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# Recombinant Soluble Interleukin-4 (IL-4) Receptor Acts as an Antagonist of IL-4 in Murine Cutaneous Leishmaniasis

ANDRÉ GESSNER,[1]* KLAUS SCHRÖPPEL,[1] ANTJE WILL,[1] KARL-HEINZ ENSSLE,[2] LEANDER LAUFFER,[2] AND MARTIN RÖLLINGHOFF[1]

*Institut für Klinische Mikrobiologie und Immunologie der Universität Erlangen-Nürnberg, 91054 Erlangen,[1] and Behringwerke AG, 35001 Marburg,[2] Federal Republic of Germany*

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**This study was performed to evaluate the soluble interleukin-4 receptor (sIL-4R) as a potential antagonist of interleukin-4 (IL-4) in an infectious disease. It is shown that antigen-triggered proliferation and cytokine secretion of *Leishmania major*-specific, cloned Th2 cells in vitro can be inhibited dose dependently by recombinant murine, but not control human, sIL-4R. In vivo, we found that endogenous synthesis of IL-4 mRNA is upregulated during the first week of infection, while an increase of IL-4R mRNA occurred later after infection of BALB/c mice with *L. major*. To interfere successfully with the IL-4 ligand-receptor interaction, we therefore chose to treat infected BALB/c mice with recombinant sIL-4R during the onset (e.g., days 0 to 7) of the immune response. Treatment with murine, but not with human, sIL-4R during the first week of infection rendered BALB/c mice clinically resistant to *L. major*, led to a 7- to 12-fold reduction of the parasite load in spleen and lymph nodes at 7 weeks of infection, shifted the pattern of cytokines towards a Th1 type, and provided durable resistance against reinfection. Thus, it could be demonstrated that the balance among sIL-4R, membrane-bound IL-4R, and their ligand IL-4 can be modulated in vivo, thereby modifying the antiparasitic immune response. These results suggest a therapeutic value of sIL-4R in diseases in which neutralization of IL-4 is desirable.**

The outcome of many infections is determined by functionally distinct T-helper (Th)-cell populations (Th1 and Th2) secreting different patterns of lymphokines (2, 12, 20, 22, 24, 27). In the model of murine cutaneous leishmaniasis, the development of either protective or disease-promoting immunity is determined within the first few days after infection. This is demonstrated by the fact that treatment inducing protection in BALB/c mice has to be performed prior to or during the first week after parasite inoculation (2, 3, 14, 25, 26, 29, 31).

Interleukin-4 (IL-4), a product of disease-promoting Th2 cells, is of central importance for the clinical course of murine cutaneous leishmaniasis. The application of neutralizing monoclonal antibody (MAb) against IL-4 leads to healing of BALB/c mice if the treatment is given during the first week after infection. The altered clinical course is accompanied by a reduced parasite load in organs of mice, the development of predominantly $CD4^+$ T cells of the Th1 type, and the establishment of protective immunity against reinfection (3, 25).

Two groups have reported the presence of a soluble, high-affinity, IL-4-binding protein in biological fluids of mice (6, 7). This molecule has been identified as the soluble form of the IL-4 receptor (sIL-4R) that retains the ligand-binding domain (19) and thus binds IL-4 with an affinity comparable to that of the membrane-bound form of IL-4R (7, 19). Furthermore, it has been shown that the recombinant extracellular domain of IL-4R inhibits IL-4 functions in vitro (8, 18). These studies have been extended to an in vivo model by Fanslow et al. (5), who have used recombinant sIL-4R to neutralize IL-4 bioactivity in a murine model of allotransplantation, leading to prolonged graft survival. Summarizing these data, sIL-4R obviously fulfills all criteria for a naturally occurring molecule displaying antagonistic properties to IL-4. Thus, this molecule should allow immune intervention with an autologous protein in an infectious disease, the course of which is aggravated by IL-4. We therefore investigated the effect of recombinant sIL-4R on antigen-stimulated murine Th cells in vitro and on the clinical course and development of an immune response in susceptible BALB/c mice after infection with *Leishmania major* in vivo.

