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Mice with *STAT6*-Targeted Gene Disruption Develop a Th1 Response and Control Cutaneous Leishmaniasis[1]

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The cutaneous growth of *Leishmania mexicana* was measured in STAT6-deficient mice ($STAT6^{-/-}$) and compared with that in similarly infected wild-type ($STAT6^{+/+}$) mice. Following s.c. inoculation with 5×10^6 amastigotes of *L. mexicana* into the shaven rump, $STAT6^{+/+}$ mice developed large, nonhealing cutaneous lesions, while $STAT6^{-/-}$ mice failed to develop detectable lesions during most of the course of study. As infection progressed, $STAT6^{+/+}$ mice infected with *L. mexicana* displayed significantly higher titers of *Leishmania*-specific IgG1 and IgE compared with $STAT6^{-/-}$ mice, which conversely produced significantly higher titers of *Leishmania*-specific IgG2a, indicating development of a Th1-like response in the latter group. At 12 wk postinfection, *Leishmania* Ag-stimulated lymph node cells from $STAT6^{-/-}$ mice produced significantly higher amounts of IL-12 and IFN-γ than those from $STAT6^{+/+}$ mice as measured by ELISA. However, there was no significant difference in IL-4 production between the two groups. Semiquantitative RT-PCR of transcript levels in intact draining lymph nodes and skin from inoculation sites confirmed a similar pattern of cytokines in vivo as that observed in stimulated lymph node cells in vitro. These results indicate that STAT6-mediated IL-4 signaling is critical for progression of *L. mexicana* infection in genetically susceptible mice and demonstrate that in the absence of STAT6, susceptible mice default toward a Th1-like response and control cutaneous *L. mexicana* infection. ***The Journal of Immunology*, 1998, 161: 6180–6188.**

The leishmaniases comprise a group of diseases caused by the intracellular protozoan parasite *Leishmania*. In humans, cutaneous *Leishmania major* infection commonly manifests as a localized self-healing skin lesion, whereas the localized cutaneous infection caused by *Leishmania mexicana* is often associated with chronic infection of the ear pinna (1). While the majority of mouse strains resolve lesions following cutaneous *L. major* infection, almost all strains develop nonhealing lesions when infected with *L. mexicana* (2). This may be due in part to the parasite species initiating infection, since *L. mexicana* has been shown to be under different genetic and immunoregulatory controls than those associated with *L. major* (3). However, genetically controlled immunoregulatory factors operating within an individual mouse strain may also influence cutaneous lesion growth (2).

Many studies have indicated that control of *L. major* lesion growth in genetically resistant mice such as C3H/HeN and C57BL/6 strains is associated with the expansion of the $CD4^+$ Th1 cell subset and the production of cytokines such as IL-12, IFN-γ, and IL-2 (4, 5). On the other hand, nonhealing responses in susceptible BALB/c mice have been related to the expansion of the $CD4^+$ Th2 cell subset and the production of cytokines such as IL-4 and IL-10 (4, 5). The disease-exacerbating role of IL-4 has been shown to be due to its ability to inhibit macrophage leishmanicidal activity and down-regulate the development of a Th1-like response (6, 7). This has been evident in studies that demonstrated that genetically susceptible mice lacking IL-4 are protected from cutaneous infection with *L. major* (8) as well as *L. mexicana* (9, 10). However, other studies suggest that the inability of the host to generate an IL-12-initiated Th1-like response and produce IFN-γ rather than the induction of a Th2-like response and IL-4 production may be the crucial factor in determining susceptibility to *L. major* (11), *Leishmania amazonensis* (12), *L. mexicana* (13), and *Leishmania panamensis* (13).

IL-4 signals through its receptor using two pathways (14). One pathway responsible for IL-4-mediated growth involves phosphorylation of IL-4-induced phosphotyrosine substrate (also termed insulin receptor substrate-2 (IRS-2)[3]) or the antigenically related IRS-1 and its association with phosphatidylinositol 3-kinase (14, 15). The other pathway responsible for IL-4-mediated differentiation events involves phosphorylation of Janus kinases JAK1 and JAK3 and subsequent activation of STAT6, a signal transducer and activator of transcription (14, 16). Recent studies show that STAT6 is essential in the IL-4 signaling mechanism for development to the Th2 subset (17, 18). Furthermore, IL-13, a cytokine closely related to IL-4 in biologic function (19), has been shown to share receptor components with IL-4 (20) and also signal through the STAT6 pathway (21).

The purpose of this study was to explore the role of STAT6-mediated IL-4 signaling in cutaneous lesion formation and in the immune response following *L. mexicana* infection. To approach this question, we compared cutaneous lesion growth following local *L. mexicana* inoculation in C57BL/6 × 129/Sv mice homozygous for the disrupted STAT6 gene ($STAT6^{-/-}$) with that in wild-type ($STAT6^{+/+}$) counterparts of matched age and sex. In addition, we analyzed Ab profiles in sera and cytokine responses in the draining lymph nodes and skin from inoculation sites in *L. mexicana*-infected $STAT6^{+/+}$ and $STAT6^{-/-}$ mice.

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[3] Abbreviations used in this paper: IRS-2, insulin receptor substrate-2; LmAg, *Leishmania mexicana* antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Materials and Methods

Animals

Breeding pairs of *STAT6*−/− (C57BL/6 × 129/Sv) mice generated by gene disruption, as described previously (22), were provided by Dr. James Ihle (St. Jude Children's Research Hospital, Memphis, TN). The mice were bred and maintained in the facility at the Harvard School of Public Health (Boston, MA) according to the guidelines for animal research. Wild-type $STAT6^{+/+}$ of the same strain combination, age, and sex were used as controls in all experiments.

Parasites

L. mexicana parasites (MYNC/BZ62/M379) obtained from Dr. James Alexander (University of Strathclyde, Glasgow, U.K.) were maintained in the shaven rumps of BALB/c mice. Amastigotes for use in experimental studies were isolated from lesions and enumerated using a Neuebauer hemocytometer (Reichert-Jung, Horsham, PA) as previously described (23).

Infection

Eight- to 12-week-old, sex-matched $STAT6^{+/+}$ and $STAT6^{-/-}$ mice were infected with 5×10^6 amastigotes of *L. mexicana* by s.c. inoculation into the shaven rump. Disease progression was monitored by measuring lesion diameter at 2-wk intervals up to 12 wk postinfection. At this time, lesions from $STAT6^{+/+}$ mice and inoculation sites from $STAT6^{-/-}$ mice were excised, and the histopathology was examined using hematoxylin and eosin stains on paraffin sections.

