IL-4-deficient Balb/c Mice Resist Infection with *Leishmania major*

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Summary

Mice with a genetically engineered deficiency for either IL-4 or IFN-γR1 (single mutants), and IL-4/IFN-γR1 (double mutants) on the Balb/c and 129Sv background were used to study the course of infection with *Leishmania major*. In contrast to genetically resistant 129Sv wild-type mice, IL-4/IFN-γR1 double mutant mice developed fatal disease with parasite dissemination to visceral organs similar to mice lacking IFN-γR1 only. Balb/c mice, which are exquisitely susceptible to *L. major*, were rendered resistant to infection by disruption of the IL-4 gene. As compared to homozygous IL-$4^{+/+}$ mice, heterozygous IL-$4^{+/-}$ animals consistently developed smaller lesions with less ulceration and necrosis, indicating the likelihood of gene-dosage effects. This implicates that the magnitude of the IL-4 response determines the severity of disease. $CD4^+$ T cells of IL-4-deficient mice showed impaired Th2 cell development, as assessed by quantitative RT-PCR of characteristic cytokines. Development of resistance is not explained by default Th1 development, because this was observed only at very late stages of infection. Moreover, the induction of inflammatory cytokines (e.g., IL-1α, IL-1β, TNF-α, IL-12) together with iNOS in the lesion and draining lymph nodes was not altered in the absence of IL-4.

Cutaneous infection of mice with *Leishmania major* is a well-established experimental model of chronic human disease caused by an intracellular parasite (1). The infection of mice of different genetic backgrounds with *L. major* results in one of two contrasting patterns of disease. In Balb/c and a few other mouse strains, local infection is not effectively controlled by the immune response and the disease disseminates to involve visceral organs with eventually fatal outcome. Infection of most other strains (e.g., C57Bl/6, C3H/HeN, 129Sv) causes a localized lesion that heals spontaneously after a few weeks. The resolution of lesions is accompanied by the development of complete resistance to reinfection. MHC class II–restricted $CD4^+$ T cells are of critical importance for the development of clinical disease (2–5) , whereas MHC class I–restricted $CD8^+$ T cells are of minor importance during primary infection (6). Cure and exacerbation of infection with *L. major* has become a paradigm of the role of different T helper subsets during the infection. Resistant strains develop predominantly Th1 responses, as revealed by high levels of IFN-γ but undetectable IL-4. Susceptible strains develop predominantly Th2 responses, characterized by high levels of IL-4 and strong antibody production (7, 8). Depletion of IL-4 by neutralization with mAb makes susceptible mice resistant (9–11) and depletion of IFN-γ by either mAb neutralization or gene disruption renders resistant mice susceptible to infection (12–14). Neutralization of IL-12, a cytokine promoting Th1 and suppressing Th2 development, abrogates resistance, whereas the supplementation of rIL-12 to susceptible mice allows them to resolve infection (15–17). Moreover, resistant mice with an IL-4 transgene expressed at low levels in B cells fail to clear infection (18). Almost all of these immune interventions that change the disease phenotype have been shown to be associated with a switch in T helper subset development. In this article, we examine infection with *L. major* in mice deficient for IL-4 and IFN-γR1 on both a genetically susceptible and resistant background.

Materials and Methods

Mice, Parasites, and Infection. Breeding pairs of homozygous IFN-γR1-deficient (IFN-$\gamma R1^{-/-}$) and control IFN-$\gamma R1^{+/+}$ mice (129/Sv/Evans)(19) were provided by M. Aguet (Genetech, San Francisco, CA). IFN-$\gamma R1^{-/-}$ mice were bred with IL-$4^{-/-}$ mice (129/Sv/Evans) (20) to obtain IL-4/IFN-γR1 double deficient mice. Mice were genotyped routinely by PCR of tail biopsies. Primers used to distinguish the mutant and wild-type IL-4 and IFN-γR1 loci were IL-4 for: GTGAGCAGATGACAT-

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GGGGC, IL-4rev: CTTCAAGCATGGAGTTTTCCC and IFN-γR1for: AGATCCTACATACGAAACATACGG, IFN-γR1rev: TCATCATGGAAAGGAGGGATACAG, respectively. IL-4-deficient mice (F2:129Sv × C57Bl/6) were backcrossed for six generations (F6) onto Balb/c mice. Heterozygous IL-$4^{+/-}$ mice (Balb/c F6) were intercrossed to obtain the mice used for experiments. Congenic IL-$4^{-/-}$ Balb/c mice were obtained by gene targeting in a Balb/c ES cell line as described (32). All mice were maintained under specific pathogen-free conditions in barrier facilities at the Max-Planck-Institute for Immunobiology (Freiburg, Germany). 8–15-wk-old mice of both sexes were used for infections and housed in filter top cages. For some experiments blinded groups of mutant and wild-type Balb/c mice were sent to the Institute of Clinical Microbiology (University Erlangen) and infected there. The origin and propagation of the cloned virulent strain (MHOM/IL/81/FEBNI) of *L. major* has been described elsewhere (21). Stationary phase promastigotes were collected from in vitro culture in biphasic Novy-Nicolle-McNeal (NNN) blood agar medium and, for infection, 2×10^6 parasites were injected subcutaneous into the right hind footpad. The clinical course developing after infection was monitored by measuring the footpad swelling with a metric caliper (Kroepelin, Schluchtern, Germany).

