Cure of Progressive Murine Leishmaniasis: Interleukin 4 Dominance Is Abolished by Transient CD4$^+$ T Cell Depletion and T Helper Cell Type 1–selective Cytokine Therapy

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Summary

Progressive infection with *Leishmania major* in susceptible BALB/c mice is mediated by interleukin (IL)-4–producing T helper cell type 2 (Th2) CD4$^+$ T cells that, once established, become resistant to Th1-deviating therapies with recombinant (r)IL-12 and/or neutralizing anti–IL-4 antibodies. We sought to restore protective immunity in advanced leishmaniasis by depletion of Th2-biased CD4$^+$ populations and by cytokine-directed reconstitution of Th1 cellular responses during lymphocyte recovery. Treatment with cytolytic GK1.5 anti-CD4 mAb alone did not reverse disease in 3 wk–infected BALB/c mice, but GK1.5 combined with anti–IL-4 antibody and intralesional rIL-12 cured cutaneous lesions in 80% of mice and established a Th1-polarized cytokine response to *L. major* antigen protective against reinfection. The curative effects of GK1.5 were not replaced by cytotoxic anti-CD8 monoclonal antibody 2.43 or nondepleting anti-CD4 mAb YTS177, confirming that depletion of CD4$^+$ cells was specific and essential for therapeutic effect. Finally, combined CD4$^+$ depletion and IL-4 neutralization were curative, indicating that neither increased parasite burden nor altered accessory cell function independently biased towards Th2 reconstitution in advanced leishmaniasis. Advanced leishmaniasis can be cured by T cell depletion and cytokine-directed recovery of Th1 cellular responses, suggesting novel interventions for other immune-mediated diseases and identifying distinct roles for CD4$^+$ T cell and non-T cell in the maintenance of Th2 and Th1 phenotypes.

Key words: T helper cells type 2/immunology • interferon type II • interleukin 12 • T helper cells type 1/immunology • immunity/cellular

After repeated stimulation, CD4$^+$ T cells differentiate into functionally distinct subsets defined by the patterns of cytokine they produce (1). Although this provides an efficient mechanism for tailoring specific host responses to distinct infectious threats, inappropriate T cell functional responses may cause pathology. For instance, Th1-type T cells that produce proinflammatory cytokines normally involved in antimicrobial defense, such as IFN-γ and TNF-α, also mediate autoimmune disease when they acquire self-reactivity (2). Th2-type T cells that produce variable combinations of IL-4, IL-5, and IL-13 and are important for resistance against intestinal helminths (3) will mediate allergic diseases when sensitized against common environmental antigens (4, 5). Some immune-mediated pathologies can be prevented experimentally if the opposite cytokine polarity is preselected by treatment with differentiation-biasing cytokines or anticytokine antibodies. For instance, immune deviation towards Th2 responses prevents the expression of autoimmune encephalomyelitis (6, 7), whereas deviation towards Th1-biased antigen-specific responses blocks the expression of allergic disorders (8). Once established, progressive forms of Th1 or Th2 immunopathology become increasingly resistant to immunologic redirection by in vivo cytokine therapy. An alternative approach to the therapy of immune-mediated diseases is the depletion of immunopathogenic CD4$^+$ T cell populations by treatment with cytotoxic anti-CD4 antibody, an intervention already under study in humans with severe rheumatoid arthritis (9). However, T cell depletion only transiently suppresses autoimmunity in mice and humans, and disease typically relapses with the recovery of Th1 CD4$^+$ T cells (10, 11, 12). These findings suggest that CD4$^+$ depletion alone has limited efficacy if it does not alter the intrinsic immune biases originally responsible for disease onset. In these studies, we address the possibility that the recovery of CD4$^+$ T cells after transient depletion restores susceptibility to phenotype-biasing cytokine therapy, and thereby permits the stable reversal of an immunopathogenic T cell response.

We tested this hypothesis using a mouse model of progressive cutaneous leishmaniasis that is mediated by CD4$^+$ Th2 responses that become solidly resistant to anti-CD4 mAb or cytokine therapies once disease is established for

>2 wk. Cutaneous infection of different inbred strains of mice with *Leishmania major* provides a well-characterized system suitable for the study of polarized cytokine responses that mediate widely divergent disease outcomes (13). Disease-resistant strains of mice cure subcutaneous infection with *L. major* due to the IL-12–dependent expansion of Th1 type $CD4^+$ T cell responses that produce IFN-γ, a proinflammatory cytokine essential for nitric oxide–dependent killing of the intracellular amastigote forms of *Leishmania*. In contrast, disease-susceptible BALB/c rapidly expand $CD4^+$ T cells populations committed towards production of the Th2 cytokine IL-4 (14). IL-4 critically disrupts IFN-γ–dependent activation of tissue macrophages that are the obligate cellular target for this parasite (15). Survival of the pathogen leads to progression of cutaneous lesions and permits eventual visceral dissemination.

