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The Relative Contribution of IL-4 Receptor Signaling and IL-10 to Susceptibility to *Leishmania major*[1]

Nancy Noben-Trauth,[2]* Rosalia Lira,[3]† Hisashi Nagase,* William E. Paul,‡ and David L. Sacks†

The roles of IL-10 and IL-4 receptor signaling were evaluated in a murine model of *Leishmania major* infection. In previous studies the *L. major* substrain LV39 caused progressive, nonhealing lesions in BALB/c mice deficient for IL-4R α-chain (IL-4Rα), while substrain IR173 was highly controlled. To explore whether IL-10 is responsible for inducing susceptibility to LV39, wild-type and IL-4R$\alpha^{-/-}$ mice were treated with anti-IL-10R mAb, and in a genetic approach, the IL-4R$\alpha^{-/-}$ mice were crossed with BALB/c IL-10$^{-/-}$ mice. In contrast to the lack of resistance conferred by IL-4Rα gene deletion, partial resistance to LV39 was conferred by IL-10 gene deletion or treatment of BALB/c mice with anti-IL-10R mAb. Lesion sizes and LV39 parasite numbers were further and dramatically reduced in both anti-IL-10R-treated IL-4R$\alpha^{-/-}$ mice and IL-4Rα × IL-10 double knockouts. Anti-IL-10R mAb treatment further suppressed parasite growth in IL-4R$\alpha^{-/-}$ mice infected with *L. major* IR173. Production of IFN-γ was only increased relative to wild-type or littermate controls in IL-4R$\alpha^{-/-}$ mice with complementary defects in IL-10. Comparisons of IFN-γ-treated infected macrophages in vitro indicated that LV39 required 25- to 500-fold greater concentrations of IFN-γ than IR173-infected macrophages to achieve a similar efficiency of parasite killing. These studies suggest that regardless of parasite substrain, IL-10 is as important as IL-4/IL-13 in promoting susceptibility to *L. major* and even more so for those substrains that are relatively resistant to IFN-γ mediated killing. *The Journal of Immunology,* **2003, 170: 5152–5158.**

Acquired resistance to *Leishmania major* is associated with the development of a Th1-dominated response, with IFN-γ as the key effector cytokine able to activate macrophages to kill intracellular parasites. In contrast, susceptibility to *L. major* has been attributed to a Th2 response dominated by high levels of IL-4 and low amounts of IFN-γ, establishing this as the prototypic model to study polarized Th2 responses in vivo (1, 2). In BALB/c mice, *L. major* is able to stimulate a burst of IL-4 production in the draining lymph node (LN)[4] as early as 16 h following s.c. infection (3). Much of the early IL-4 is thought to derive from an oligoclonal population of CD4$^+$ T cells with the Vβ4 Vα8 TCR that recognize the *Leishmania* Ag *Leishmania* homolog of receptors for activated C kinase (4, 5). The critical role played by early IL-4 production in exacerbating infection is supported by the observation that BALB/c mice treated with anti-IL-4 Abs at the time of challenge exhibit a healing phenotype (6, 7). This conclusion is further supported by the fact that some *L. major* strains, including IR173, are at least partially controlled in IL-4$^{-/-}$ mice (8) and even more effectively controlled in IL-4R$\alpha^{-/-}$ mice (9). Despite the reversal of BALB/c susceptibility in these settings, the resistance was not associated with a significant increase in IFN-γ production. More significantly, infection of BALB/c IL-4$^{-/-}$ or IL-4R$\alpha^{-/-}$ mice with certain other *L. major* substrains, such as LV39 (9) or NIH/Seidman (10), failed to reverse the nonhealing phenotype, suggesting that additional or alternative disease-promoting factors to IL-4 or IL-13 exist in the *L. major* murine model.

The in vitro activities of IL-10 in suppressing IFN-γ responses and deactivating macrophages to IFN-γ-mediated intracellular killing are well documented (11). A role for IL-10 in promoting susceptibility to *L. major* infections in the mouse remained unsupported, however, until a recent report indicating that resistant mice expressing an IL-10 transgene under the control of the MHC II Ea promoter were more susceptible (12), and conversely, that BALB/c IL-10 knockout (KO) mice were more resistant, although they still harbored high numbers of parasites (13).

Thus, there are accumulating data to challenge an underlying principle of the Th1/Th2 paradigm as it relates to resistance and susceptibility to *L. major*, i.e., as counter-regulatory populations of IFN-γ- or IL-4-producing cells. In an attempt to reconcile these apparent differences, the relative contribution of IL-10 and IL-4 receptor signaling to BALB/c susceptibility was investigated using 1) single- or double-deficient IL-4Rα and/or IL-10 KO mice, 2) treatment of wild-type and IL-4R$\alpha^{-/-}$ mice with anti-IL-10R mAb, and 3) infection with *L. major* substrains that are divergent in the degree to which IL-4Rα signaling is required to promote susceptibility. The results conclusively show that IL-10 is the additional factor controlling LV39 susceptibility in BALB/c IL-4R$\alpha^{-/-}$ mice, and that even for the IR173 substrain, IL-10 controls the survival of residual parasites in IL-4R$\alpha^{-/-}$ mice. The spectrum of type 2 cytokines required to promote susceptibility to *L. major* substrains was investigated in the context of the differential sensitivity of the parasite strains to IFN-γ-mediated killing in vitro.

Materials and Methods

Mice

BALB/c IL-4R$\alpha^{-/-}$ mice were generated as previously described (14) and bred under specific pathogen-free conditions in the National Institute of

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[4] Abbreviations used in this paper: LN, lymph node; cRPMI, complete RPMI; KO, knockout; pms, promastigotes; SLA, soluble *L. major* Ag.