## MATERIALS AND METHODS

**Mice and parasites.** Female mice of the inbred strain BALB/c were obtained from Charles River Breeding Laboratories, Sulzfeld, Federal Republic of Germany (FRG) and used at 6 to 12 weeks of age. *L. major* promastigotes were grown in vitro in blood agar cultures as described before (28). Stationary-phase promastigotes were washed in phosphate-buffered saline (PBS), and $2 \times 10^6$ organisms were injected in a volume of 50 μl intradermally into the right hind footpad.

**Measurement of lesions and determination of parasite burden.** Lesion development was monitored by measuring the thickness of the infected and noninfected contralateral footpad with a vernier caliper (Kroeplin, Schlüchtern, FRG) and was calculated as described previously (thickness of infected foot/thickness of control foot) (32). At indicated time points of infection, mice were bled and sacrificed, the popliteal lymph nodes and spleens were removed, and single cell suspensions were prepared and cultivated in vitro for 48 h in the presence of *L. major* antigen. Parts of the organs were frozen immediately (liquid nitrogen) and stored at −70°C until use. A limiting-dilution in vitro culture assay was used to quantitate the number of viable *L. major* parasites in spleens and lymph nodes of mice (32). Organs were homogenized, and serial twofold dilutions of the homogenates were plated in 96-well flat-bottom microtiter plates containing 50 μl of blood agar and 100 μl of complete medium (Click's-RPMI 1640 medium

* Corresponding author. Mailing address: Institut für Klinische Mikrobiologie und Immunologie, University of Erlangen, Wasserturmstr. 3, 91054 Erlangen, Federal Republic of Germany. Phone: 0049 9131/852580. Fax: 0049 9131/852573.

supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 100 μg of penicillin per ml, 160 μg of gentamicin per ml, 13 mM $NaHCO_3$, and $5 \times 10^{-5}$ M 2-mercaptoethanol). After incubation at 28°C for 2 weeks, the wells were assessed microscopically for growth of *L. major* promastigotes. Minimal estimates of the number of viable *L. major* promastigotes were analyzed with an ELIDA program (C. Taswell, Harvard University, Boston, Mass.) by applying Poisson statistics and the $\chi^2$ minimization method (30).

**Cytokine assays.** The concentrations of IL-4, IL-5, IL-10, and gamma interferon (IFN-γ) in supernatants of stimulated lymph node, spleen, or cloned T cells were measured by specific two-site enzyme-linked immunosorbent assays (ELISAs) with reference standard curves, using known amounts of the respective murine recombinant cytokines. The antibody pairs for the detection of IL-4 (BVD4-1D11 and biotinylated BVD6-24G2; detection limit, 10 pg/ml), IL-5 (TRFK 5 and biotinylated TRFK 4; detection limit, 30 pg/ml), and IL-10 (JES5-2A5 and biotinylated SXC-1; detection limit, 20 pg/ml) were purchased from Dianova, Hamburg, FRG. IFN-γ was determined with microtiter plates coated with affinity-purified R4-6A2 MAb and rabbit anti-mouse IFN-γ antiserum for detection (10). The IFN-γ ELISA was further developed with swine anti-rabbit antiserum conjugated with peroxidase (Dianova).