Preparation of soluble L. mexicana *Ag*

Soluble *L. mexicana* Ag (LmAg) for use in ELISA and T cell proliferation assays was prepared from stationary phase promastigotes of *L. mexicana.* Promastigotes were washed twice in ice-cold PBS and resuspended in a hypotonic buffer consisting of 10 mM Tris-HCl and 2 mM EDTA, pH 7.8. Following a 20-min incubation on ice, the promastigotes were disrupted using a sonicator and then were centrifuged for 30 min at 10,000 × *g* at 4°C. The protein concentration was determined using a Bradford assay (24).

Leishmania-*specific ELISA*

Peripheral blood was collected at 2-wk intervals from tail snips of the *L. mexicana*-infected $STAT6^{+/+}$ and $STAT6^{-/-}$ mice. Blood was centrifuged at 200 × *g*, and serum was collected and tested for specific Ab content. The *Leishmania*-specific levels of the Th2-associated Ab IgG1 and the Th1-associated Ab IgG2a (25) were measured by ELISA as described previously (10). Briefly, each well of a polystyrene microtiter plate (Corning, Corning, NY) was coated with 0.5 μg of LmAg in PBS, pH 9.4, by overnight incubation at 4°C. Plates were washed with PBS and 0.05% Tween 20 (PBS/Tween 20; Sigma, St. Louis, MO) and were blocked with nonfat powder milk for 1 h at 37°C. Serially diluted serum samples (1/100 starting dilution in PBS/Tween 20) were added to the plates and incubated for 2 h at 37°C. Bound Abs were detected by incubation with either goat anti-mouse IgG1 or goat anti-mouse IgG2a horseradish peroxidase conjugate (1/5000 dilution in 25% goat serum and 75% PBS; Southern Biotechnologies, Birmingham, AL). After a further 1-h incubation at 37°C, H_2O_2 substrate solution (Kirkegaard & Perry, Gaithersburg, MD) was added to the plates. The reaction was stopped by 5% phosphoric acid after approximately 10 min, and the A_{450} was read on a microplate reader (Molecular Devices, Menlo Park, CA).

For determining *Leishmania*-specific and total serum levels of the Th2-associated Ab IgE (26), flat-bottom Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 0.5 μg/well of rat anti-mouse IgE mAb (clone 4818-01R; BioSource, Camarillo, CA) diluted in carbonate buffer, pH 9.6. The plates were washed four times with PBS/Tween 20 and were blocked with 10% FCS in PBS/Tween 20 incubated for 2 h at 37°C. After washing, 100 μl of diluted serum samples and purified mouse IgE standards (2 μg/ml starting concentration; clone 27-74; PharMingen, San Diego, CA) were added to the plates in duplicate and incubated overnight at 4°C. Plates were washed three times, and 0.05 μg of biotinylated anti-mouse IgE mAb (clone R35-72; PharMingen, San Diego, CA) was added per well to determine total serum IgE. Similarly, 0.01 μg of biotinylated LmAg was added per well to determine *Leishmania*-specific IgE. For biotinylation, LmAg (2 mg/ml) in sodium bicarbonate buffer, pH 8.5, was incubated with biotin (long arm) *N*-hydroxy succinimide ester (Vector, Burlingame, CA) for 2 h at room temperature. The reaction was stopped by addition of 5 μl of ethanolamine, and the complex was dialyzed overnight with PBS/0.05% sodium azide. After a 2-h incubation at 37°C with the appropriate secondary Ab, plates were incubated with 100 μl of streptavidin-peroxidase conjugate (1/1000 dilution in 10% FCS in PBS/Tween 20; Sigma) for 1 h at 37°C. Finally, the plates were washed three times, and H_2O_2 substrate solution (Kirkegaard & Perry) was added. Again, the reaction was stopped by 5% phosphoric acid after approximately 10 min, and the A_{450} was read on a microplate reader (Molecular Devices, Menlo Park, CA).

T cell proliferation assay

T cell proliferation assays were performed as previously described (10). $STAT6^{+/+}$ and $STAT6^{-/-}$ mice were sacrificed at 12 wk postinfection, and the spleens and draining inguinal lymph nodes were removed aseptically. Single cell suspensions were prepared by gentle teasing in RPMI 1640 medium supplemented with 10% FCS (heat inactivated; HyClone, Walkersville, MD), 10,000 U/ml penicillin and 10,000 μg/ml streptomycin (Life Technologies, Grand Island, NY), and 0.05 mM β-ME (Life Technologies). The cells were centrifuged at 200 × *g* for 5 min. Erythrocytes from the spleen were lysed by resuspending cells in Boyle's solution (0.17 M Tris and 0.16 M ammonium chloride). Following two washes, the viable lymphocytes and splenocytes were counted by trypan blue exclusion with a Neuebauer hemocytometer. Lymph node cell suspensions were adjusted to 3×10^6 cells/ml, and spleen cell suspensions were adjusted to 5×10^6 cells/ml. Aliquots (100 μl) of the adjusted cell suspension were added in triplicate to the wells of sterile 96-well flat-bottom tissue culture plates (Costar, Cambridge, MA) containing 100 μl of LmAg (20 μg/ml), Con A (1 μg/ml) as a positive control, or supplemented medium as a negative control. Following incubation at 37°C for 72 h in 5% CO_2, cells were pulsed with 1 μCi of [^{3}H]thymidine and further incubated at 37°C for 12 h. Pulsed cells were harvested onto filter paper (Tomtec, Hamden, CT), and [^{3}H]uptake was measure by liquid scintillation on a beta scintillation counter (Wallac, Gaithersburg, MD). Supernatants were collected from parallel cultures after 72 h of incubation for ELISA quantification of cytokine production (see below).