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Allergy and Infectious Disease Animal Care Unit. IL-$10^{-/-}$ mice were backcrossed to the BALB/c strain for eight generations (15) and were provided by D. Rennick (DNAX Research Institute). Crosses between IL-$4R\alpha^{-/-}$ and IL-$10^{-/-}$ mice were screened for the mutated loci by PCR analysis of tail DNA. BALB/c and C57BL/6 mice were purchased from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD)

Parasite infection, lesion measurement, and parasite quantitation

L. major substrains LV39 (MRHO/SU/59/P), IR173 (WHOM/IR/IR/173), and clone V1 (WHOM/IL/80/Friedlin) promastigotes (pms) were cultured at 26°C in medium 199 supplemented with 20% heat-inactivated-FCS (HyClone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5 μg/ml hemin (in 50% triethanolamine), and 1 μg/ml 6-biotin (in 95% ethanol, pH 7.2; M199/S). Infective stage metacyclic pms were isolated from stationary culture (5–6 days old) by negative selection using peanut agglutinin (Vector Laboratories, Burlingame, CA). Mice were infected with 10^5 purified metacyclics in the left hind footpad. Lesion size was measured with a metric caliper and calculated by subtracting the size of the contralateral noninfected footpad. Parasites from the infected footpad lesions were quantitated by homogenizing the tissue using a Teflon-coated microtissue grinder in a microfuge tube containing 200 μl of M199/S. The tissue homogenates and cell suspensions of draining LN cells were serially diluted in a 96-well, flat-bottom microtiter plate containing biphasic medium prepared using 50 μl of NNN medium with 30% defibrinated rabbit blood and overlaid with 50 μl of M199/S. The number of viable parasites was determined from the reciprocal of the highest dilution at which pms could be detected after 7 days of incubation at 26°C.

Culture medium

Complete RPMI (cRPMI) consisted of RPMI 1640 medium (Biofluids, Rockville, MD) supplemented with 10% FBS (Life Technologies, Gaithersburg, MD), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.05 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Antibodies

Hybridomas secreting anti-IL-10R mAb (1B1.3a) (16) and a rat IgG1 isotype control (GL113) were provided by K. Moore (DNAX, Palo Alto, CA) and purified from ascites (Harlan Bioproduct, Madison, WI). Anti-CD4 mAb (GK1.5) was provided by the Biological Resources Branch of the National Cancer Institute.

Cell stimulation and cytokine ELISA

For Ag-specific responses, draining LN cells were cultured at 3×10^6/ml in 24-well plates in cRPMI containing 25 μg/ml soluble leishmania Ag (SLA) obtained from LV39 or IR173 pms. Supernatants were collected at 48 h and assayed for cytokine production. IL-4, IFN-γ (Endogen, Woburn, MA), and IL-10 (R&D Systems, Minneapolis, MN) ELISAs were performed according to the manufacturer's directions.

In vitro killing assays

Peritoneal macrophages were obtained from BALB/c and IL-$4R\alpha^{-/-}$ mice and resuspended at 2.5×10^5/ml in cRPMI medium. The cells were plated in 0.4 ml on eight-chamber Lab-Tek Permanox tissue culture slides (Miles Laboratories, Elkhart, IN) and were allowed to adhere overnight at 37°C in 5% CO_2. Nonadherent cells were removed by gently washing. LV39 and IR173 metacyclic pms were opsonized with 5% normal mouse serum for 30 min at 37°C. The adherent macrophages were infected with 1×10^6 pms/ml at an approximate *Leishmania*/macrophage ratio of 3/1 in 0.2 ml of cRPMI. After 30-min incubation at 37°C, free pms were removed by washing with cRPMI, and the cultures were incubated for an additional 72 h at 35°C. At this time, 0.1 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) and concentrations of recombinant mouse IFN-γ (Genzyme, Cambridge, MA) ranging from 0.0001–5 ng/ml were added. The cultures were incubated for an additional 72 h at 35°C, and the slides were fixed in absolute methanol for 5 min and stained with Diff-Quick solutions (DADE International, Miami, FL). The numbers of infected and noninfected macrophages and the number of amastigotes were counted. The percent killing relative to untreated macrophages was calculated on the basis of the comparison of total amastigotes per 100 macrophages.

Statistical analysis

Statistical analysis for parasite numbers and lesion sizes was performed by Student's *t* test. Differences were considered significant if $p < 0.05$.

Results

Susceptibility of IL-4Rα$^{-/-}$ mice to L. major *substrain LV39 is reversed by treatment with anti-CD4 or anti-IL-10R Abs*

We previously showed that infection with *L. major* substrains LV39 and IR173, although very similar in disease progression in BALB/c mice, produce very different outcomes in BALB/c IL-$4R\alpha^{-/-}$ mice (9). We have extended these studies to other *L. major* substrains, including the V1 clone of *L. major* Freidlin. As shown in Fig. 1, LV39 infected BALB/c IL-$4R\alpha^{-/-}$ mice maintained a susceptible phenotype throughout infection, comparable to that of wild-type mice. In contrast and as previously reported (9), parallel infection with *L. major* IR173 in the IL-$4R\alpha^{-/-}$ mice showed lesion progression similar to that of genetically resistant C57BL/6 mice. The Friedlin/V1 infection in IL-$4R\alpha^{-/-}$ mice was more severe than that in resistant mice, although by day 42 there was clear evidence for control, and in a subsequent experiment with Friedlin/V1 parasites, the disease progression in IL-$4R\alpha^{-/-}$ mice was similar to that in IR173 mice. Consistent with our previous findings, (9), despite their resistant phenotypes, neither IR173- nor V1-infected IL-$4R\alpha^{-/-}$ mice up-regulated IFN-γ in response to SLA stimulation (data not shown).