**RNA isolation, reverse transcription, and competitive PCR.** After RNA extraction by use of acidic guanidinium thiocyanate (4) from pooled organs of three mice for each time point, cDNAs were synthesized in 20-μl reactions containing 1 μg of RNA, 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 1 mM (each) deoxynucleoside triphosphate, 2.5 mM oligo(dT), 32 U of RNAguard, and 17 U of avian myeloblastosis virus reverse transcriptase at 42°C for 90 min (16). For PCR, cDNA was amplified in a 40-μl reaction volume containing 50 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 10 mM (each) deoxynucleoside triphosphate, 1 U of *Taq* DNA polymerase, and 100 nM primers for 35 cycles (1 min of denaturation at 94°C, 1 min of annealing at 58°C, 1 min of extension at 72°C). The reagents were purchased from Pharmacia, Freiburg, FRG. Samples were analyzed on a 1.5% agarose gel. Primers used were as follows: IL-4R sense primer 5'-GGCCTGGCAGTGGCAT GGGAGGCC-3', IL-4R antisense primer 5'-TTATCGTGC CCGCTGGGGCCCTGC-3' (amplified fragment, 274 bp) (21); actin sense primer 5'-CACCCGCCACCAGTTCGCCA 3', actin antisense primer 5'-CAGGTCCCGGCCAGCCAG GT-3' (amplified fragment, 574 bp) (9); and IL-4 sense primer 5'-TCTCAACCCCCAGCTAGTTGTCAT-3', IL-4 antisense primer 5'-CCAGGCATCGAAAAGCCCG-3' (amplified fragment, 320 bp) (9). For the construction of control fragments (c.f.) and cloning into plasmids, *Escherichia coli* DNA was PCR amplified under conditions of low stringency (annealing at 37°C for 5 cycles and at 60°C for 35 cycles) to obtain nonsense PCR products flanked with the primers of interest (11). Appropriate fragments which differed in size between 50 and 200 bp with the specific PCR product were purified and cloned into the pSPT18 plasmid. These plasmid DNAs of known molarity were used as c.f. for competitive PCR amplification. The c.f. for the IL-4R was a kind gift of T. Blankenstein, Berlin, Germany (21). The relative amount of distinct cDNA molecules was determined with competitive PCR as reported recently (11). Briefly, each cDNA was first amplified in the presence of diluted actin c.f. (1:10 in a first approach followed by 1:2-dilution intervals for exact monitoring) of known concentration to determine the titer of c.f. needed to compete successfully with this cDNA. Relative concentrations of IL-4 and IL-4R cDNA were then determined in actin-standardized cDNA samples, using the same two-step protocol. All reactions were repeated three times independently. The relative amount of cDNA molecules was calculated on the basis of equivalent numbers of actin cDNA molecules (numbers represent the ratio between IL-4 or IL-4R and actin cDNA molecules).

**Recombinant murine and human sIL-4R.** DNAs encoding the murine (13, 19) and human (15) IL-4R were obtained from Immunex Co., Seattle, Wash. The regions encoding the extracellular domains were excised and cloned by use of a PCR-based strategy into mammalian immunoglobulin fusion expression vectors (33) yielding plasmid pmusolil4rg2b, which encodes the protein smuIL-4R-Fc consisting of the extracellular region of the murine IL-4R linked to hinge CH2 and CH3 domains of a murine immunoglobulin G2b molecule and plasmid phusolil4rg2b coding for the respective human IL-4R-Fc fusion protein. The sequences of the PCR-derived regions of the expression plasmids were confirmed by dideoxy chain termination sequencing. The plasmids were stably transfected into BHK-21 cells (ATCC CCL10). smuIL-4R-Fc and shuIL-4R-Fc were purified as disulfide-linked dimeric proteins from transfectant supernatants, using protein A-Sepharose affinity chromatography. Five hundred microliters of PBS or PBS containing 250 μg of chimeric murine or human IL-4R-Fc protein was injected intraperitoneally on days 0, 2, 4, and 6 after infection.

**In vitro restimulation of spleen and lymph node cells.** Total spleen and lymph node cells were cultured for 48 h in vitro at a density of $4 \times 10^6$ or $2 \times 10^6$/ml, respectively, in the presence or absence of *L. major* freeze-thawed promastigote antigen (LmAg; $5 \times 10^6$ cell equivalents per ml), prepared as described previously (28).

**Cell proliferation tests with murine helper T-cell clones.** The Th2 cell clone L1/1 (17) and the Th1 cell clone C57/6 (kindly provided by M. Lohoff, Erlangen, FRG) are specific for the protozoan parasite *L. major* and were established from infected BALB/c (L1/1) or C57BL/6 mice, respectively. The T-cell clones were propagated in Click's-RPMI 1640 medium supplemented with 10% fetal calf serum and stimulated weekly with irradiated (2,500 rads) syngeneic spleen cells and antigen. All cell proliferation tests were performed in 96-well flat-bottom microtiter plates (Nunc, Wiesbaden, FRG). T cells were pulsed after 48 h of culture with [$^3$H]thymidine (18.5 kBq per well; New England Corp., Dreieich, FRG) for 16 h and processed for β-scintillation counting.