Determination of Parasite Load. For quantitation of parasite number, a limiting dilution in vitro culture was performed essentially as described (22). Briefly, organs were homogenized and serial twofold dilutions were plated in three replicates at each dilution in 96-well plates containing 50 μl NNN blood agar and 100 μl complete medium (Click's RPMI, supplemented with 10% FCS, 2 mM l-Glutamine, 10 mM Hepes, 160 μl/ml gentamycin, and 100 μl/ml penicillin (all from Biochem, Berlin, Germany) and 5×10^{-5} M 2-ME. The plates were incubated at 28°C for 2 wk and assessed for parasite growth microscopically.

Collection of Lymph Nodes and Cell Separation. For analysis of cytokine production and transcripts, popliteal lymph nodes (PLN) were excised at designated times, teased to a single-cell suspension and $CD4^+$ T lymphocytes were purified by incubation with magnetized anti-CD4-antibodies using the MACS® system according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). FACS® analysis of the resulting $CD4^+$ cell population revealed >90% purity (data not shown). Cells were always kept at 4°C and collected before and after $CD4^+$ selection for either RNA preparation or in vitro restimulation.

Stimulation of Cells and Cytokine Production. $CD4^+$ cells were plated at a concentration of 4×10^5 per well (200 μl) in flat-bottomed 96-well tissue culture plates and stimulated with solid-phase coupled anti-CD3 mAb 145-2C11 (20 μg/ml) and rmIL-2 (250 U/ml; Genzyme, Munich, Germany). Supernatants were harvested after 48 h, pooled and stored at −20°C until used. Cytokine levels in the supernatants were determined by ELISA with paired monoclonal antibodies for IL-4, IL-5, IL-10, and IFN-γ (all from PharMingen, San Diego, CA).

RT-PCR. At designated times after infection, total cellular RNA was prepared from homogenized tissue (e.g., infected and contralateral uninfected footpad) and from PLN cells prepared as described above using Trizol[RM] (GIBCO BRL, Gaithersburg, MD). Random hexamer-primed reverse transcription (RT) was performed with Superscript-RT (GIBCO BRL) using 3 μg total RNA in a 30 μl reaction volume containing 0,04 u (1.2 ng) random hexamers (Pharmacia, LKB, Freiberg, Germany), 0.4 mM dNTP (Promega, Zürich, Switzerland) 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 0.5 units RNAsin (Promega). After 90 min of incubation at 37°C, samples were heated at 94°C and then quickly chilled on ice. Samples were diluted 1:10 with H_2O to a concentration of 10 ng/μl cDNA equivalents assuming a 1:1 ratio of reverse transcription. Cycling conditions for all PCR amplifications of cytokines were 94°C for 2 min before 35 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 min. Cycling conditions for the amplification of β2-microglobulin (β2-mgl) and hypoxanthin-phosphoribosyl-transferase (HPRT) consisted of 30 and 32 cycles, respectively. The templates for PCR consisted of 2 to 4 μl of the diluted RT reaction.

Construction of a PCR MIMIC for the Quantitative Analysis of Cytokine Transcripts. A competitive PCR MIMIC control vector (pNIL) containing a multispecific primer cassette was constructed essentially as described (23) to enable quantitative analysis of IL-12p40, IL-12p35, IL-10, IL-13, and iNOS transcripts. A PCR MIMIC control vector, pMus (24), for the quantitation of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL6, TNF-α, IFN-γ, and β2-microglobulin transcripts was kindly provided by D. Shire, Sanofi, Toulouse. PMCQ, a MIMIC control vector for LTα (TNF-β) (25), was kindly provided by T. Blankenstein (Max Delbrick Center, Berlin). Primer sequences and the lengths of amplified cDNA fragments and MIMIC controls are given in Table 1 below. Primers were designed to span introns.

Quantitation of Cytokine Transcripts. The level of individual transcripts was determined by competitive PCR as described (23, 26) using the multispecific MIMIC control vectors described above. PCR reactions with equal aliquots (usually 4 μl) of the diluted RT-reaction containing approximately 40 ng cDNA equivalents and oligonucleotide specific primers were added to serial fourfold dilutions of known molarity (5×10^6 to 7.5×10^1 molecules) of the appropriate MIMIC plasmid. PCR co-amplification was performed using the cycling conditions described above. Specific primers compete for annealing and amplification of the control fragment and cellular cDNA, resulting in fragments that differ 50–100 bp in size. The PCR products derived from the MIMIC template and the cDNA were resolved on an agarose gel, and the relative ethidium bromide staining intensities of the target and the MIMIC DNAs were compared. Equal band intensities of both fragments allow the quantitation of cDNA relative to the competitor. For numerical calculation, the ratio was formed between the dilutions of the respective cDNA and competitor fragment. Before quantitation of individual transcripts, cDNAs were adjusted to equal concentrations of both β2-microglobulin (β2-mgl) and HPRT.