It has been shown that anti-CD4 treatment of BALB/c IL-4-deficient mice promotes resistance to *L. major* LV39 infection

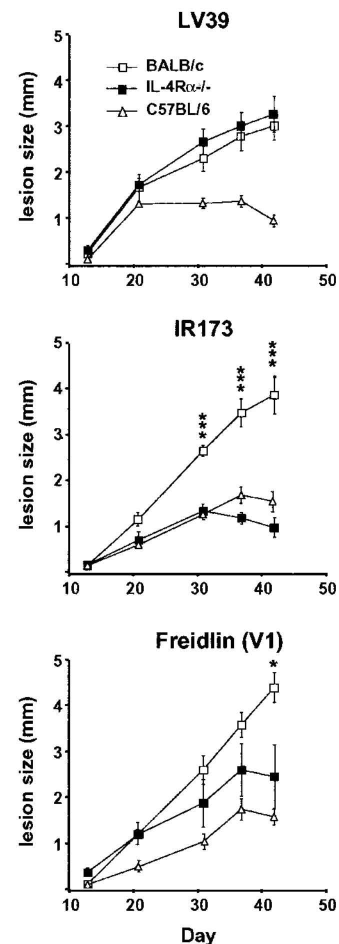

FIGURE 1. IL-$4R\alpha^{-/-}$ mice remain susceptible to *L. major* LV39, but control IR173 and V1 infections. BALB/c, IL-$4R\alpha^{-/-}$, and C57BL/6 mice were infected with 10^5 *L. major* LV39, IR173, or V1 pms in the left hind footpad, and lesion development was monitored by measurment with a metric caliper during the 6-wk infection. Asterisks indicate significant differences by Student's *t* test (*, $p < 0.05$; ***, $p < 0.001$).

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(17). To confirm that a $CD4^+$ cell-derived factor is also responsible for susceptibility to LV39 infection in IL-4R$\alpha^{-/-}$ mice, $CD4^+$ T cells were transiently depleted in BALB/c and IL-4R$\alpha^{-/-}$ mice by injecting anti-CD4 mAb (GK1.5) on the day of infection with LV39. When examined 6 wk postchallenge, CD4 depletion reduced the number of parasites in the lesion by 1000- to 2000-fold in both BALB/c and IL-4R$\alpha^{-/-}$ mice (Fig. 2*A*), which correlated with small lesion progression (data not shown).

The levels of IFN-γ, IL-4, and IL-10 from SLA-stimulated draining LN cells in anti-CD4-treated mice were measured 6 wk after infection (Fig. 2*B*). Again, IFN-γ levels in the IL-4R$\alpha^{-/-}$ mice were not up-regulated compared with those in BALB/c mice. In contrast, CD4-depleted BALB/c and IL-4R$\alpha^{-/-}$ mice each had increased levels of IFN-γ and little or no detectable IL-4 after infection with *L. major* LV39. Anti-CD4 treatment also significantly decreased the levels of IL-10 in both BALB/c and IL-4R$\alpha^{-/-}$ mice.

The results with anti-CD4 treatment provide evidence that a CD4-derived factor may be suppressing the IFN-γ response in IL-4R$\alpha^{-/-}$ mice and is responsible for their inability to control infection with LV39. Our initial results using the anti-IL-10 mAb (clone JES5-A) indicated that IL-10 production was not efficiently neutralized by this Ab in vivo (data not shown). Therefore, we explored IL-10 neutralization by weekly injections of an mAb to the IL-10R (clone 1B1.3a) (16), which has recently been shown to promote sterile cure following healing in *L. major*-infected C57BL/6 mice (18) and in *L. donovani*-infected mice (19). Six weeks following infection, the number of LV39 pms per milligram of tissue was decreased ~600-fold in the anti-IL-10R-treated BALB/c mice, although four of the six treated mice still harbored $>10^4$/mg of tissue (Fig. 3*A*). The control treated IL-4R$\alpha^{-/-}$ mice remained susceptible to LV39 infection; however, anti-IL-10R treatment rendered them highly resistant, with no parasites detected within the tissue sampled from the inoculation site in the eight mice tested. In the draining LN site, the parasite numbers in BALB/c and IL-4R$\alpha^{-/-}$ mice treated with anti-IL-10R showed 1000- to 3000-fold decreases, with lower numbers than those in resistant C57BL/6 mice (Fig. 3*B*). The Ag-specific IFN-γ produced by LN cells was significantly up-regulated after anti-IL-10R treatment in both BALB/c and IL-4R$\alpha^{-/-}$ mice (Fig. 3*C*).

IL-4Rα and IL-10 double-KO mice are highly resistant to L. major *LV39*

To further examine the role of IL-10 in promoting susceptibility to *L. major* LV39, we crossed the genetically pure BALB/c IL-4R$\alpha^{-/-}$ strain to the IL-$10^{-/-}$ strain (15) that had been backcrossed onto the BALB/c background. To facilitate breeding and to obtain adequate numbers of double-homozygous knockouts, we used offspring from (IL-4R$\alpha^{+/-}$, IL-$10^{+/-}$) × (IL-4R$\alpha^{-/-}$, IL-$10^{+/-}$) matings. The various genotypes and the parental strain of IL-$10^{-/-}$ mice were infected with *L. major* LV39. After 6 wk of infection, the numbers of LV39 pms per milligram of footpad lesion tissue (Fig. 4*A*) and draining LN site (Fig. 4*B*) were comparable among the various heterozygous controls (IL-4R$\alpha^{+/-}$, IL-$10^{+/+}$ in *lane 1*; IL-4R$\alpha^{+/-}$, IL-$10^{+/-}$ in *lane 2*). Again, the