## RESULTS AND DISCUSSION

**Inhibitory effect of sIL-4R on Th2 cells.** IL-4 is one of the most important factors in the differentiation and proliferation of Th2 cells. However, no data have been reported so far about the effect of sIL-4R on the proliferation and function of Th2 cells after stimulation with their antigen. In a first set of experiments, we therefore examined the effects of several sIL-4R preparations on the proliferation and cytokine production of murine *L. major*-specific Th2 and Th1 cell clones. Cells of the Th2 cell clone L1/1 and the Th1 clone C57/6 were cultivated with antigen-presenting cells and the *Leishmania* antigen preparation LmAg in the presence or absence of increasing concentrations of either MAb directed against murine IL-4 (11B11) or recombinant murine or human IL-4R. As depicted in Fig. 1A, cell proliferation was inhibited dose dependently with comparable efficacy by either anti-IL-4 MAb, murine IL-4R, or murine IL-4R-Fc but not by the respective human control proteins. Thus, stimulation of cells via their T-cell receptor, leading to autocrine stimulatory production of

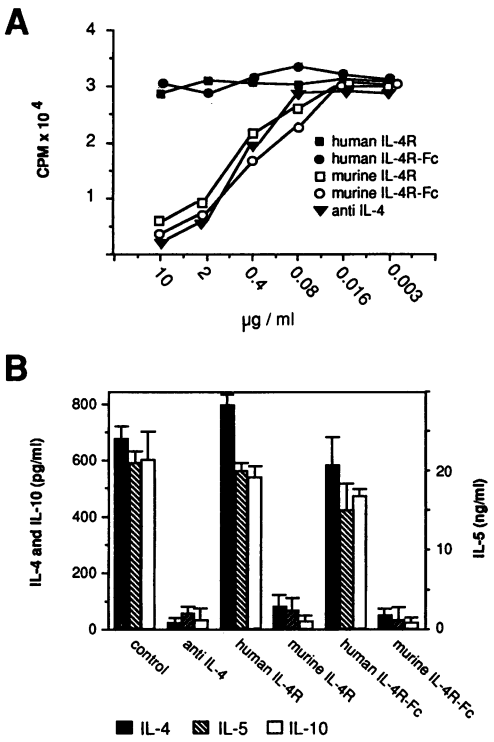


FIG. 1. Effect of sIL-4R on proliferation and cytokine synthesis of an *L. major*-specific Th2 cell clone. Cells of the Th2 cell clone L1/1 were stimulated for 48 h with antigen-presenting cells and LmAg in the presence or absence of different concentrations of murine or human sIL-4R proteins or anti-IL-4 MAb 11B11. (A) Effect on cell proliferation as determined by the incorporation of [$^3$H]thymidine. (B) Effect on synthesis of cytokines IL-4, IL-5, and IL-10 as measured by ELISAs in cell supernatants.

IL-4, can be effectively blocked by sIL-4R. To determine the effect of sIL-4R on the synthesis of Th2 cytokines, IL-4, IL-5, and IL-10 were measured in the supernatants of L1/1 cells 48 h after stimulation with LmAg. In the presence of optimal concentrations of murine sIL-4R proteins or anti-IL-4 MAb (e.g., 10 µg/ml), but not of human sIL-4R, the amounts of Th2 cytokines secreted were reduced to less than 10% (Fig. 1B). As expected, neither the proliferative response nor the production of cytokines by the Th1 clone C57/6 was influenced by sIL-4R (data not shown). These experiments therefore demonstrate the specific neutralizing capacity of sIL-4R for IL-4 in vitro, leading to suppression of Th2 cell functions.

**Monitoring IL-4 and IL-4R expression in BALB/c mice after infection with *L. major*.** Since recombinant sIL-4R was found to be capable of inhibiting IL-4 function in vitro, we planned to examine the in vivo effect of this molecule on the immune response against the parasite *L. major*. Because sIL-4R has to compete with the membrane-bound IL-4R for binding of IL-4, we first analyzed the expression of IL-4 and IL-4R in spleens and lymph nodes of BALB/c mice during the early course of infection with *L. major*. Splenic and lymph node cells from infected mice were harvested at different times after infection and stimulated in vitro with LmAg. Culture supernatants were collected 48 h later and assayed for IL-4. As shown in Fig. 2A, secretion of IL-4 was absent in cell cultures from uninfected mice (day 0) but maximal if cells were taken from mice 7 days after infection and gradually declined thereafter. In addition, the expression of mRNA for IL-4 and its receptor was analyzed