Results and Discussion

IFN-γ but not IL-4 Determines the Outcome of L. major Infection in Genetically Resistant Mice. Mice (129Sv) deficient for either IL-4 (IL-$4^{-/-}$) or IFN-γR1 (IFN-$\gamma R1^{-/-}$), and (IL-4/IFN-γR1) doubly deficient mice were infected with *L. major* promastigotes subcutaneously in one hind footpad, and the clinical course of infection was monitored for up to 9 wk. Disease outcome with respect to the size of footpad lesion and parasite load is shown in Fig. 1 *A* and Table 2, respectively. In the absence of an intact IFN-γR1, mice with an otherwise resistant genetic background (129Sv) were unable to control the growth of *L. major* . Already at day 24 after infection, the number of viable parasites was >5,000-fold increased in the draining popliteal lymph

Table 1. *Primer Sequences for the Amplification of cDNA and Competitor Control Fragments*

Gene	Primer sequence 5′	3′	5′	3′	cDNA	MIMIC
β2-mgl	for:	TGACCGGCTTGTATGCTATC	rev:	CAGTGTGAGCCAGGATATAG	222	320
IL-1α	for:	CAGTTCTGCCATTGACCATC	rev:	TCTCACTGAAACTCAGCCGT	218	320
IL-1β	for:	TTGACGGACCCCAAAAGATG	rev:	AGAAGGTGCTCATGTCCTCA	204	320
IL-2	for:	GACACTTGTGCTCCTTGTCA	rev:	TCAATTCTGTGGCCTGCTTG	227	320
IL-3	for:	GACCCTCTCTGAGGAATAAG	rev:	CTCCAGATCGTTAAGGTGGA	232	320
IL-4	for:	TCGGCATTTTGAACGAGGTC	rev:	GAAAAGCCCGAAAGAGTCTC	216	320
IL-5	for:	TCACCGAGCTCTGTTGACAA	rev:	CCACACTTCTCTTTTTGGCG	201	320
IL-6	for:	GTTCTCTGGGAAATCGTGGA	rev:	TGTACTCCAGGTAGCTATGG	208	320
TNF-α	for:	TCTCATCAGTTCTATGGCCC	rev:	GGGAGTAGACAAGGTACAAC	212	320
IFN-γ	for:	GCTCTGAGACAATGAACGCT	rev:	AAAGAGATAATCTGGCTCTGC	227	320
TGF-β	for:	ACCGCAACAACGCCATCTAT	rev:	GTAACGCCAGGAATTGTTGC	200	320
TNF-β/LT-α	for:	GGGAACAGGGGAAGGTTGAC	rev:	CTTTCTTCTAGAACCCCTTGG	205	290
IL-10	for:	AGCCGGGAAGACAATAACTG	rev:	CATTTCCGATAAGGCTTGG	189	280
IL-12p35	for:	GATGACATGGTGAAGACGGCC	rev:	GGAGGTTTCTGGCGCAGAGT	402	280
IL-12p40	for:	CTGGCCAGTACACCTGCCAC	rev:	GTGCTTCCAACGCCAGTTCA	384	220
IL-13	for:	CTCACTGGCTCTGGGCTTCA	rev:	CTCATTAGAAGGGGCCGTGG	420	300
iNOS	for:	AGCTCCTCCCAGGACCACAC	rev:	ACGCTGAGTACCTCATTGGC	481	320

nodes of IFN-γR1$^{-/-}$ compared to control wild-type mice. From week 6 to 8 after infection, IFN-γR1$^{-/-}$ mice died with fulminant visceral leishmaniasis. Parasitized livers of these mice showed large areas of confluent inflammatory infiltrates consisting mainly of polymorphonuclear cells in necrotic liver parenchyma (Fig. 2 *b*). Infection of IFN-γR1/IL-4 doubly deficient mice resulted in fatal disease (e.g., parasite load, tissue damage) indistinguishable from IFN-γR1$^{-/-}$ mice (Table 2, Figs. 1 *A* and 2 *c*), whereas IL-4$^{-/-}$ mice controlled the infection as effectively as the 129Sv wild-type mice, which had only small foci of inflammatory infiltrates of mainly mononuclear cells in liver tissue. These results confirm an essential role of IFN-γ/IFN-γR1 for the elimination of *L. major* (12, 13). In terms of the disease outcome in genetically resistant mice, the presence or absence of IL-4 was inconsequential. The absence of IL-4, in particular, did not change the severity of disease in double-mutant mice below that seen in single IFN-γR1 mutant animals. These data are novel and would not have been predicted based on data demonstrating that in vivo neutralization of IL-4 reverses the exacerbative effect of neutralization of IFN-γ with monoclonal antibodies (27).

The Absence of IL-4 Confers Resistance to Infection with L. major in Susceptible Mice. To study the influence of IL-4 in a genetically susceptible strain, the disrupted IL-4 locus was backcrossed for 6 generations (F6) onto Balb/c mice. As expected, IL-4 competent Balb/c mice infected with *L. major* developed progressive disease, as assessed by parasite load (Table 2), footpad swelling (Fig. 1 *B*), ulceration and necrosis of the lesion (Fig. 2 *e*). In contrast, IL-4$^{-/-}$ Balb/c mice (F6) were resistant to progressive infection and no signs of necrosis of the lesion were observed. Interestingly, as compared to homozygous IL-4$^{+/+}$ mice, heterozygous IL-4$^{+/-}$ (F6) animals consistently developed smaller lesions (Fig. 1 *B*) with less ulceration and necrosis, indicating the likelihood of gene-dosage effects. A correlation between the magnitude of the IL-4 response and of the severity of disease was established earlier by comparing different inbred strains covering the range of susceptibility to disease (28). Likewise, Balb/c × C57BL/6 F1 hybrid mice are intermediate in the severity of disease compared to the susceptible Balb/c and resistant C57Bl/6 parents (29). The fact that the F2 generation can be divided into three groups in conformity with a 1:2:1 ratio suggests that genetic susceptibility to *L. major* is under the control of a single genetic locus. While the exact location of the putative resistance gene(s) is unknown, there is evidence from linkage studies that it maps with high concordance to genes at the mid to the distal end of chromosome 11 (30, 31), a region including the IL-4 gene family cluster and IL-12p40. Considering these results, we could not exclude the possibility that the resistance of IL-4$^{-/-}$ Balb/c mice (F6) is influenced by the presence of a resistance gene(s) carried over by breeding the mutant IL-4 locus from a resistant (129Sv) onto a susceptible strain. To test this possibility, we followed *L. major* infection in IL-4$^{-/-}$ Balb/c mice that were obtained from targeting in Balb/c ES cells (32). As shown in Fig. 1 *b*, these mice resisted infection comparable to IL-4$^{-/-}$ Balb/c (F6) backcrosses. At day 42 after infection, when the acute inflammatory footpad swelling reaction de-