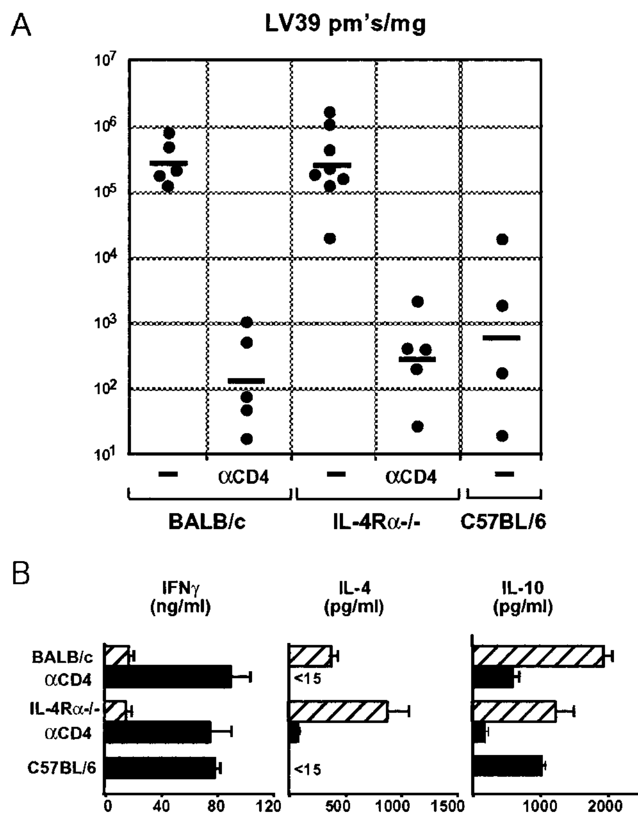

FIGURE 2. Depletion of $CD4^+$ T cells in IL-4R$\alpha^{-/-}$ mice leads to control of *L. major* LV39 and increased IFN-γ secretion. *A*, BALB/c and IL-4R$\alpha^{-/-}$ mice were injected i.p. with 1 mg of anti-CD4 (GK1.5) on days −1 and 0 of infection with 10^5 *L. major* LV39 pms in the left hind footpad. The numbers of parasites in the lesion were quantitated 6 wk after infection, as described in *Materials and Methods*. Each circle represents parasite numbers from an individual mouse tissue sample. Horizontal bars represent the geometric means for each group. *B*, Draining LN cells from 6-wk infected mice were stimulated with SLA and assayed for IFN-γ, IL-4, and IL-10 by ELISA.

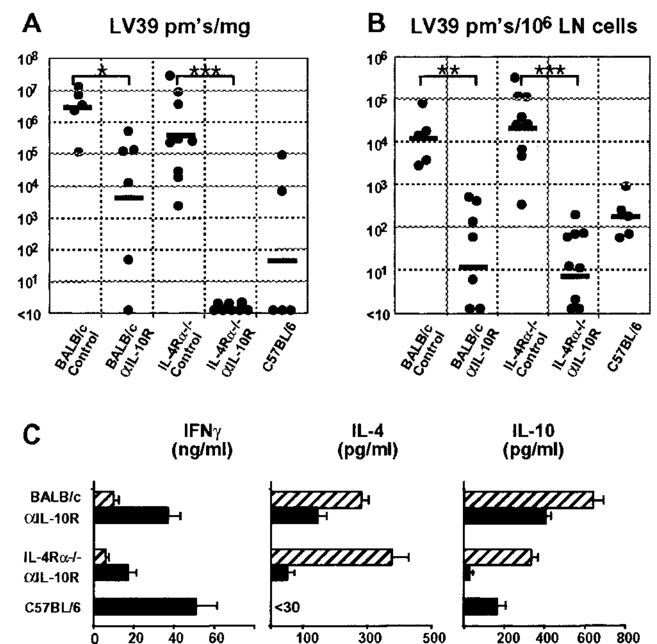

FIGURE 3. Anti-IL-10R mAb treatment leads to highly controlled growth of LV39 parasites in IL-4R$\alpha^{-/-}$ mice and increased IFN-γ secretion after treatment. BALB/c and IL-4R$\alpha^{-/-}$ mice were treated i.p. with 1 mg of anti-IL-10R mAb (1B1.3a) or control mAb (GL113) on days −1 and 0 and weekly thereafter during infection with 10^5 *L. major* LV39 pms. The numbers represent the LV39 pms per milligram of infected footpad tissue (*A*) and LV39 pms per 10^6 LN cells (*B*) of individual mice taken 6 wk after infection. *C*, IFN-γ, IL-4, and IL-10 secretion were measured by ELISA in supernatants from stimulated LN cells. Asterisks indicate significant differences by Student's *t* test. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

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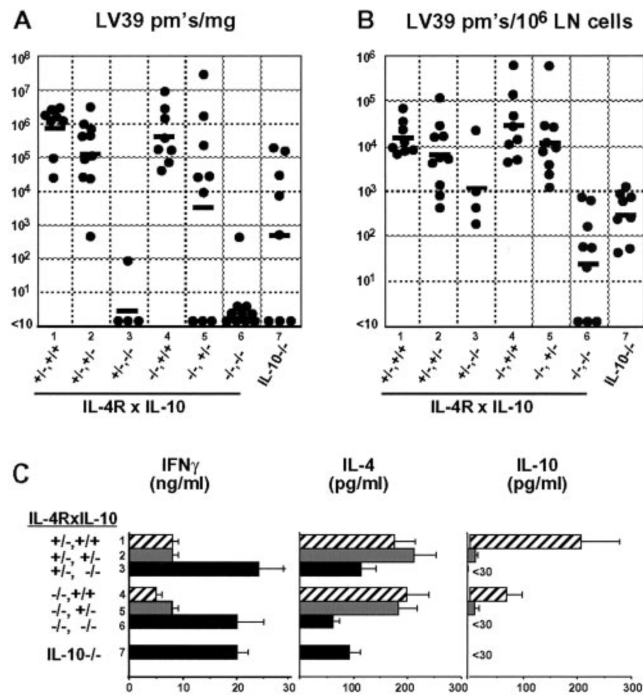