Cytokine ELISA

IL-12, IFN-γ, and IL-4 production by Con A- and LmAg-stimulated cells and nonstimulated cells from *L. mexicana*-infected $STAT6^{+/+}$ and $STAT6^{-/-}$ mice were measured by capture ELISA as previously described (10). Maxisorp multititer plates (Nunc) were incubated overnight at 4°C with 2 μg/ml of capture mAb (rat anti-mouse IL-12, clone C15.6, or rat anti-mouse IFN-γ, clone R4-6A2 (both from PharMingen); or rat anti-mouse IL-4, clone 1D11 (from Endogen, Cambridge, MA)) in PBS, pH 9.0. The plates were blocked with 10% FCS in PBS, pH 7.4, for 1 h at 37°C, after which murine recombinant standards of IL-12 (0–10 ng/ml; PharMingen), IFN-γ (0–30.0 ng/ml; PharMingen), or IL-4 (0–1.5 ng/ml; Endogen) and the cultured supernatant samples were added in duplicate and incubated overnight at 4°C. The plates were washed three times in PBS/Tween 20 and incubated for 1 h at 37°C with 1 μg/ml biotinylated anti-IL-12 (clone C17.8; PharMingen), anti-IFN-γ (clone XMG1.2; PharMingen), or anti-IL-4 (clone 24G2; Endogen). To detect the biotinylated Abs, streptavidin-linked alkaline phosphatase (1/5000 dilution in 10% FCS in PBS; PharMingen) was added after washing and incubated for 45 min in the dark at 37°C. After a final washing in PBS/Tween 20, 100 μl of *p*-nitrophenylphosphatase substrate (Sigma) in glycine buffer was added to each well. The A_{405} values of the plates were measured on a microplate reader (Molecular Devices), and the concentration of the samples was calculated using the standard curve.

RT-PCR

Total cellular RNA was extracted from snap-frozen lymph nodes and skin sections from $STAT6^{+/+}$ and $STAT6^{-/-}$ mice using RNAzol (Tel-Test, Friendswood, TX). The quality and the quantity of the RNA were confirmed by running 2.0 μg on formaldehyde gels. cDNA synthesis using 2.5 μg of RNA was performed according to the manufacturer's protocol (Super Amp II system cDNA kit, Life Technologies). A published RT-PCR technique was used to measure relative differences in transcript levels of IL-12, IL-2, IFN-γ, TNF-α, IL-1β, TGF-β, IL-10, and IL-4 against levels of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (27–29). The GeneAmp 9600 system was used to establish logarithmic ranges of PCR amplification as a function of cycle number and cDNA dilution, and the hot start technique was used to increase specificity (30). Reaction conditions included 1.25 μl of cDNA, 1 μM of each 5′ and 3′ primer, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 800 μM deoxynucleotide triphosphates (200 μM of each), and 0.625 U of AmpliTaq DNA polymerase in a total volume of 25 μl. [^{32}P]dCTP (150,000 cpm)

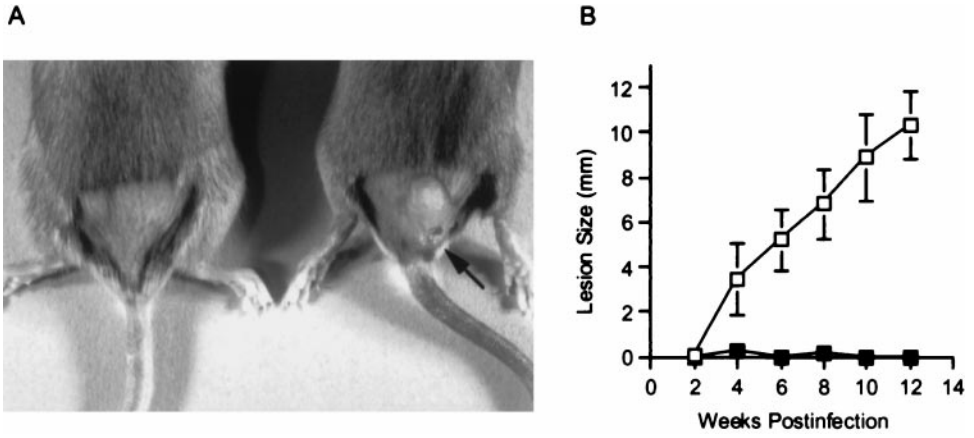

FIGURE 1. Course of *L. mexicana* infection following infection with 5×10^6 amastigotes in $STAT6^{+/+}$ and $STAT6^{-/-}$ mice. *A*, The visual difference in cutaneous lesion growth between $STAT6^{+/+}$ and $STAT6^{-/-}$ mice is demonstrated at 12 wk postinfection. *B*, Progression of lesion growth was measured by mean lesion diameter on the shaven rump of the infected $STAT6^{+/+}$ (open symbol) and $STAT6^{-/-}$ (closed symbol) mice. Results are representative of four experiments with three to five animals per group. Data are expressed as the mean ± SE.

was included for quantitative PCR studies. The thermal cycling parameters were denaturation at 94°C for 15 s, annealing at 50–70°C for 20 s, and extension at 72°C for 60 s (with a final extension of 7 min at the end of all cycles). For IFN-γ and IL-4 primers the touchdown PCR technique (28) was employed, with a gradual decrease in the annealing temperature from 70 to 60°C over 10 cycles and an additional 22 or 28 cycles, respectively, with a subsequent annealing temperature of 56°C. Accession numbers, primer sequences, annealing temperatures, and number of cycles were previously reported (28, 29) or were as follows: GAPDH (M32599): sense, 5′-CAT CAA GAA GGT GGT GAA GCA GGC; antisense, 5′-TTG TGA GGG AGA TGC TCA GTG TTG G (56°C, 23 cycles); IL-1β (M13177): sense, 5′-TAA TGG TGG ACC GCA ACA ACG C; antisense, 5′-TCC CAG ACA GAA GTT GGC ATG GTA G (55°C, 28 cycles); and TGF-β (L03799): sense, 5′-TTA CTG CTA TGG ACA AGG CAC GGG; antisense, 5′-ATT GAG GGC AAG ACG TGT ACG AGT G (56°C, 28 cycles).

PCR products (10 μl) were separated on 1–2% agarose gels, and incorporation of [^{32}P]dCTP into PCR product bands was quantified from dried gels on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), as previously described (27). PCR amplification with the GAPDH reference gene was performed to assess variations in cDNA or total RNA loading between samples. Normalized values were derived by dividing the mean of the triplicate ^{32}P values measured for the transcript of interest by the mean of triplicate GAPDH values for the sample. Mean relative transcript levels per group were then determined from cDNA panels that included a negative control in which water was used for the PCR instead of cDNA.

Statistical significance

Student's unpaired *t* test was used to determine the statistical significance of the values obtained. Differences in Ab endpoint titers were determined using the Mann-Whitney *U* prime test.

Results

Growth of cutaneous lesions following L. mexicana *infection in* STAT6$^{+/+}$ *and* STAT6$^{-/-}$ *mice*

Following s.c. inoculation of 5×10^6 *L. mexicana* amastigotes, $STAT6^{+/+}$ mice developed progressive nonhealing lesions, reaching a size of almost 1 cm in diameter (Fig. 1, *A* and *B*). In sharp contrast, $STAT6^{-/-}$ mice developed either no lesions during the period of study or slight lesions that were completely healed by 12 wk postinfection (Fig. 1, *A* and *B*). Similar results were obtained in five separate sets of experiments.