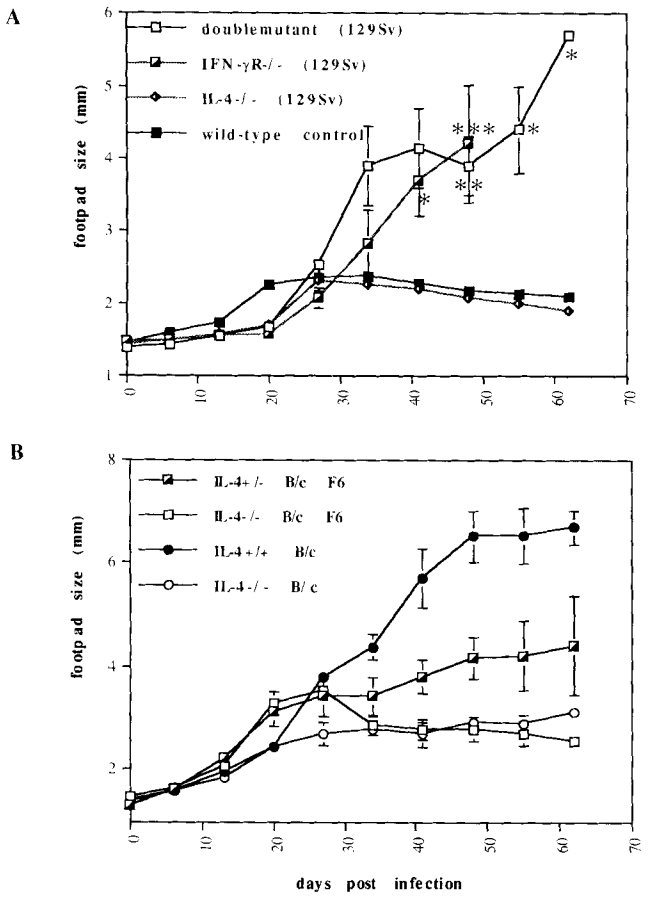

Figure 1. Course of *L. major* infection in IFN-γ-receptor 1 and IL-4-deficient mice. Mice (5 per group) were infected in the hind footpad with 2×10^6 promastigotes of *L. major* and the course of disease monitored by using metric calipers to measure footpad swelling. Infected mice included genetically resistant mice deficient for IL-$4^{-/-}$, IFN-γ$R^{-/-}$, IL-4/IFN-γR doublemutant, and wild-type controls (all 129Sv)(*A*), and genetically susceptible Balb/c mice (IL-$4^{+/+}$) with a homozygous (IL-$4^{-/-}$) or heterozygous (IL-$4^{+/-}$) disruption of the IL-4 gene. The IL-4 mutation was derived either in 129Sv ES cells and heterozygous mice were backcrossed for six generations to Balb/c (B/c F6) mice, or in Balb/c ES cells and heterozygous mice backcrossed for two generations to Balb/c mice (IL$4^{-/-}$ B/c). Resistant IL-$4^{-/-}$ B/c mice were maintained for more than 5 mo. 1 out of 5 mice developed progressive lesion size after 3 mo. Representative results (mean + SE) from one of two independent experiments are shown. * indicates death of individuals.

Table 2. *Parasite Load in Lesions and Lymphoid Tissues of Mice Deficient for IL4 and/or IFN-γR1 Infected with L. Major*

		Day 24			Day 42	
Strain	No.	PLN	spleen	footpad	PLN	spleen
IL-4 wt	1	2^{23}	2^{9}	2^{20}	2^{21}	2^{13}
Balb/c	2	2^{22}	2^{11}	2^{22}	2^{21}	2^{10}
IL-4 ko	1	2^{18}	2^{9}	2^{20}	2^{16}	2^{9}
Balb/c	2	2^{18}	2^{9}	2^{19}	2^{16}	2^{7}
wildtype	1	2^{13}	2^{9}	2^{16}	2^{9}	2^{4}
129 Sv	2	2^{18}	2^{6}	2^{17}	2^{10}	2^{3}
IL-4 ko	1	2^{15}	2^{7}	2^{15}	2^{10}	2^{7}
129 Sv	2	ND	ND	ND	2^{10}	2^{4}
IFNγR ko	1	2^{27}	2^{13}	2^{21}	2^{23}	2^{19}
129 Sv	2	2^{26}	2^{16}	2^{20}	2^{24}	2^{19}
IFNγR/IL-4 doubly ko	1	2^{30}	2^{15}	2^{22}	2^{24}	2^{24}
129 Sv	2	ND	ND	ND	2^{24}	2^{18}