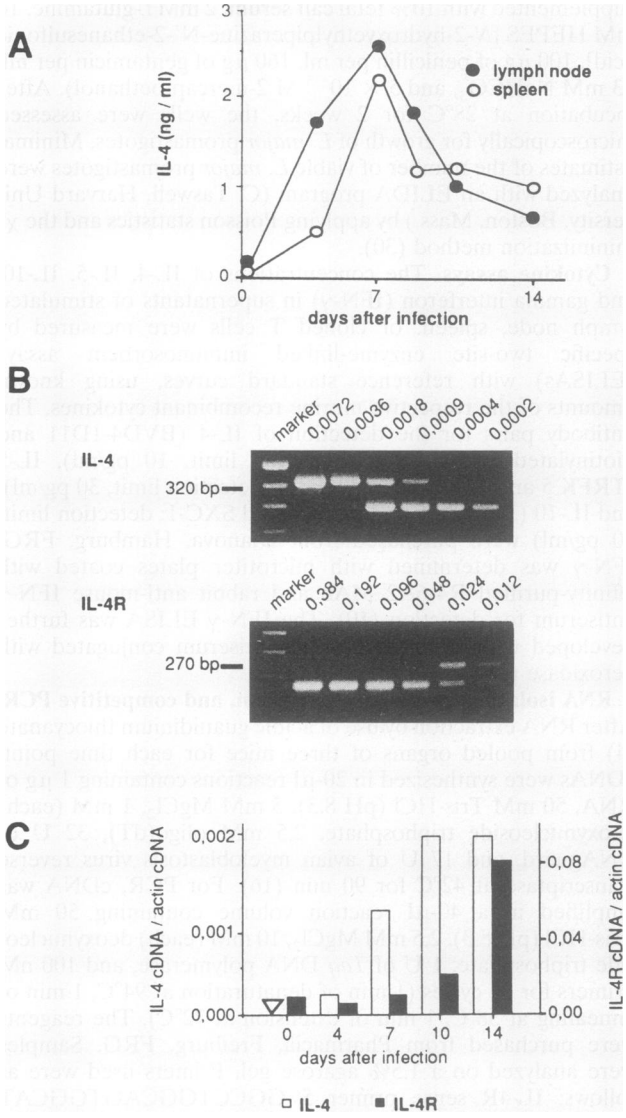


FIG. 2. Expression of IL-4 and IL-4R in organs of BALB/c mice during the first 2 weeks after infection with *L. major*. (A) IL-4 production in lymph node or spleen cell cultures from infected BALB/c mice after restimulation in vitro with LmAg. Mice were infected with $2 \times 10^6$ promastigotes injected into the right hind footpad. Lymph nodes and spleens were harvested on days after infection as indicated, and single cell suspensions were cultivated for 48 h in the presence of LmAg ($5 \times 10^6$ cell equivalents per ml). The IL-4 concentration in the supernatants were determined by an ELISA. Each value represents the mean of three mice. (B) Quantification of mRNA for IL-4 (amplified fragment, 320 bp; c.f., ca. 410 bp) and IL-4R (amplified fragment, 270 bp; c.f., ca. 210 bp) in actin-standardized cDNAs from lesion-draining lymph nodes by competitive PCR. Gels show results with cDNAs from lymph nodes taken on day 10 after infection. (C) Graph displays relative concentrations of IL-4 mRNA (open bars) and IL-4R mRNA (filled bars) to actin mRNA in lymph nodes of mice during the course of infection. Reverse transcription and PCR were performed with pooled RNAs from three mice for each time point and repeated three times independently.

in the lesion-draining lymph nodes by competitive PCR, using reverse transcribed RNA. As shown for cDNA prepared from lymph nodes of BALB/c mice 10 days after infection with *L. major* in Fig. 2B, the relative concentrations of IL-4 and IL-4R