Analysis of the histopathology of inoculation sites in L. mexicana*-infected* STAT6$^{+/+}$ *and* STAT6$^{-/-}$ *mice*

At 12 wk postinfection, skin lesions from $STAT6^{+/+}$ mice demonstrated extensive s.c. tissue destruction with diffuse inflammatory infiltrate consisting of heavily parasitized macrophages, eosinophils, and lymphocytes (Fig. 2*A*). On the other hand, skin from the inoculation sites of $STAT6^{-/-}$ mice at the same time point displayed preserved skin structure with some inflammatory foci comprised primarily of lymphocytes and macrophages and only a few parasites (Fig. 2*B*).

Ab response to L. mexicana *in* STAT6$^{+/+}$ *and* STAT6$^{-/-}$ *mice*

Serum IgG1, IgG2a, and IgE levels were determined in *L. mexicana*-infected $STAT6^{+/+}$ and $STAT6^{-/-}$ mice at 4, 6, 8, and 10 wk

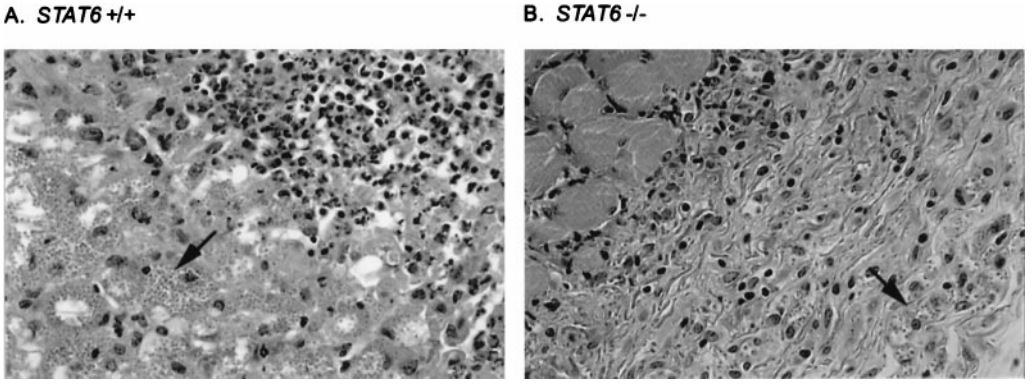

FIGURE 2. Analysis of the histopathology of skin inoculation sites from *L. mexicana*-infected $STAT6^{+/+}$ and $STAT6^{-/-}$ mice at 12 wk postinfection. *A*, Hematoxylin-eosin-stained skin lesions from $STAT6^{+/+}$ mice showed extensive tissue destruction with inflammatory infiltrate comprising of parasitized macrophages, neutrophils, eosinophils, and lymphocytes. *B*, Similarly stained skin from the inoculation sites of $STAT6^{-/-}$ mice displayed a more preserved skin structure, with lymphocytes and some macrophages with few intracellular parasites. Arrows indicate *L. mexicana* amastigotes. Original magnification, ×125.

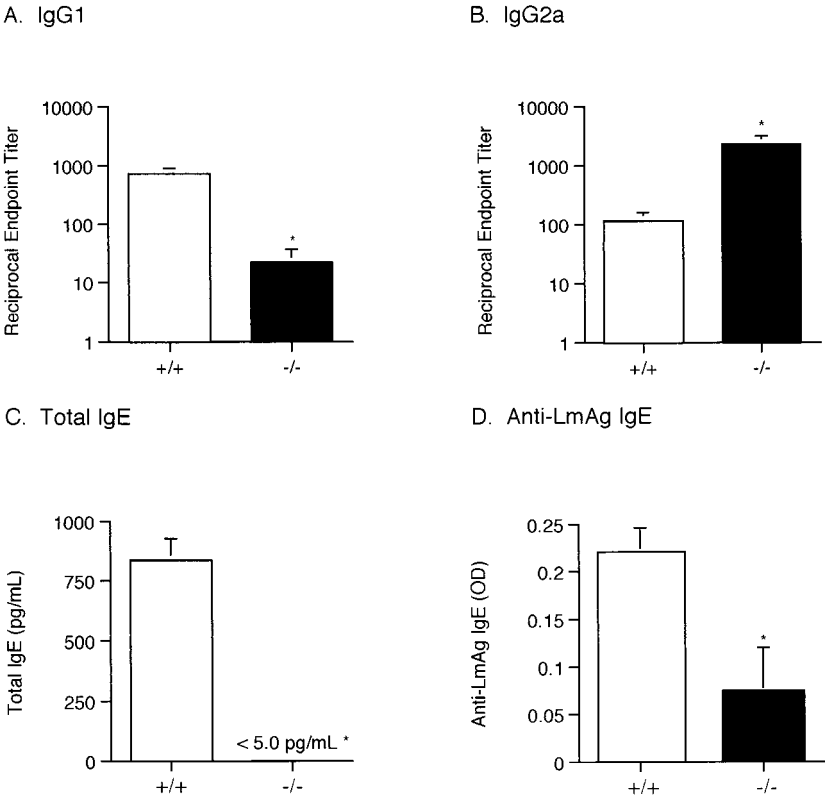

FIGURE 3. Ab profiles of *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice at 8 wk postinfection. *A* and *B*, *L. mexicana*-specific IgG1 and IgG2a production in *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice at 8 wk postinfection is presented as reciprocal end-point titers on a log scale. The bar graph shows the mean ($n = 8$ animals) of two separate experiments ($n = 3$ and $n = 5$, respectively). *C*, Total IgE production was measured (picograms per milliliter) in sera of *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice. Six animals were analyzed in each group. *D*, *L. mexicana*-specific IgE production in *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice was measured as the OD at A_{405}. Six animals were analyzed in each group. Data are expressed as the mean ± SE. Asterisks indicate statistically significant differences between groups ($p < 0.05$). Similar results were found at 4, 6, and 10 wk postinfection.

postinfection. Similar results were found at all time points. Ab data from serum collected at 8 wk postinfection are shown and are representative of the results (Fig. 3). At 8 wk postinfection, the *STAT6*$^{-/-}$ mice produced two-log fold less IgG1 compared with wild-type *STAT6*$^{+/+}$ mice ($p < 0.001$; Fig. 3*A*). Furthermore, the *STAT6*$^{-/-}$ mice produced significantly lower levels of total IgE ($p < 0.0005$; Fig. 3*C*) and threefold less *L. mexicana*-specific IgE ($p < 0.0005$; Fig. 3*D*) compared with *STAT6*$^{+/+}$ mice. Conversely, the *STAT6*$^{-/-}$ mice produced nearly two-log fold higher IgG2a titers than *STAT6*$^{+/+}$ mice ($p < 0.002$; Fig. 3*B*).