Groups of mice ($n = 5$) were infected with 2×10^6 *L. major* promastigotes into the right hind footpad. The animals were killed at day 24 or 42 and the parasite load was determined in spleens, popliteal lymph nodes, and footpads. Serial twofold dilutions of organ homogenates were plated in micro-titer plates and cultured for two weeks and assessed for parasite growth. Shown are values of two representative individuals of each group. Numbers indicate the reciprocal of dilution in which at least two out of three replicates were positive.

clined, histological examination revealed typical granulation tissue with proliferated fibroblasts, collagen deposition and scar formation, together, indicating concomitant repair of the lesion (Fig. 2 *f*). The number of viable parasites in the draining popliteal lymph node (PLN) of IL-$4^{-/-}$ Balb/c mice was 32-fold reduced compared to Balb/c wild-type controls. However, parasite elimination in IL-$4^{-/-}$ Balb/c mice was not as efficient as in genetically resistant 129Sv mice. A cohort of infected IL-$4^{-/-}$ Balb/c mice was maintained for more than 5 mo, confirming their capacity to control the infection for long periods. In accordance with previous studies that used mAbs to neutralize IL-4 in Balb/c mice, this finding shows that a null-mutation of the IL-4 gene confers resistance to *L. major* infection in susceptible mice. However, by using the same strain of IL-$4^{-/-}$ Balb/c mice to study the outcome of infection with *L. major*, other investigators arrived at opposite conclusions. The knockouts remained susceptible (33). While we presently do not understand the reason for this discrepancy, it should be noted, that the other report shows that both parasitemia and lesion size were already declining in IL-$4^{-/-}$ Balb/c mice, while they were still progressing in Balb/c wild-type controls at a later time point of infection (33).

The Absence of IL-4 in Balb/c Mice Infected with L. major Impairs Th2 Development without Preferential Expansion of Th1 Development In Vivo. The development of either protective immunity or progressive disease after experimental infection of C57Bl/6 and Balb/c mice with *L. major* has been clearly correlated with either the expansion of Th1 or Th2 cells, respectively. IL-4-deficient mice have impaired Th2 but enhanced Th1 responses, when infected with a variety of pathogens (20, 34–38). The developmental pathway of T helper subsets in IL-$4^{-/-}$ and IL-$4^{+/+}$ Balb/c mice at day 22 post infection with *L. major* was determined by competitive RT-PCR of a panel of characteristic Th1 and Th2 cytokines from both total popliteal lymph node (PLN) cells and purified $CD4^+$ PLN cells before and after restimulation with anti-CD3 (Fig. 3, Table 3). In infected IL-$4^{+/+}$ Balb/c mice, both Th2 (e.g., IL-4, IL-10, IL-13) and Th1 cytokines (e.g., IFN-γ, TNF-β) were strongly elevated. IL-5, an-