FIGURE 4. Genetic deficiency of both IL-4Rα and IL-10 leads to resistance to *L. major* LV39. *A*, BALB/c IL-4R$\alpha^{-/-}$ mice were crossed to IL-10$^{-/-}$ mice on a BALB/c background. The indicated genotypes were litters obtained from (IL-4R$\alpha^{+/-}$, IL-10$^{+/-}$) $\times$ (IL-4R$\alpha^{-/-}$, IL-10$^{-/-}$) crosses. The BALB/c IL-10$^{-/-}$ parental strain was used as a comparison. The mice were infected with 10^5 *L. major* LV39 pms in the left hind footpad, and lesions were quantitated for LV39 pms per milligram of tissue (*A*) and LV39 pms per 10^6 LN cells (*B*) 6 wk after infection. *C*, IFN-γ, IL-4, and IL-10 were quantitated from SLA-stimulated LN cells as described at 6 wk postinfection.

single knockouts for IL-4R$\alpha^{-/-}$ obtained from this cross (IL-4R$\alpha^{-/-}$, IL-10$^{+/+}$, *lane 4*) remained susceptible, with parasite numbers equivalent to those in the heterozygous controls. Interestingly, IL-4R$\alpha^{-/-}$ mice, which were heterozygous for the IL-10 locus (IL-4R$\alpha^{-/-}$, IL-10$^{+/-}$, *lane 5*), produced a considerable range of pms per milligram, but had an average 100-fold fewer parasites than the IL-4R$\alpha^{-/-}$, IL-10$^{+/+}$ mice (Fig. 4*A*, *lane 4*). However, this was not observed in the draining LN (Fig. 4*B*). In the limited number of IL-4R$\alpha^{+/-}$, IL-10$^{-/-}$ mice (*lane 3*) available from the cross, a reduction in parasites in the footpad and a moderate reduction in the draining LN were observed compared with each of the heterozygous control groups. However, the IL-10$^{-/-}$ parental strain (IL-10$^{-/-}$, *lane 7*), which is homozygous wild type for IL-4Rα, did not exhibit this level of control.

The most striking reduction in the number of *L. major* LV39, at both the injection site and the draining LN, occurred in the group that was double homozygous for deletion of both IL-4Rα and IL-10 loci (*lane 6*). In nine of the 10 mice shown there were no detectable parasites in the tissue sampled from the lesion (Fig. 4*A*, *lane 6*), and the number of parasites in the LN was reduced ~1000-fold compared with that in IL-4R$\alpha^{-/-}$, IL-10$^{+/+}$ controls (Fig. 4*B*, *lanes 4* and *6*). Two subsequent infections produced similar results.

IFN-γ, IL-4, and IL-10 levels in SLA-stimulated, draining LN cells of the double-deficient homozygous mice and their genetic controls were measured (Fig. 4*C*). Interestingly, IFN-γ levels were increased in each group that was IL-10$^{-/-}$ regardless of the genotype of the IL-4Rα locus. However, IFN-γ levels did not necessarily translate into clearance of LV39 parasites, since the parental stock of IL-10$^{-/-}$ mice had ~200 times greater numbers of LV39 pms compared with the double-homozygous KO mice (Fig. 4*A*).

When lesion development during *L. major* LV39 infection was compared between the parallel infections of anti-IL-10R-treated mice (Fig. 5*A*) and offspring of the IL-4Rα $\times$ IL-10 genetic cross (Fig. 5*B*), the double-homozygous KO mice showed the greatest control over lesion progression, with an early and slight inflammation that began to resolve starting at 2 wk. In two additional infections with IL-4Rα $\times$ IL-10 crossed mice, the double-KO mice had smaller lesions than either the C57BL/6 mice or the IL-10 or IL-4Rα single-KO mice (data not shown).

Statistical comparisons of parasite numbers in the footpad and draining LN and lesion sizes between groups are shown in Table I. The groups that displayed statistically significant differences at the $p < 0.001$ level for all three parameters were the IL-4R$\alpha^{-/-}$ mice treated with anti-IL-10R and the IL-4Rα $\times$ IL-10 double-KO mice. It is worth noting that by lesion size alone both the BALB/c and IL-4R$\alpha^{-/-}$ mice treated with anti-IL-10R displayed relatively resistant phenotypes (Fig. 5*A*) despite a clear difference in parasite load within the lesion site (Fig. 3*A*). This illustrates the fact that lesion scores may not always adequately reflect immunity, particularly when IL-10 deficiencies and possible dysregulated inflammation are concerned.

IL-10 is also a cofactor in BALB/c susceptibility to L. major *IR173*

In contrast to *L. major* LV39 parasites, the growth of substrain IR173 is well controlled in IL-4R$\alpha^{-/-}$ mice, and we were interested to learn whether in mice with intact IL-4R signaling, IL-10

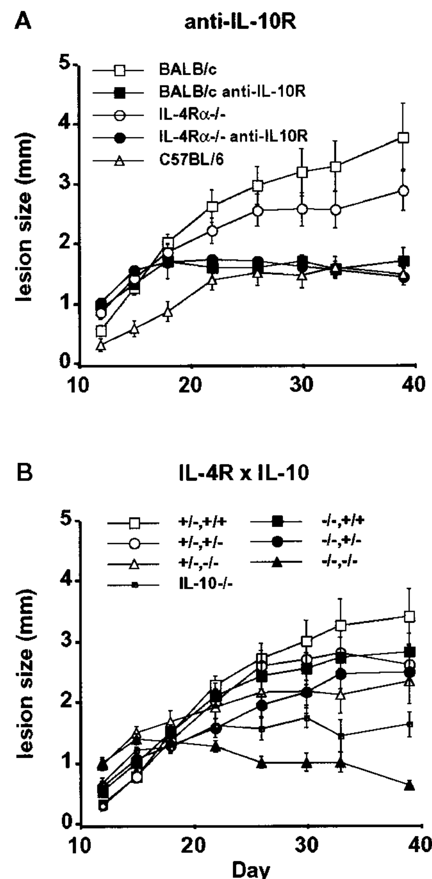

FIGURE 5. IL-4Rα mice treated with anti-IL-10R and IL-4Rα $\times$ IL-10 double-deficient mice are highly resistant to *L. major* LV39. Footpad lesion sizes of mice treated with anti-IL-10R (*A*) or genetic crosses of IL-4R$\alpha^{-/-}$ $\times$ IL-10 and infected with *L. major* LV39 (*B*) were measured with a metric caliper as described in *Materials and Methods*.