Although the development of Th2 responses and progressive disease in *L. major*–infected BALB/c mice is prevented by early treatment with anti–IL-4 antibody or rIL-12, these interventions do not reverse T cell polarity or the disease outcome once infection is established for >2 wk (16, 17). The resistance of Th2 cells to immune deviation may reflect downregulation of IL-12 receptor function and continual self-renewal due to the biasing effects of IL-4 on the expansion of precursor $CD4^+$ T cells committed towards Th2 development (18). Whether factors extrinsic to $CD4^+$ T cell function independently contribute to the phenotypic stability of Th2 responses in established progressive leishmaniasis is unresolved. Increased parasitic burdens potentially affect T cell differentiation by altering the costimulatory competence of heavily parasitized accessory cells (19) or by qualitatively perturbing T cell activation due to antigen excess (20). Consistent with this, the Th2 response of progressively infected BALB/c mice can be reversed by IL-12 or anti–IL-4 mAb immunotherapy if the infectious burden is first reduced by treatment with the leishmanicidal antibiotic, sodium stibogluconate (21). Spontaneous or antibiotic-induced cure of visceral leishmaniasis in humans also results in deviation towards Th1 immune responses (13). The successful treatment of progressive leishmaniasis with CpG-enriched oligonucleotides (22), which act directly on accessory cell populations (23), further supports a critical role for T cell extrinsic factors in the experimental reversal of established Th2 cytokine phenotypes.

In the study presented here, we examined whether antibody-mediated removal of dysfunctional Th2 $CD4^+$ T cell populations and their cytokine products is sufficient to restore Th1 differentiating capacity in advanced murine leishmaniasis in the absence of antibiotic therapy. We hypothesized that transient depletion of $CD4^+$ T cells not only removes established Th2 cell populations responsible for disease progression, but also results in the subsequent outgrowth of $CD4^+$ T cells that are susceptible to the differentiating effects of Th1-selective rIL-12 or anti–IL-4 mAb therapies. Our findings demonstrate that $CD4^+$ depletion combined with IL-4 neutralization is sufficient to promote stable deviation towards a Th1 phenotype, with subsequent decreases in parasite load and curing of cutaneous lesions. This suggests a novel approach to the immunotherapy of Th2-dependent diseases, and also demonstrates that the immune environment of progressively infected BALB/c mice is preferentially supportive of de novo Th1 responses after the removal of Th2 $CD4^+$ T cell activities that are intrinsically resistant to phenotypic reversal.

Materials and Methods

Mice. 4–6-wk-old female C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory and housed in the Case Western University animal facility under specific pathogen-free conditions.

Parasite Cultivation and Mouse Infection. *L. major* (WHO strain WHOM/IR/−/173) were grown in M199 medium (GIBCO BRL) containing antibiotics, supplemental glutamine, and 30% FCS (HyClone Labs.), as previously described (24). Stationary phase promastigotes were injected into the hind feet of recipient mice at a dose of 2×10^6 organisms per footpad to initiate infection. The course of infection was monitored by measuring the thickness of footpad swelling weekly using a dial gauge caliper. Soluble *L. major* antigen was made as previously described (24).

Reagents. Hybridomas producing cytotoxic anti-CD4 mAb GK1.5 (rat IgG2b), anti-CD8 mAb 2.43 (rat IgG2b), neutralizing anti–I-A^d/I-E^d M5/114 (rat IgG2b), and neutralizing anti–IL-4 mAb 11B11 (rat IgG1) were obtained from the American Type Culture Collection. Noncytotoxic anti-CD4 mAb YTS 177.9 (rat IgG2a) was obtained from Dr. Shixin Qin (Leukosite Inc., Boston, MA). Antibodies were generated using serum-free media supplemented with 1% Nutridoma-NS (Boehringer Mannheim) or by induction of nude mouse ascites, and were purified using HiTrap Protein G columns (Pharmacia). Recombinant IL-12 was generated in dihydrofolate reductase–deficient CHO-DUXB11 cells transfected with mouse p35 and p40 IL-12 cDNA coexpressing dihydrofolate reductase (Genetics Institute), and subsequently was incubated in increasing concentrations of methotrexate to amplify transgene expression, as previously described (25). IL-12 was purified from serum-free culture supernatant by sequential Mono Q and heparin-Sepharose (Pharmacia) affinity chromatography, and the specific IFN-γ–inducing activity (1.1×10^6 U/mg) was determined in cultures of normal splenocytes, as previously described (26). As indicated in the figure legends, we used rIL-12 (sp. act. 2.1×10^6 IFN-γ–inducing U/mg) provided by Dr. M. Gately (Hoffmann-LaRoche, Nutley, NJ) as an alternative.