Analysis of IL-12, IFN-γ, and IL-4 production in vitro by LmAg-stimulated lymph node cells from STAT6$^{+/+}$ *and* STAT6$^{-/-}$ *mice following* L. mexicana *infection*

At 12 wk postinfection, lymph node cells from *L. mexicana*-infected *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice displayed significant LmAg-induced proliferative responses. There was no significant difference in LmAg-specific proliferation of lymph node cells between the two groups ($p < 0.375$), indicating that differences in cytokine levels in vitro measured by ELISA were not reflective of a higher proliferative response of one group over the other (data not shown).

The supernatants from the LmAg-stimulated lymphocyte proliferation assays were analyzed by ELISA for the Th1-like cytokines IL-12 and IFN-γ, and the Th2-like cytokine IL-4. LmAg-stimulated lymphocytes from *STAT6*$^{-/-}$ mice produced over fivefold higher levels of IL-12 protein than *STAT6*$^{+/+}$ mice ($p < 0.005$; Fig. 4*A*). Furthermore, the unstimulated production of IL-12 from lymphocytes of the *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice was not significantly different from that of the LmAg-stimulated lymphocytes of each group, indicating comparable constitutive secretion ($p > 0.375$ and $p > 0.10$, respectively). On the other hand, unstimulated lymphocytes from both *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice produced undetectable levels of IFN-γ. LmAg-stimulated lymphocytes of

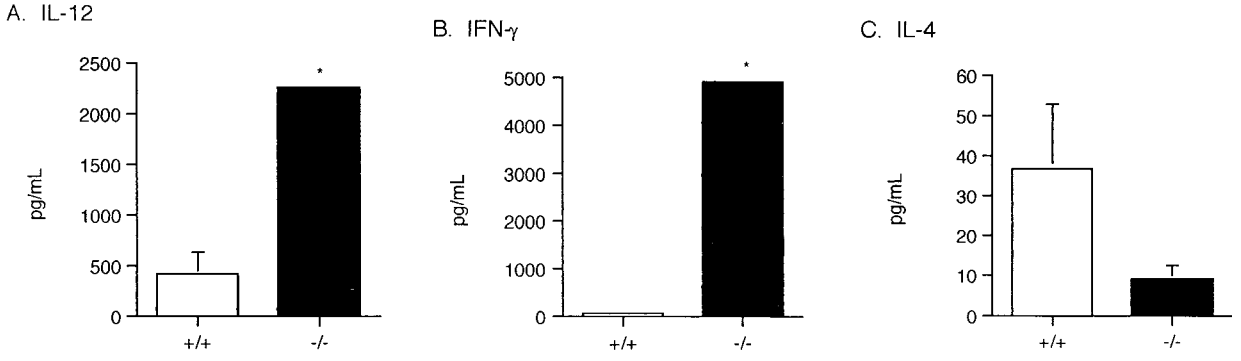

FIGURE 4. In vitro cytokine production of LmAg-stimulated lymphocytes from *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice. In vitro LmAg-induced (20 μg/ml) IL-12 (*A*), IFN-γ (*B*), and IL-4 (*C*) production by lymphocytes in *STAT6*$^{+/+}$ (white columns) and *STAT6*$^{-/-}$ (black columns) mice was measured at 12 wk postinfection. Three to six animals were analyzed in each group. Data are expressed as the mean ± SE. Asterisks indicate statistically significant differences between groups ($p < 0.05$).