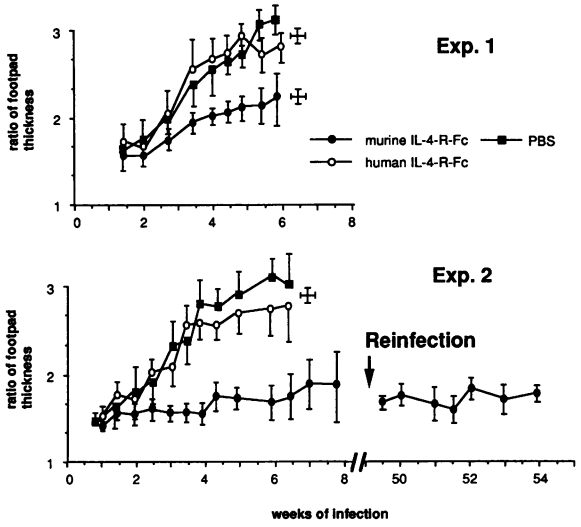


FIG. 3. Influence of sIL-4R chimeric proteins on clinical course of murine cutaneous leishmaniasis. Female BALB/c mice were infected as described in the legend to Fig. 2 and were treated with PBS, human IL-4R-Fc, or murine IL-4R-Fc as described in Materials and Methods. Data shown represent the mean values of footpad swelling of 6 (experiment 1; $P = 0.07$) or 12 (experiment 2; $P < 0.05$) mice per group. Four mice were reinfected with $2.5 \times 10^6$ *L. major* promastigotes 50 weeks after the primary infection and treatment with murine IL-4R-Fc.

mRNA were determined with cDNAs previously standardized for the concentration of actin mRNA, as described in Materials and Methods. While no IL-4 mRNA was found at day 0 of infection, it became detectable by day 3 after infection and its concentration increased 8- to 10-fold between days 7 and 14 after infection (Fig. 2C).

The mRNA for IL-4R appeared to be constitutively expressed in the lymph nodes. A significant upregulation became detectable as late as day 14 after infection (Fig. 2C). Thus, the expression of IL-4 and IL-4R mRNAs followed different kinetics, with an increase of IL-4 mRNA clearly before that of IL-4R mRNA. In vitro, it has been shown with murine lymphocytes that IL-4 induces the expression of its own receptor on the levels of mRNA and cell surface expression (23). The delay of IL-4R mRNA upregulation in *L. major*-infected mice might therefore reflect the first example of an important role of IL-4 in IL-4R upregulation in vivo.

**Treatment of *L. major*-infected BALB/c mice with recombinant sIL-4R leads to an improved clinical course, reduced parasite burden, and predominance of Th1 cytokine synthesis.** Since recombinant sIL-4R showed very pronounced effects in vitro (Fig. 1), it was of great interest to test whether similar effects could also be observed in vivo in *L. major*-infected BALB/c mice. As production of IL-4 is upregulated already during the initial phase of leishmaniasis, the effect of recombinant sIL-4R in vivo was tested by treating mice during the first week after infection. BALB/c mice were infected with *L. major* promastigotes and subsequently treated intraperitoneally with 250 μg of recombinant murine sIL-4R per dose or with equally prepared but not cross-reactive human IL-4R as a control on days 0, 2, 4, and 6 after infection. As depicted in Fig. 3, application of the murine IL-4R, but not of the human control protein, significantly ameliorated the clinical course of the infection measured as an increase of footpad swelling. In 7 of 12 BALB/c mice, treatment with the murine IL-4R resulted in complete resolution of the disease (experiment 2). These permanently cured mice remained free of obvious disease and were resistant to reinfection with $2.5 \times 10^6$ stationary-phase promastigotes (Fig. 3). These results are therefore similar to those after treatment with either IL-12 or anti-IL-4 in which 66 or 85% of treated BALB/c mice displayed permanent clinical cure, respectively (14, 25).

In previous studies performed with heterologous anti-IL-4 antibodies to neutralize IL-4 in vivo, the correlation between the clinical benefit of such treatment and the elimination of *Leishmania* spp. has not been analyzed (3, 25). In the present study, the parasite load, as monitored by limiting-dilution analysis, was reduced 11-fold in footpad tissue, 7.3-fold in the spleens, and 12-fold in the lymph nodes of sIL-4R-treated mice 6 weeks after infection (Table 1), supporting the therapeutic benefit of the recombinant soluble murine IL-4R in vivo. Parasite burdens were further reduced more than 10-fold in spleens and lymph nodes and to very low levels in footpad tissue after 50 weeks of infection in sIL4-R-treated mice (Table 1), suggesting that the induced antileishmanial effects were sustained. The observed persistence on a low level in these mice, however, represents a more general phenomenon, since several different strains of mice which were resistant to *L. major* infection also harbored persistent parasites 1 year after recovery from infection (1).