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Table I. *Statistical analysis of LV39 parasite numbers in the footpad and LN sites and footpad lesion sizes*

Group			pm/mg	pm/10^6 LN	Lesion Size (d39)
Anti-IL-10R treatment					
BALB/c control	vs	IL-4R$\alpha^{-/-}$ control	NS	NS	NS
BALB/c control	vs	BALB/c anti-IL-10R	*	**	***
IL-4R$\alpha^{-/-}$ control	vs	IL-4R$\alpha^{-/-}$ anti-IL-10R	***	***	***
IL-4Rα × IL-10 genetic cross					
+/−, +/+	vs	+/−, +/−	NS	NS	NS
+/−, +/+	vs	−/−, +/+	NS	NS	NS
+/−, +/+	vs	+/−, −/−	***	*	NS
+/−, +/+	vs	IL-10$^{-/-}$	**	***	**
+/−, +/+	vs	−/−, −/−	***	***	***
−/−, +/+	vs	−/−, +/−	NS	NS	NS
−/−, +/+	vs	IL-10$^{-/-}$	**	***	**
−/−, +/+	vs	−/−, −/−	***	***	***
−/−, +/−	vs	−/−, −/−	**	***	***
IL-10$^{-/-}$	vs	−/−, −/−	*	*	***

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

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deficiency is a sufficient condition to alter the normal susceptibility phenotype, and whether it would promote even greater resistance in IL-4R$\alpha^{-/-}$ mice. As shown in Fig. 6*A*, lesions from BALB/c mice contained an average of ~7 million IR173 pms/mg tissue compared with ~3000 pms/mg in the IL-4R$\alpha^{-/-}$ mice, although the parasite loads were highly variable in this group. After weekly injections of anti-IL-10R mAb, the parasite load was reduced 300-fold in the BALB/c mice (24,000 pms/mg) while the numbers in IL-4R$\alpha^{-/-}$ mice were further reduced to undetectable levels in five of seven animals. In the IL-10$^{-/-}$ mice, three of seven animals had a striking reduction in parasite burden, and on the average, there was a significant 70-fold reduction.

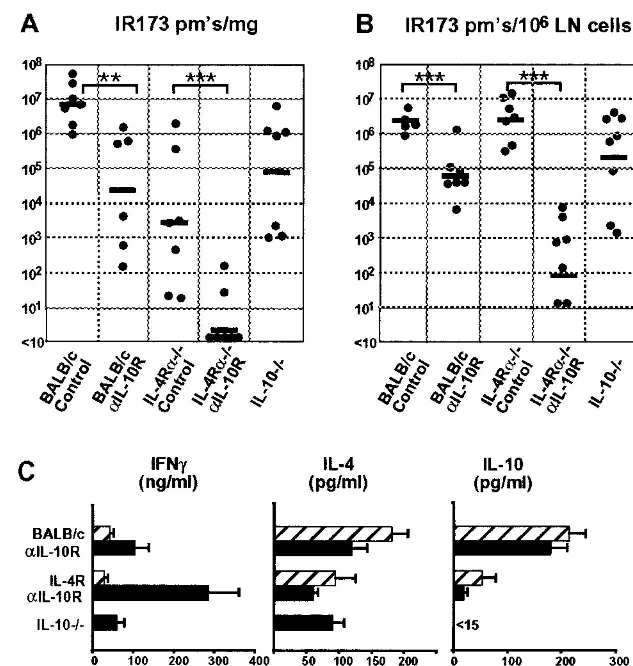

FIGURE 6. Anti-IL-10R mAb treatment resolves IR173 parasite growth in IL-4R$\alpha^{-/-}$ mice. *A*, The mice were infected with 10^5 *L. major* IR173 pms in the left hind footpad, and lesions were quantitated for IR173 pms per milligram of tissue (*A*), or LN cells were removed at 48 h and assayed for IFN-γ, IL-4, and IL-10 by ELISA (*B*). Asterisks indicate significant differences by Student's *t* test (**, $p < 0.01$; ***, $p < 0.001$).

Interestingly, the number of IR173 parasites at the LN site (Fig. 6*B*) was not altered in the IL-4Rα-deficient mice, which is consistent with our previous results (9). Anti-IL-10R treatment, however, led to a significant decrease in IR173 parasite number in the LN of both BALB/c and IL-4R$\alpha^{-/-}$ mice (Fig. 6*B*). Thus, even when a resistant phenotype seems firmly established in terms of lesion development, the full potential of host resistance is not realized unless IL-4/IL-13 and IL-10 are inhibited and may not be appreciated unless parasite burdens in the lesion and disseminating sites are carefully compared.

Anti-IL-10R treatment also induced a striking increase in IFN-γ levels, particularly in IL-4R$\alpha^{-/-}$ mice (Fig. 6*C*). These data suggest that in contrast to IL-4R signaling, inhibition of IL-10 activity alone is sufficient to alter the disease outcome following infection with either LV39 or IR173 *L. major* substrains, perhaps because of its common effect on the IFN-γ response.