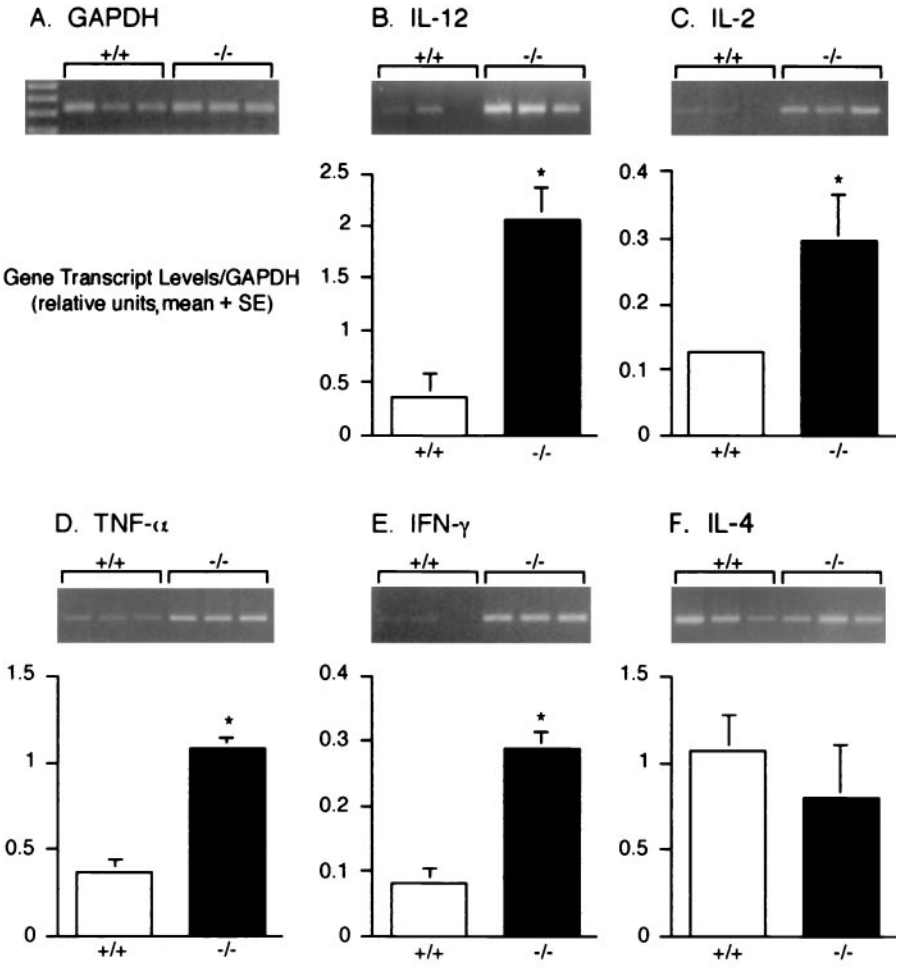

FIGURE 5. In vivo transcript analysis by RT-PCR in intact lymph nodes of *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice at 12 wk postinfection. Three animals were analyzed in each group. *A*, The reference gene, GAPDH, was used to assess variation between RNA and cDNA loading. Results for IL-12 (*B*), IL-2 (*C*), TNF-α (*D*), IFN-γ (*E*), and IL-4 (*F*) are shown. Agarose gel (1.5%) stained with ethidium bromide was used to visualize PCR products. Representative gels for each gene are shown. In all cases the first three columns are the products from *STAT6*$^{+/+}$ mice, and the last three columns are the products from *STAT6*$^{-/-}$ mice. The intensities of the bands were quantified as incorporated [^{32}P]dCTP on a PhosphorImager. The bar graph shows normalized values for each gene in *STAT6*$^{+/+}$ (white columns) and *STAT6*$^{-/-}$ (black columns) mice derived by taking the ratio of the mean of triplicate values for each animal. Data are expressed as the mean ± SE. Asterisks indicate statistically significant differences between groups ($p < 0.05$).

the *STAT6*$^{-/-}$ mice produced significantly higher levels of IFN-γ than those of the similarly infected *STAT6*$^{+/+}$ mice ($p < 0.005$; Fig. 4*B*). Although the LmAg-stimulated lymph node cells from the *STAT6*$^{+/+}$ mice produced higher quantities of the IL-4, on the average, than those of the *STAT6*$^{-/-}$ mice (Fig. 4*C*), the difference was not statistically significant ($p < 0.10$).

Cytokine production of spleen cells from *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice was also assayed. Analysis of the supernatants from LmAg-stimulated splenocytes displayed similar patterns of cytokine production as those found in lymphocytes from peripheral lymph nodes (IL-12: 0.58 ± 0.15 pg/ml in *STAT6*$^{+/+}$ mice, 1.71 ± 0.43 pg/ml in *STAT6*$^{-/-}$ mice ($p < 0.05$); IFN-γ: 3,681.00 ± 1,862.00 pg/ml in *STAT6*$^{+/+}$ mice, 15,070.00 ± 1,230.00 pg/ml in *STAT6*$^{-/-}$ mice ($p < 0.025$); IL-4: 97.17 ± 50.44 pg/ml in *STAT6*$^{+/+}$ mice, 33.88 ± 33.41 pg/ml in *STAT6*$^{-/-}$ mice ($p < 0.375$)).

Analysis of in vivo cytokine transcript levels in draining lymph nodes and skin from STAT6$^{+/+}$ *and* STAT6$^{-/-}$ *mice infected with* L. mexicana

To determine the in vivo cytokine expression after cutaneous *L. mexicana* infection, total RNA from the draining inguinal lymph nodes and skin from cutaneous inoculation sites was extracted at 12 wk postinfection for semiquantitative RT-PCR analysis. Compared with those from *STAT6*$^{+/+}$ mice, draining lymph nodes from *STAT6*$^{-/-}$ mice had sixfold higher relative transcript levels of IL-12 ($p = 0.009$), over twofold higher relative transcript levels of IL-2 ($p = 0.049$), nearly threefold higher relative transcript levels of TNF-α ($p = 0.002$), and threefold higher relative transcript levels of IFN-γ ($p = 0.004$; Fig. 5, *B*, *C*, *D*, and *E*, respectively). However, both *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice displayed similar levels of IL-4 transcripts in their lymph nodes ($p = 0.500$; Fig. 5*F*). No significant differences were found in comparing the normalized relative transcript levels of IL-1β, TGF-β, and IL-10 in the draining lymph nodes from *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice (data not shown).

To compare the local inflammatory response in skin to the immune response in the draining lymph nodes, we analyzed cytokine transcript levels in skin from the inoculation site. Skin from *L. mexicana*-infected *STAT6*$^{-/-}$ mice had threefold higher levels of IL-12 transcripts ($p = 0.049$), over twofold higher levels of TNF-α transcripts ($p = 0.007$), and eightfold higher levels of IFN-γ transcripts than that from *STAT6*$^{+/+}$ mice ($p = 0.045$; Fig. 6, *B*, *C*, and *D*, respectively). Although relative IL-4 transcript levels in cells from *STAT6*$^{+/+}$ skin lesions appeared to be higher than in cells from *STAT6*$^{-/-}$ skin, the difference did not reach statistical significance when normalized against the reference gene GAPDH ($p = 0.147$; Fig. 6*E*). In addition, there was no significant difference in the relative gene transcript levels of IL-1β, TGF-β, and IL-10 in cells from the skin of *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice (data not shown). Hence, the local immune response in the skin was similar to the immune response in the draining lymph nodes.

Taken together, *STAT6*$^{-/-}$ mice infected with *L. mexicana* develop an enhanced Th1-like cytokine response (IL-12, IL-2, and IFN-γ) with no significant changes in the Th2-like cytokine, IL-4. This default toward a Th1-like response may have prevented lesion progression in *STAT6*$^{-/-}$ mice.