In the model of murine cutaneous leishmaniasis, protective immunity has been shown to be associated with the development of a Th1-type T-cell response, while progression of disease correlates with the predominance of Th2 cells (2, 20, 22). To evaluate if the sIL-4R treatment leads to predomi-

TABLE 1. Effect of sIL-4R treatment of *L. major*-infected BALB/c mice on parasite load in footpads, spleens, and lymph nodes[a]

| Time (wk) of infection | Organ[b] | No. of viable leishmania ± SD | |
|---|---|---|---|
| | | Control[c] | Murine IL-4 receptor[d] |
| 6 | Footpad | $0.9 \times 10^5 \pm 0.3 \times 10^5$ | $0.08 \times 10^5 \pm 0.04 \times 10^5$ |
| | Lymph node | $1.06 \times 10^5 \pm 0.2 \times 10^5$ | $0.09 \times 10^5 \pm 0.04 \times 10^5$ |
| | Spleen | $11.2 \times 10^5 \pm 2.1 \times 10^5$ | $1.54 \times 10^5 \pm 0.6 \times 10^5$ |
| 50 | Footpad | Dissemination: animals died | 0.05–250 |
| | Lymph node | | $1 \times 10^3 \pm 2.6 \times 10^3$ |
| | Spleen | | $1.4 \times 10^4 \pm 3.5 \times 10^4$ |

[a] Mice were infected with $2 \times 10^6$ promastigotes injected into the right hind footpad. After 6 and 50 weeks of infection, mice were killed and numbers of viable *L. major* parasites in organs were quantified by a limiting-dilution in vitro culture assay. Each value represents the mean ± standard deviation of three to five animals.
[b] For footpads, numbers of viable parasites per milligram of tissue are given. For lymph nodes and spleens, numbers of parasites per organ are given.
[c] Mice received 500 μl of PBS containing 250 μg of chimeric human IL-4R-Fc intraperitoneally on days 0, 2, 4, and 6 after infection.
[d] Mice received 500 μl of PBS containing 250 μg of chimeric murine IL-4R-Fc intraperitoneally on days 0, 2, 4, and 6 after infection.

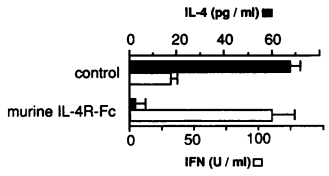


FIG. 4. Effect of sIL-4R treatment on LmAg-specific IL-4 and IFN-γ synthesis in BALB/c mice 6 weeks after infection. BALB/c mice were infected as described in the legend to Fig. 2 and treated with human sIL-4R-Fc (control) or murine sIL-4R-Fc. At day 42 after infection, lymph nodes were harvested, and their cells were stimulated with LmAg in vitro for 48 h. Concentrations of IL-4 and IFN-γ in the supernatants were measured by ELISA.

nance of Th1 cells, the cytokine response elicited by antigen-specific T cells was analyzed 6 weeks after infection. Lymph node and spleen cells from *L. major*-infected BALB/c mice were stimulated with LmAg in vitro. In the supernatants of these cultures, we determined the concentrations of IL-4 as a marker cytokine for Th2 cells and of IFN-γ, a product of activated Th1 cells. Earlier studies have documented the functional importance of the presence of IFN-γ to the capacity to heal *L. major* infection (2, 3, 20, 22). As depicted in Fig. 4, lymph node cells from IL-4R-treated BALB/c mice produced less than 1/10 of IL-4 but nearly sixfold more IFN-γ compared with cells from control-treated and equally infected mice. Thus, it can be concluded that the ameliorated clinical course and the enhanced elimination of the parasite correlated with the predominant Th1-type T-cell response in IL-4R-treated mice.

The results presented here demonstrate the immunomodulatory capacity of murine recombinant sIL-4R in a parasitic infection. The data are in line with a report by Fanslow et al. (5), who showed a prolonged cardiac allograft survival in mice treated with recombinant sIL-4R. Since there is a naturally occurring form of the sIL-4R which has been detected in the blood and urine of mice and in supernatants of cultured T and B cells, it will be of special interest to determine whether this sIL-4R is secreted from cells containing the alternatively spliced transcript for the sIL-4R or represents a portion of the full-length receptor that has been cleaved from the cell surface. The evaluation of the expression and the immunomodulatory role of the naturally produced sIL-4R will possibly uncover novel ways of immune intervention in infectious diseases.

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