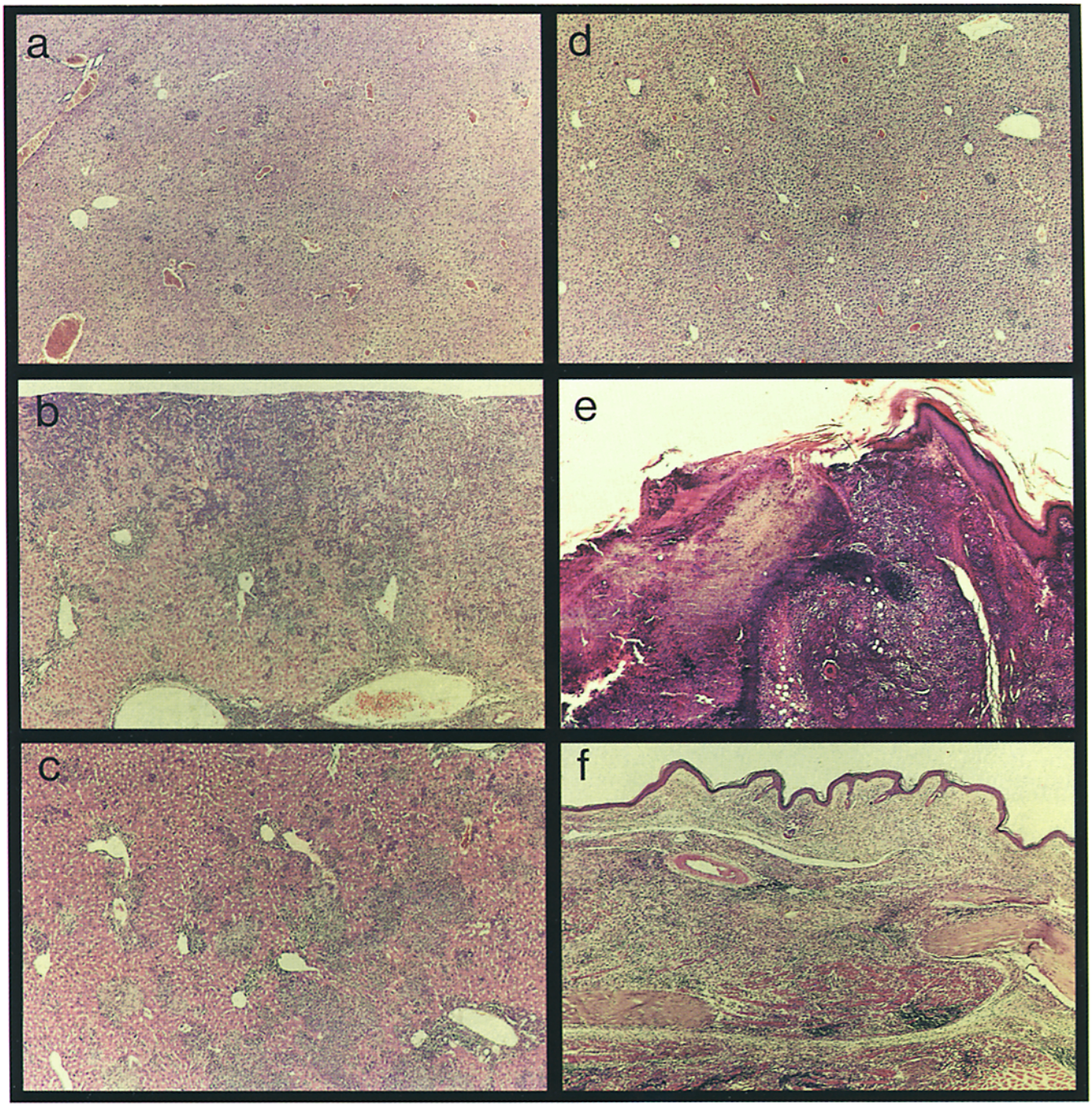

Figure 2. Histopathology of *L. major* infected mice. At day 42 after infection, livers of IFN-γR1-deficient (*b*) and IFN-γR1/IL-4 doubly deficient (*c*) mice (129Sv) show large areas of confluent inflammatory infiltrates consisting mainly of polymorphonuclear cells in necrotic liver parenchyma, whereas livers of both genetically resistant 129Sv (*a*) and susceptible Balb/c wild-type mice (*d*) contain only small foci of inflammatory infiltrates of mainly mononuclear cells. Footpads of Balb/c wild-type mice (*e*) show ulceration and necrosis adjacent to acute inflammation of the dermal connective tissue with mainly polymorphonuclear infiltrates (*e*). In contrast, photographs of sectioned footpads of IL-4−/− Balb/c mice (*f*) reveal a chronic inflammatory reaction with granulation tissue containing mononuclear inflammatory cells, spindle shaped fibroblasts and collageneous fibers indicating scar formation and concomitant repair of the lesion. Sections were prepared 6 wk after infection and stained with hematoxylin and eosin. Original magnification 50×.

other Th2 cytokine, was poorly expressed after infection. With the exception of TNF-β, enhanced transcriptional levels of individual Th1 and Th2 cytokines were found mainly in the $CD4^+$ population, consistent with the MHC class II/$CD4^+$ T cell dependence of the infection and the identification of $CD4^+$ T cells as the primary source of these cytokines (8). No such induction of Th2 cytokines was observed in $CD4^+$ T cells of infected IL-$4^{-/-}$ mice, which contained 8–16-fold lower levels of IL-10 and IL-13 transcripts (Fig. 3, Table 3). More interestingly, levels of