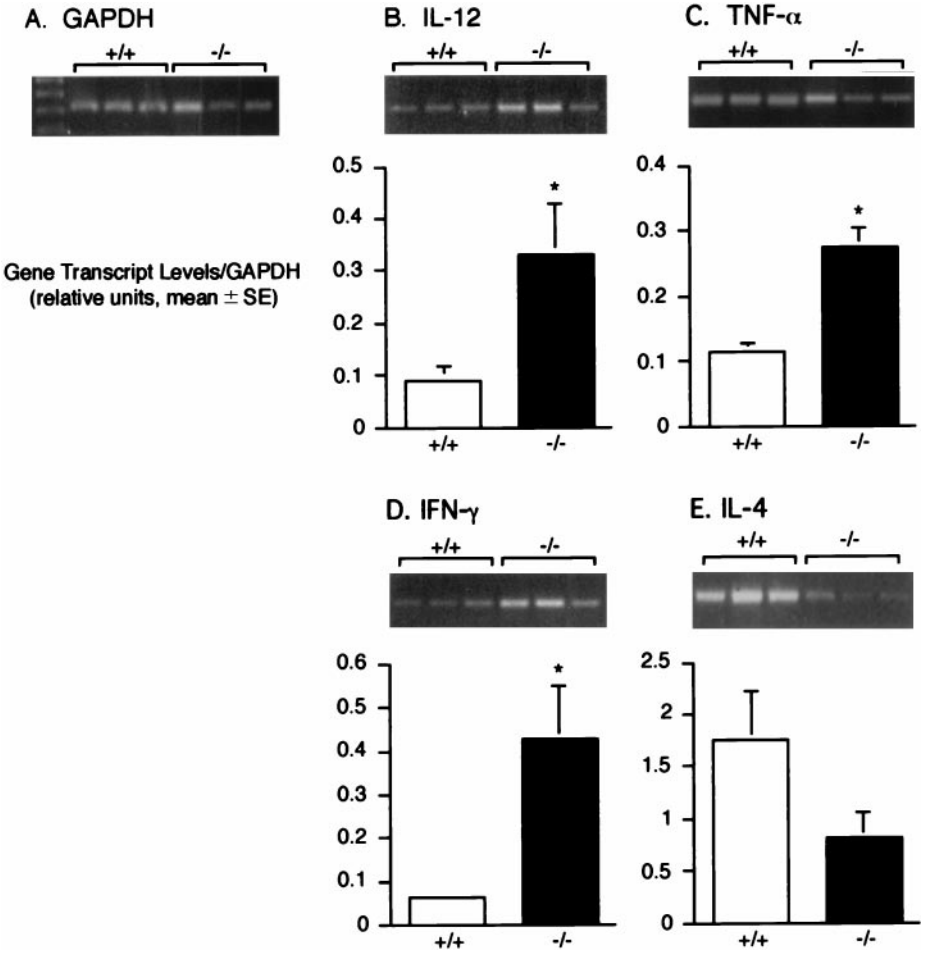

FIGURE 6. In vivo transcript analysis by RT-PCR in skin cells at the inoculation site of *STAT6*$^{+/+}$ and *STAT6*$^{-/-}$ mice at 12 wk postinfection. Three animals were analyzed in each group. *A*, The reference gene, GAPDH, was used to assess variation between RNA and cDNA loading. Results for IL-12 (*B*), TNF-α (*C*), IFN-γ (*D*), and IL-4 (*E*) are shown. Agarose gel (1.5%) stained with ethidium bromide was used to visualize PCR products. Representative gels for each gene are shown. In all cases, the first three columns are the products from *STAT6*$^{+/+}$ mice, and the last three columns are from *STAT6*$^{-/-}$ mice. The intensities of the bands were quantified as incorporated [^{32}P]dCTP on a PhosphorImager. The bar graph shows normalized values for each gene in *STAT6*$^{+/+}$ (white columns) and *STAT6*$^{-/-}$ (black columns) mice derived by taking the ratio of the mean of triplicate values for each animal. Data are expressed as the mean ± SE. Asterisks indicate statistically significant differences between groups ($p < 0.05$).

Discussion

The novel finding in this study is that *STAT6*$^{-/-}$ mice are protected from the cutaneous lesions that are seen in *STAT6*$^{+/+}$ mice following local inoculation with *L. mexicana*. This protective response was associated with increased Th1-like cytokine production evident in LmAg-stimulated draining lymph nodes, in intact lymph nodes, as well as in the skin from the inoculation site itself. These findings support the conclusion that STAT6-mediated IL-4 signaling is critical for the suppression of the Th1-like responses that are required for control of cutaneous lesions after *L. mexicana* infection.

The proposed mechanisms underlying the development of nonhealing lesions in genetically susceptible mice following *L. major* infection have included the presence of an IL-4-driven Th2-like response suppressing Th1 cell development (31, 32) and a failure to produce IL-12 (33, 34) and mount an IL-12-induced Th1-like response (11). Studies in IL-4-deficient mice have failed to resolve this controversy. Some investigators have shown IL-4-deficient BALB/c mice to be resistant to *L. major* infection (8), while others have found these mice to maintain susceptibility (35). Despite the absence of IL-4 in the former study, these mice did not default to a Th1-like response, suggesting that the lack of IL-4 and an IL-4-induced Th2-like response protected the mice (8).

On the other hand, genetically resistant mice lacking IL-12 (36, 37) or IFN-γ (38) defaulted to a Th2-like response and were highly susceptible to cutaneous *L. major* infection. Interestingly, IFN-γR-deficient 129/Sv/Ev mice were also susceptible to *L. major*, but did not develop a Th2-like response (39). The susceptible mice lacking IFN-γR defaulted toward a Th1-like response implying that IFN-γ, although important in resistance to *L. major*, was not necessary for a Th1-like response and that IL-12, instead, may be the critical cytokine responsible for Th1 cell development.

Previous studies have clearly demonstrated that protective immunity against the *L. mexicana* complex, which includes *L. mexicana* and *L. amazonensis* strains, is ultimately dependent upon generation of a Th1-like response and IFN-γ production (10, 12, 13). Lymph node cells from genetically susceptible mice produced little or no IFN-γ and low levels of IL-4 following *L. amazonensis* (12) and *L. mexicana* infection (10, 13). Nonetheless, IL-4-deficient C57BL/6 × 129/Sv mice develop a Th1-like response, as measured by an increase in IFN-γ production, and cure *L. mexicana* infection (10). Our results with *STAT6*$^{-/-}$ mice extend earlier findings and indicate that increased Th1-like responses in *L. mexicana*-infected *STAT6*$^{-/-}$ mice may be due to the absence of IL-4-mediated suppression of IFN-γ production. The implications of IFN-γ in the development of a Th1-like response and resistance to *L. major* are based upon observations that have showed impaired Th1-like responses following treatment of genetically resistant C3H/HeN mice with anti-IFN-γ Abs (40). However, treatment with recombinant IFN-γ failed to promote Th1 cell expansion and cure *L. major* infection in susceptible BALB/c mice (41).

Recent studies have shown that IL-12 is critical for the development of Th1-like CD4$^+$ T cell responses following *L. major* infection in resistant mice (36, 37) and that treatment of susceptible BALB/c mice with rIL-12 cures cutaneous *L. major* infection (42). Furthermore, in previously reported studies, anti-IFN-γ Ab had no effect on IL-12-induced Th1 cell differentiation in vitro

(43), whereas addition of rIL-12 during specific priming of $CD4^+$ T cells from transgenic mice expressing an Ag-specific TCR-$\alpha\beta$ resulted in the development of the Th1-like phenotype (44). Previous studies using the *L. major* model have indicated that genetic susceptibility of BALB/c mice to this strain of *Leishmania* is due to a loss of the ability to generate an IL-12-induced Th1-like response (11, 45). In the present study $STAT6^{-/-}$ mice produced significantly higher levels of IL-12 than $STAT6^{+/+}$ and subsequently developed a Th1-like response. Therefore, these results indicate that diminished levels of IL-12, and not unresponsiveness to this cytokine, may be the mechanism responsible for susceptibility to *L. mexicana*.