L. major *substrain LV39 is more resistant to killing by activated macrophages*

In an attempt to address the differences between LV39 and IR173 infections in IL-4R$\alpha^{-/-}$ mice, we explored the differential sensitivities of LV39 and IR173 amastigotes to intracellular killing by macrophages activated by IFN-γ in vitro (Fig. 7). Peritoneal macrophages from BALB/c and IL-4R$\alpha^{-/-}$ mice with established intracellular infections with either LV39 or IR173 were treated with LPS and increasing amounts of IFN-γ. In these experiments the *L. major* Freidlin V1 was included as another substrain that is controlled in IL-4R$\alpha^{-/-}$ mice (Fig. 1). The number of remaining parasites and NO activity were measured after 4 days. As shown in Fig. 7, the LV39-infected BALB/c macrophages required a 500-fold greater concentration of exogenous IFN-γ to clear 50% of intracellular parasites compared with IR173, which were, in turn, more resistant to killing than Friedlin clone V1. The LV39-infected IL-4R$\alpha^{-/-}$ macrophages required ~25-fold more IFN-γ to achieve a similar percent killing. The NO_2/NO_3 levels were equivalent in both in vitro infections, and IL-10 was below the detection threshold in all groups (data not shown).

Discussion

There is abundant evidence that a predominant Th2 response and secretion of type 2 cytokines determine susceptibility to *L. major* (2), and *L. major* infection in BALB/c mice remains one of the

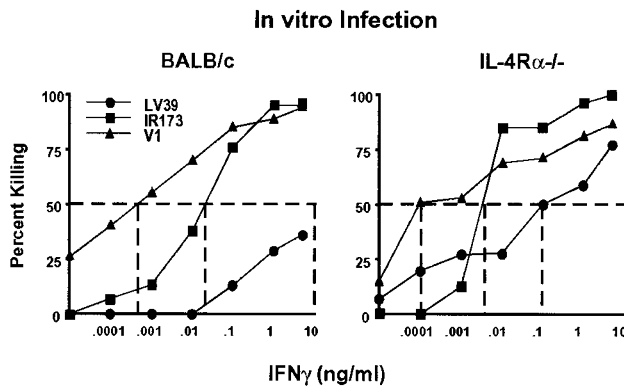

FIGURE 7. Macrophages infected with *L. major* LV39 pms are more resistant to IFN-γ-mediated killing than IR173- or V1 (Friedlin)-infected macrophages. Peritoneal macrophages were isolated from BALB/c or IL-4R$\alpha^{-/-}$ mice and infected with *L. major* substrains LV39, IR173, and V1 as described in *Materials and Methods*. After 72 h, 0.1 μg/ml of LPS and increasing concentrations of recombinant mouse IFN-γ were added and cultured for an additional 72 h. The numbers of infected and noninfected cells and total amastigotes were counted, and the percent killing relative to untreated macrophages was calculated on the basis of comparison of total amastigotes per 100 macrophages.

most popular models to explore the factors controlling Th2 development in vivo. While much of the work has focused on IL-4, roles for IL-13 and IL-10 have recently been reported (12, 13, 20). In previous studies we found that, depending on the *L. major* substrain, IL-4 KO mice and even IL-4R$\alpha^{-/-}$ mice deficient in both IL-4 and IL-13 activities remained fully susceptible to *L. major*. Thus, under certain conditions IL-4R signaling is not required for and may not even contribute to Th2 polarization and *L. major* susceptibility in vivo, challenging one of the basic tenets of the Th1/Th2 subset differentiation scheme. In studies designed both to identify the key susceptibility determinant in these mice and to reconcile the conflicting data that have accrued in the *L. major*-BALB/c model, genetic or applied defects in IL-10 were superimposed on IL-4R$\alpha^{-/-}$ mice, which were challenged with *L. major* substrains that differ dramatically in their sensitivity to the effects of defective IL-4/IL-13 activities. In this study we identified IL-10 as the key susceptibility factor in LV39-infected IL-4R$\alpha^{-/-}$ BALB/c mice. We furthermore determined that regardless of substrain, both IL-4R signaling and IL-10 contribute to susceptibility, but that in relative terms IL-10 appears to exert the more profound effect on promoting parasite growth and disease.

The evidence that IL-10 is the additional factor controlling susceptibility in LV39-infected IL-4R$\alpha^{-/-}$ mice was obtained from studies in IL-4R$\alpha^{-/-}$ mice treated with IL-10R mAb, and in double-KO mice homozygous for deletions in both IL-4Rα and IL-10, which in each case displayed a resistance phenotype comparable to that of C57BL/6 mice. The results further imply that there is a cumulative effect of IL-4/IL-13 and IL-10, since neither IL-10$^{-/-}$ mice nor wild-type mice treated with anti-IL-10R mAb approached the striking level of resistance displayed by IL-4Rα mice that were also deficient in IL-10 or IL-10R signaling. In infections involving *L. major* substrain IR173, in which the absence of IL-4Rα was sufficient to control these parasites, anti-IL-10R treatment caused a further and again striking reduction in the number of parasites in the lesion and draining LN. The idea that IL-4 might act in concert with IL-13 to produce an additive effect on *L. major* susceptibility has already been supported (20), and the current data extend this concept as it applies to IL-10 and to the cumulative effect of three disease-promoting cytokines.

The disparate outcomes in LV39- vs IR173-infected IL-4$^{-/-}$ or IL-4R$\alpha^{-/-}$ mice initially suggested that distinct susceptibility factors are somehow induced by and act on these closely related pathogens. On closer examination, however, both *L. major* substrains appear to be influenced by the same set of conditions. The strain differences in this view are due to the different thresholds of type 2 cytokines required to inhibit the induction or expression of immunity, with the absence of IL-4, IL-13, or IL-10 alone sufficient to alter the balance in favor of control of IR173, whereas for LV39 the absence of IL-4 and IL-13 requires a complementary defect in IL-10 for the normal susceptibility phenotype to be reversed. For both strains, however, the absence of all three cytokines seems necessary for the expression of a fully resistant phenotype. These data illustrate the utility of a careful measure of parasitic load and of parallel comparison with C57BL/6 mice to refine the concept of resistance as it applies to disease outcome in immune-manipulated BALB/c mice.