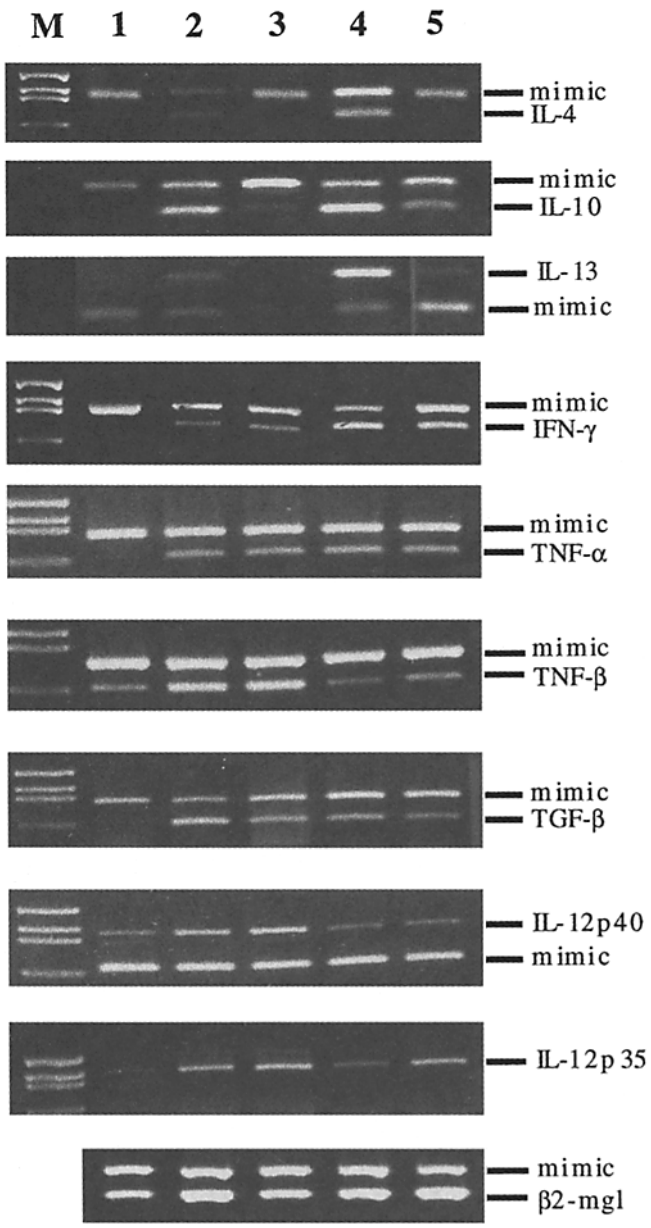

Figure 3. Cytokine mRNA expression 22 d after infection with *L. major*. Groups of five IL-4-deficient (IL-$4^{-/-}$ Balb/c F6) and control (IL-$4^{+/+}$ Balb/c F6) mice were infected with *L major*. On day 22 post-infection popliteal lymph node cells were harvested, pooled, and $CD4^+$ T cells isolated with anti-CD4 magnetic beads. The levels of the indicated cytokine mRNA transcripts were determined by competitive RT-PCR after standardization for the expression of the constitutively transcribed β2-microglobulin gene. Constant amounts of cDNA samples were amplified in the presence of serial fourfold dilutions of a multispecific internal plasmid control ranging from 5×10^6 to 7.5×10^1 molecules . Competitor construct (mimic) and transcript amplicons were separated in an agarose gel and visualized by ethidium bromide staining. Equal densities of target cDNA and competitor fragment were used to calculate the amount of initial target gene given by the molar amount of competitor plasmid used for amplification. All PCR results shown were repeated two to three times independently to confirm results. The figure represents one single dilution taken from the titration to compare expression of the indicated cytokine in the different samples. The gel was photographed with a CCD high resolution video camera system (GDS 5000; UVP Cambrdige, UK) and the image printed using Adobe Photoshop™ 3.0. Lane *1*, non-infected mice; lanes *2–5*, infected mice. Lanes *1*, *2*, and *4*, IL-$4^{+/+}$ Balb/c; lanes *3* and *5*, IL-$4^{-/-}$ Balb/c mice. Lanes *1–3*, total PLN; lanes *4* and *5*, $CD4^+$ PLN T cells. *M*, DNA size marker.

IFN-γ transcripts were comparable in both groups of mice. To follow whether IL-12, a cytokine which promotes Th1 development and hence IFN-γ responses, was altered in the absence of IL-4 we determined transcript levels of both chains of the IL-12 heterodimer, IL-12p40 and IL-12p35, at day 22 post infection. IL-12 mRNA levels were very low in PLN, with no difference comparing wild-type and mutant mice.

Further, upon stimulation of PLN $CD4^+$ T cells from infected IL-$4^{+/+}$ mice with anti-CD3 significant amounts of both Th1 (e.g., IFN-γ) and Th2 cytokines (e.g., IL-4, IL-10) were secreted into supernatants (Fig. 4 *a*). Supernatants from $CD4^+$ T cells of IL-$4^{-/-}$ mice contained reduced levels of IL-10, which indicates reduced Th2 development, whereas levels of IFN-γ were comparable to controls. Elevated (fourfold) levels of IFN-γ indicating Th1 default development in IL-$4^{-/-}$ mice were found only at late stages of infection (9 wk) (Fig. 4 *b*), suggesting that Th1 default development is not responsible for the resistance of IL-$4^{-/-}$ mice. Hence, IL-$4^{+/+}$ Balb/c mice exacerbate infection despite the presence of a powerful IFN-γ response. This may explain why continuous administration of exogenous IFN-γ did not ameliorate *L. major* infection in Balb/c mice (9). Indeed, it has become evident from other studies that susceptible and resistant mice do not considerably differ in IFN-γ levels or the number of IFN-γ secreting cells within the first 3 to 4 wk of infection (6, 13). Mutually exclusive Th responses have been observed only at very late stages of infection (4). Taken together, these results clearly demonstrate that the key player in susceptibility to *L. major* is IL-4: the more the worse. Balb/c mice do not develop fatal disease because of a loss of the ability to induce IFN-γ responses, as suggested recently by elegant in vitro studies (39), but by their failure to downregulate the production of IL-4.

The Absence of IL-4 Has No Consequence on Transcript Levels of iNOS IL-1, TNF, and TGF-β in the Lesion and Lymphoid Tissues of Infected Mice. Macrophage activation and the subsequent production of pro-inflammatory cytokines (e.g., IL-1, TNF), together with anti-microbial products such as the radical nitric oxide (NO), has been suggested to be an important effector pathway in Leishmania defense (40–43). IFN-γ is a key inducer of NO production (19, 44), whereas IL-4, IL-10, IL-13, and TGF-β are inhibitory cytokines (45–50). For instance, neutralization of TGF-β allows otherwise susceptible mice to resist *Leishmania braziliensis* infection (51). The activation state of macrophages and their defense potential were studied directly in the lesion by RT-PCR amplification of a panel of pro- and anti-inflammatory cytokines and the inducible nitric oxide synthase (iNOS). Infected and contralateral uninfected footpads were removed at day 22 of infection, when the le-

Table 3. *Quantification of Transcripts of a Variety of Cytokines in Popliteal Lymph Nodes of IL-4-deficient Mice on Day 22 after Infection with L. Major*