In addition to its ability to down-regulate IL-12 and IFN-γ production, IL-4 has been shown to also inhibit the production of the inflammatory cytokines IL-1β and TNF-α from macrophages (46). Several studies have demonstrated that TNF-α plays a protective role in immunity against *L. major* infection (47). For example, lymph node cells from mice resistant to *L. major* produce high levels of TNF-α when stimulated in vitro, whereas cells from susceptible strains under the same conditions produce low levels (48). TNF-α has been shown to induce parasite killing by macrophages in the presence of IFN-γ by increasing nitric oxide production (49). Recently, it was shown that mice deficient in both TNF-α receptors, p55 and p75, were able to control *L. major* infection, but failed to resolve lesions (50). Although the role of the p75 TNF-α receptor was not found to be essential in *L. major* infection, it was concluded that the p55 receptor may be required for optimal macrophage activation (50). In previous studies IL-4-deficient mice infected with *L. major* displayed similar levels of TNF-α transcripts as wild-type mice (8, 35). In contrast, we found that *L. mexicana*-infected $STAT6^{-/-}$ mice displayed significantly higher relative levels of TNF-α in the skin and lymph nodes compared with wild-type mice. These differences are most likely due to the ability of IL-13 to inhibit the production of TNF-α using the STAT6 pathway in IL-4-deficient mice and the inability of IL-13 to do so in $STAT6^{-/-}$ mice (21, 51). The different species of parasite used in the experiments may also play a part in the observed differences.

IL-4 signals through two distinct pathways, one of which involves phosphorylation of IRS-1 and IRS-2 (14, 15) and the other of which involves JAK1 and JAK3 and subsequent activation of STAT6 (14, 16). IRS-1 and IRS-2 are interchangeable in the former pathway and play an important role in proliferative responses to IL-4 (15, 52). However, IRS-2 couples more sensitively to the IL-4R system than IRS-1 (52, 53). The IL-4R system is comprised of the IL-4R α-chain that governs the nature of the signal and the common γ-chain that is necessary for generation of the signal (14). The proximal region of the IL-4R α-chain includes the tyrosine residues that when phosphorylated signal the IRS pathway for IL-4-mediated proliferative responses (14, 15, 52). On the other hand, the more distal region of the IL-4R α-chain contains the STAT6 binding sites that are responsible for IL-4-mediated differential events (14, 53). Previous studies have demonstrated that although B and T cells from $STAT6^{-/-}$ mice maintain the ability to proliferate in response to IL-4, presumably through the IRS pathway, they have lost other functions of IL-4, such as inducing Th2 development, up-regulation of MHC class II, and CD23 and Ab class switching to the IgE isotype (17, 18, 22). The current study suggests that the STAT6-mediated pathway for IL-4 signaling, not the IRS-mediated pathway, plays the critical role for suppression of the Th1-like response and consequent lesion growth following *L. mexicana* infection.

IL-13 is a cytokine that exhibits similar functions as IL-4 (19, 54, 55). IL-13 also shares the IL-4R α-chain and signaling pathway through STAT6 with IL-4 (20, 56, 57). Mice lacking STAT6 have been shown to have impaired IL-13-mediated functions, including up-regulation of MHC class II expression and inhibition of nitric oxide production by activated macrophages (21). However, it is unlikely that IL-13 is important in the down-regulation of Th1-like responses, since IL-4-deficient mice on the same genetic background have been shown to develop Th1-like responses and control cutaneous *L. mexicana* infection despite the presence of IL-13 and an intact signaling pathway (10).

IL-4 and, to a lesser extent, IL-13 (58) have been demonstrated to enhance Ab class switching to the IgE isotype (26) by a mechanism involving STAT6 (17, 18, 22, 59). Consistent with observations made in other studies (17, 18, 22, 59), the present study showed that $STAT6^{-/-}$ mice infected with *L. mexicana* also fail to produce IgE. IgG1 production has also been shown to be regulated by, although not completely dependent upon, IL-4 and IL-4 signaling (25, 60). In this study, data showed decreased levels of IgG1 in $STAT6^{-/-}$ mice compared with wild-type mice. This finding extended the findings of previous studies (17, 18, 22, 59), suggesting that STAT6-mediated IL-4 signaling is also important in Ab class switching to IgG1 following *L. mexicana* infection.

A recent study reported that the immunosuppressive drug leflunomide pharmacologically inhibits phosphorylation of the tyrosine residues of JAK3 and STAT6 (61). Following treatment with leflunomide, the JAK3 and STAT6 proteins remain inactive upon IL-4 binding to IL-4Rα, and STAT6 fails to bind subsequently to the IL-4-responsive genes, including those required for class switching to IgG1 (61). Therefore, treatment with leflunomide inhibits IgG1 production, a result similar to that in $STAT6^{-/-}$ mice in this study (61). These observations suggest that protection of *L. major*-infected BALB/c mice following leflunomide pretreatment as reported previously (62) may be due to the ability of this drug to inhibit STAT6-mediated IL-4 signaling pathway.

Whereas the IgE and IgG1 isotypes are associated with the development of a Th2-like response, switching to the IgG2a isotype has been shown to be increased during Th1-like responses (25). Some studies previously report only a slight increase in Ab IgG2a production in $STAT6^{-/-}$ mice compared with that in wild-type mice (17, 18). However, this and other studies demonstrate significantly higher levels of IgG2a in serum from $STAT6^{-/-}$ deficient mice (22, 59). This latter finding suggests that lack of IL-4 signaling in $STAT6^{-/-}$ mice facilitates class switching to the Th1-associated IgG2a Ab isotype.

In conclusion, *L. mexicana*-infected $STAT6^{-/-}$ mice on a genetically susceptible C57BL/6 $\times$ 129/Sv background are protected from cutaneous lesions and produce significantly higher amounts of the Th1-like cytokines in draining lymph nodes and in skin from inoculation sites than $STAT6^{+/+}$ mice. In addition, $STAT6^{-/-}$ mice have higher levels of the Th1-associated Ab IgG2a than the $STAT6^{+/+}$ mice, which, conversely, have higher levels of the Th2-associated Abs IgG1 and IgE. These findings suggest that the STAT6-mediated IL-4 signaling is responsible for the suppression of a protective Th1-like immune response in susceptible mice following *L. mexicana* infection.

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