An important aspect of these findings is that in contrast to IL-4R$\alpha^{-/-}$ mice, single-KO, IL-10-deficient mice or wild-type mice treated with anti-IL-10R mAb were more resistant to either *L. major* substrain. The ability of IL-10 KO BALB/c mice to control *L. major* Friedlin strain has been reported (13). We suggest that the more consistent ability of IL-10 inhibition to reverse BALB/c susceptibility is related to the finding that in contrast to IL-4$^{-/-}$ or IL-4R$\alpha^{-/-}$ mice, the IFN-γ response in IL-10$^{-/-}$ mice or anti-IL-10R mAb-treated mice was markedly enhanced (Figs. 3*B*, 4*C*, and 6*B*). The increased amount of IFN-γ in conjunction with the absence or defective function of such a potent deactivating cytokine would be more apt to shift the balance in favor of an ability of macrophages to kill. Why, then, was a complementary defect in IL-10 not necessary for IL-4$^{-/-}$ and IL-4R$\alpha^{-/-}$ to control IR173? While IL-10 and IFN-γ secretion by LN cells were comparable between LV39 and IR173 infections, it is possible that IFN-γ was up-regulated at the site of IR173 infection, or conversely that IL-10 was up-regulated at the site of LV39 infection. These possibilities aside, we have preliminary in vitro evidence to suggest that the *L. major* strains have intrinsic differences in their susceptibility to killing by immune-activated macrophages that may account, at least in part, for their different infection outcomes in IL-4$^{-/-}$ and IL-4R$\alpha^{-/-}$ mice. Macrophages infected with IR173 or Friedlin V1 required 25- to 500-fold lower concentrations of IFN-γ to achieve the same level of parasite killing as LV39 (Fig. 7). From these results we propose that in vivo in the absence of deactivating signals provided by IL-4 and IL-13, control of IR173 or V1 is achieved even without a significant up-regulation of IFN-γ. In addition, the levels of IL-10 produced by LN cells was significantly reduced in IL-4R$\alpha^{-/-}$ mice, which may have contributed to their enhanced resistance to these *L. major* strains. Nevertheless, complete ablation of IL-10 function by gene deletion or receptor blockade was required to release IFN-γ from suppression and to promote killing of the more NO-resistant LV39 amastigotes.

IL-10 is mainly produced by Th2 ($CD4^+$ T cells) cells, but can also be produced by B cells, macrophages, thymocytes, keratinocytes, and activated mast cell lines (11). Based on the evidence that both $CD4^+$ T cell depletion and anti-IL-10R treatments up-regulated IFN-γ and induced healing in LV39-infected IL-4R$\alpha^{-/-}$ mice, we assume that the primary source of IL-10 in vivo is $CD4^+$ T cells. Furthermore, the fact that transient anti-CD4 treatment conferred a strong resistant phenotype in LV39-infected, wild-type BALB/c mice is consistent with an effect of global depletion of Th2 cytokines, including IL-10. Nonetheless, since anti-CD4 treatment of IL-4R$\alpha^{-/-}$ mice did not achieve quite the same level of resistance as anti-IL-10R treatment, it is likely that additional sources of IL-10 exist in these mice. Kane and Mosser (13) have

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shown that IL-10 may be induced from macrophages via FcR triggering after culture with LPS and *L. major* Friedlin amastigotes. In our in vitro culture system we found no evidence for IL-10 production by peritoneal macrophages infected with amastigotes alone from substrains LV39, IR173, and clone V1 of Friedlin (Fig. 7).

The generation of a dominant Th2 response in the absence of IL-4R signaling challenges an early postulate regarding the instructional role of IL-4 in Th2 development. It is now clear, however, that IL-4Rα/STAT6 signaling is not essential for priming of $CD4^+$ T cells to produce Th2 cytokines in vivo, since in $STAT6^{-/-}$ or IL-4R$\alpha^{-/-}$ mice Th2 responses are decreased, but significant levels of IL-4 and other Th2-related cytokines are still present (21–24). The conclusion that IL-10 produced by $CD4^+$ T cells is as important as IL-4, if not more so, in the evolution of susceptibility to *L. major* infection in BALB/c mice raises the question of whether these cytokines are secreted by the same $CD4^+$ T cells or produced by discreet subpopulations that are activated by unique priming environments. It is especially important to consider the possible role of IL-10 produced by $CD4^+CD25^+$ $CD45RB^{low}$ regulatory T cells, as these have recently been shown to be activated in resistant C57BL/6 mice and to suppress the ability of $CD4^+CD25^-CD45RB^{high}$ cells to effect sterile cure (25). Consistent with their suppressive role in *L. major*-infected BALB/c mice, removal of the $CD4^+CD45RB^{low}$ subset from splenic cells resulted in the transfer of immunity to *L. major*-infected *scid* mice (26). In contrast, anti-CD25 treatment was recently found to exacerbate *L. major* infection in BALB/c mice (27), suggesting that while regulatory T cells may be activated by *L. major*, they are not responsible for susceptibility, but actually suppress the Th2 effector cells that are.

Finally, to what extent do the multiple factors controlling *L. major* susceptibility in BALB/c mice also operate in nonhealing and/or systemic forms of human disease? In this context, overproduction of IL-10 has been detected in lesional tissue from kala-azar patients (28–30) in chronic cutaneous lesions (31), and in plasma from patients with post-kala-azar dermal leishmaniasis (32) and seems to provide a much better correlate than IL-4. Whether $CD4^+$ T cells produced the IL-10 in these clinical settings has not been adequately addressed.

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