	PLN naive	PLN (p. Lm)		$CD4^+$ cells PLN (p. Lm)		CD4+ PLN (p.Lm) <CD3	
	$IL\text{-}4^{+/+}$	$IL\text{-}4^{+/+}$	$IL\text{-}4^{-/-}$	$IL\text{-}4^{+/+}$	$IL\text{-}4^{-/-}$	$IL\text{-}4^{+/+}$	$IL\text{-}4^{-/-}$
IL-4	<0.16	1.22	<0.16	4.88	<0.16	39	<0.16
IL-5	<0.16	nt	nt	0.61	<0.61	nt	nt
IL-10	<0.16	0.61	0.16	2.44	0.32	4.88	0.61
IL-13	<0.16	nt	nt	2.44	<0.16	9.76	0.61
IFN-γ	<0.16	19.6	19.6	156.3	78.1	156.3	312.5
TNF-β	78.1	312.5	312.5	78.1	78.1	nt	nt
TNF-α	<0.64	9.76	9.76	9.76	9.76	nt	nt
TGF-β	<0.64	39.2	19.6	19.6	19.6	nt	nt
IL-12p40	<0.16	0.61	0.61	<0.16	<0.16	nt	nt
iNOS	nt	0.61	0.61	nt	nt	nt	nt

Transcript levels were calculated using competitive RT-PCR as described in the legend to Fig. 3. Values represent the ratio of target gene: β2-microglobulin gene expression ($\times 10^{-4}$). *p.Lm*, post *L. major*, infection; <CD3, after stimulation with anti-CD3; *nt*, not tested.

sion size in $IL\text{-}4^{-/-}$ Balb/c mice (F6) peaked and differences in healing between mutant and wild-type mice became first evident. Transcripts encoding IL-1α, IL-1β, TNF-α, TGF-β, and iNOS were highly elevated in the infected compared to noninfected footpad and little differences were detected between $IL\text{-}4^{+/+}$ and $IL\text{-}4^{-/-}$ mice (not shown). Similarly, we found no differences in the expression of TNF-α, TGF-β , and iNOS (Fig. 3, Table 3) in draining PLN of both groups of mice. It may have been anticipated that the absence of IL-4 and a concomitant reduction of IL-10 and IL-13 results in elevated macrophage effector functions such as increased NO production (47). Comparing resistant C57Bl/6 mice and susceptible Balb/c mice, an inverse pattern of expression of iNOS and TGF-β was observed. C57Bl/6 mice expressed high levels of iNOS and low levels of TGF-β protein, whereas disease in Balb/c mice correlated with low levels of iNOS and increased TGF-β (52). Our results show that the absence of IL-4 did neither increase the mRNA expression of iNOS nor attenuate the expression of TGF-β. However, as shown for both NO and TGF-β, the regulation may occur posttranscriptionally (53) (Steven Reed, personal communication). On the other hand, the detrimental activity of IL-4 on macrophage function in susceptible Balb/c mice is possibly exerted only during the first days of infection as suggested by the inability of anti-IL-4 treatment to support a curative outcome when administered 7-14 days post infection (10). It has been shown previously that infection with promastigotes induce a rapid biphasic IL-4 response, which is curtailed by IL-12, within the first hours and few days of infection. The first but not the second burst of IL-4 is dependent on IFN-γ, (54–56). This early IL-4 response may be decisive for the fatal outcome of infection, since it is mainly found in susceptible mice. It remains to be shown

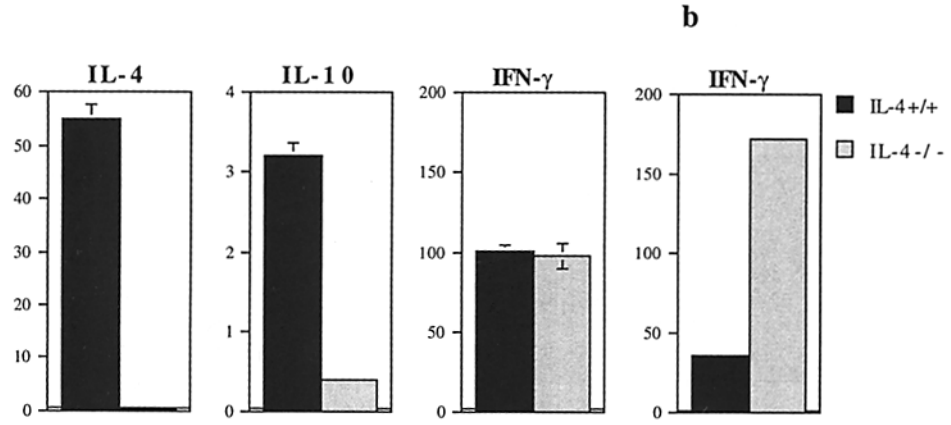

Figure 4. Cytokine production of CD4+ T cells from popliteal lymph nodes of *L. major* infected mice at day 22 (*a*) or day 63 (*b*). CD4+ T cells prepared as described in the legend to Fig. 3 were restimulated with immobilized anti-CD3 for 48 h. Designated cytokines in the supernatants were measured by ELISA using specific monoclonal antibodies (all PharMingen). Values for IL-4 and IL-10 are given as ng/ml; for IFN-γ as U/ml.

whether IL-12 and other macrophage functions are antagonized by the early IL-4 response.

As shown previously, IL-$4^{-/-}$ mice are completely resistant to *L. mexicana* infection, a different species of Leishmania, which grow cutaneously and induce nonhealing lesions in most strains of mice (57). Thus, the IL-4-deficient mice will prove valuable for elucidating the molecular mechanisms of an inappropriate immune response to a parasite with fatal consequences for the host.

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