c mice, shown by delayed footpad swelling, and disease progression (Fig. 6a), as well as reduced parasite burden (b), no effect was observed.

Anti-IL-9 vaccination leads to a shift to protective Th1/type 1 responses

To investigate possible mechanisms to explain the observed increased resistance, L. major-specific Th polarization was determined during infection. Mitogenic- or Ag-specific restimulation of CD4+ T cells, isolated from the draining lymph node of IL-9-OVA-immunized BALB/c mice, produced significantly higher IFN-γ but lower IL-4 levels compared with cells from sham-immunized mice (Fig. 3). Reduced IL-4 production was consistent with impaired Th2 effector cytokines, because IL-9 and IL-13 were significantly impaired in IL-9-vaccinated BALB/c mice (Fig. 3). As expected, the healer strain C57BL/6 showed a predominant L. major-specific Th1-polarized response with high IFN-γ and very low IL-4 production (Fig. 3) without an Ag-specific effect of IL-9 OVA vaccination. These results, first, suggest an influence on Th cell differentiation by IL-9 and, second, confirm that Th2 cells are major IL-9 producers during leishmaniasis. This resulted in slightly increased type 1 Ab responses but significantly impaired Ab-specific type 2 responses in comparison to control BALB/c mice (Fig. 4). IL-9-OVA or sham-immunized C57BL/6 mice showed no significant differences in their Ab responses. Together, these results show evidence for a shift toward Th1/type 1 responses by neutralizing endogenous IL-9 in BALB/c mice.
in IL-9-vaccinated BALB/c IL-4Rα-deficient mice compared with sham-vaccinated controls. CD4+ T cell IFN-γ, IL-4, and IL-9 responses after Ag- or CD3-specific restimulation were similar between the IL-9- and sham-immunized IL-4Rα-deficient mice, with no detectable IL-9 found (Fig. 6c). Together, these data suggest that IL-9 vaccination had no influence on the balance of Th differentiation in this mouse strain. This conclusion was confirmed, because IgG1, -2a, and IgE Ag-specific Ab responses (data not shown) and macrophage NO production were also similar (Fig. 6d). Together, these data suggest that IL-9 acts downstream from IL-4/IL-13-mediated functions.

Discussion
IL-9-deficient mice were generated by immunization with mIL-9, which was chemically complexed to OVA. This approach induced high titers of neutralizing anti-IL-9 Abs, as shown before (21). In this study, we present data showing evidence for the first time that IL-9 is a susceptible factor in L. major infection. Experimentally, this was shown by in vivo IL-9 neutralization, which substantially delayed disease progression of nonhealer BALB/c mice. Delayed leishmaniasis was accompanied by reduced parasite burden, footpad swelling, and milder histopathology during acute infection. As a consequence, IL-9-vaccinated BALB/c mice more than doubled their time span upon infection until deleterious leishmaniasis developed. Neutralization of endogenous IL-9 caused impaired Th2/type 2 responses with a shift toward protective Th1/type 1 responses, as determined by cytokine production of Ag-specific restimulated CD4+ T cells, isolated from the draining lymph nodes. This was confirmed ex vivo by reduced Ag-specific type 2 Ab isotype production in the blood of these mice. We concluded from these data that IL-9 acts downstream from Th2 cell depletion due to direct anti-IL-9-specific Ab binding to its receptor is unlikely due to the restricted surface expression of the IL-9R among T cell subpopulations (26) and the observed normal Ag-specific IgG1 and IgE responses during immunization with Aspergillus extract or infection with Trichinella in IL-9-vaccinated mice (21). Despite the fact that IL-9 can act as a growth factor for certain T cell clones (1) and IL-9 overexpression in vivo can lead to thymic lymphomas (10), a Th2-promoting effect was an unexpected finding, because no effect on Th differentiation has been found until now. In vitro and in vivo.
Th differentiation was found to be normal in a genetically engineered IL-9-deficient 129sv mouse strain (15). Together, this may indicate that the observed influence of IL-9 on Th responses is restricted to certain diseases, like L. major infection, and/or certain genetic backgrounds influencing T cell responses. In any case, the present observations provide the first demonstration that IL-9 is an important element in the physiological regulation of the Th1/Th2 balance in vivo.

With respect to the analysis of IL-9 mode of action, we are currently not able to distinguish whether IL-9 has a direct effect on early steps in L. major-induced Th differentiation or alternatively may promote Th2 effector cell expansion. The latter possibility is more attractive, because IL-9 is barely detectable at the onset of an L. major infection and is mainly produced by differentiated Th2 effector cells (see Fig. 3) during acute leishmaniasis. This may also explain why IL-9 vaccination had no measurable effect during L. major infection in C57BL/6 as well as IL-4Rα-deficient BALB/c mice. Both mouse strains showed dominant Th1 responses during infection with very little IL-9 production after CD4+ T cell restimulation (Figs. 3 and 6). Therefore, it can be envisaged that IL-9 does act downstream from IL-4-mediated Th2 differentiation, and may be an important element to maintain Th2-dominated response to L. major. Clearly, further investigations are needed to fully explore the effects of IL-9 on Th responses in L. major infection.

IL-9 vaccination resulted in improved classical macrophage effector functions. This was determined by increased NO production but reduced urea production, the latter a side product of arginase 1 catalyzation from LPS/IFN-γ-restimulated macrophage. Indeed, NO is a crucial effector molecule able to kill amastigotes within the macrophages (27). NO is catalyzed by the enzyme inducible NO

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isolated, and parasite burden (\(b\) and \(c\)) was determined after 48 h from the supernatant. Values are expressed as means ± SD. d, Thioglycollate-elicited peritoneal macrophages were LPS/IFN-\(\gamma\) stimulated, and the NO concentration in the supernatant was measured.

In conclusion, we uncovered the role of IL-9 as a susceptibility factor in Leishmaniasis. Eight IL-9-OVA-immunized IL-4Ra-deficient BALB/c mice. Eight IL-9-OVA-immunized IL-4Ra-deficient BALB/c (○) or sham-immunized IL-4Ra-deficient BALB/c (●) were injected in the left hind footpad with \(2 \times 10^6\) metacyclic promastigotes of \(L.\ major\) (MHOM/IL/81/FEBN). a. The course of the infection was monitored weekly by measuring infected and noninfected hind footpads. At wk 5 postinfection, three mice per group were sacrificed and further analyzed. b and c. The draining popliteal lymph node was isolated, and parasite burden (b) and anti-CD3 and \(L.\ major\) Ag-specific IFN-\(\gamma\), IL-4, and IL-9 production (c) was determined after 48 h from the supernatant.

FIGURE 6. No effect on leishmaniasis in IL-9-OVA-immunized IL-4Ra-deficient BALB/c mice. Eight IL-9-OVA-immunized IL-4Ra-deficient BALB/c (○) or sham-immunized IL-4Ra-deficient BALB/c (●) were injected in the left hind footpad with \(2 \times 10^6\) metacyclic promastigotes of \(L.\ major\) (MHOM/IL/81/FEBN). a. The course of the infection was monitored weekly by measuring infected and noninfected hind footpads. At wk 5 postinfection, three mice per group were sacrificed and further analyzed. b and c. The draining popliteal lymph node was isolated, and parasite burden (b) and anti-CD3 and \(L.\ major\) Ag-specific IFN-\(\gamma\), IL-4, and IL-9 production (c) was determined after 48 h from the supernatant. Values are expressed as means ± SD. d, Thioglycollate-elicited peritoneal macrophages were LPS/IFN-\(\gamma\) stimulated, and the NO concentration in the supernatant was measured.

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