Culture of Lymph Node Cells. Lymph node cells were washed three times in HBSS, counted, and suspended in DMEM/10% fetal bovine serum (FBS)[1] (DMEM supplemented with 100 μg/ml of penicillin and streptomycin, 2 mM glutamine, 0.1 mM nonessential amino acids, and 10% FBS, buffered at pH 7.4 with 10 mM Hepes). Cells were aliquoted into flat-bottomed 96-well culture plates at 10^6 cells per well and cultured for 48 h in DMEM/10% FBS. Stimuli included media alone or media containing soluble leishmania antigen at 10–20 μg/ml. Where indicated, 10 μg/ml of M-1 anti–IL-4 receptor mAb (Genzyme) was added to culture to prevent IL-4 sequestration by soluble or cell-associated IL-4 receptor (27). Anti-MHC II mAb was added at 10 μg/ml to determine the MHC II dependence of antigen-inducible cytokine responses (28).

[1]*Abbreviations used in this paper:* FBS, fetal bovine serum; iNOS, inducible nitric oxide synthase.

Cytokine ELISA Assays. Culture supernatants were assayed for murine cytokines using double sandwich mAb ELISA techniques as previously described (27). Antibodies used for capture of IL-4, IFN-γ, and IL-12 p40 were BVD-6, R46A2, and 15.6, whereas captured cytokine was detected using biotinylated antibodies BVD-4, XMG1.2, and 17.8, respectively (PharMingen). Recombinant mouse IFN-γ, IL-4, and IL-12 used as standards in ELISA were purchased from PharMingen or Genzyme.

Reverse Transcriptase PCR Analysis of Cytokine mRNA. Lymph node mRNA was isolated using STAT-60 (TelTest) and analyzed by oligo-dT primed reverse transcription and subsequent PCR. The techniques and primers used have been published previously (29).

Quantitative Parasite Cultures. Approximately 0.2–0.3 gram of footpad tissue was minced in 2 ml of M199/30 FCS medium, crushed through a No. 200 stainless steel screen (Small Parts, Inc.), and disrupted using a Ten-Broeck homogenizer. 50-μl aliquots of footpad or lymph node suspension were serially diluted fivefold in promastigote growth medium and incubated in flat-bottomed 96-well plates at 26°C in humidified room air. Individual wells were examined using an inverted microscope at 200× at 2-d intervals for the presence of motile promastigotes. Data represent the geometric mean ± SEM of the last positive reciprocal dilution for each experimental group.

Statistics. For comparisons of ELISA data, significance was assessed using the nonparametric Mann-Whitney rank sum test. Significant differences in disease outcome were determined by contingency table analysis (Fisher exact test).

Results

Cytokine-based Therapy with rIL-12 and Neutralizing Anti–IL-4 mAb in Combination Fails to Reverse Established Leishmaniasis. Since IL-4 antagonizes the immunoregulatory effects of IL-12 (30, 31), we tested whether neutralization of IL-4 by anti–IL-4 mAb 11B11 would restore the therapeutic benefit provided of rIL-12 in BALB/c mice infected with *L. major* for 2 wk, a time at which these agents are individually ineffective (16, 17, 32). Mice treated daily with 1 μg of intraperitoneal rIL-12 on days 7–21 only demonstrated nonsignificant decreases in footpad swelling (Fig. 1 A). In contrast, lesional development was significantly delayed in mice injected intraperitoneally with 1 mg of anti–IL-4 mAb 11B11 in combination with intraperitoneal rIL-12 treatments on days 7, 14, and 21 of infection. However, all mice treated with this combination still displayed progressive footpad swelling leading to ulceration and limb necrosis. Similar results were obtained in a second experiment where 1 mg of anti–IL-4 mAb was administered intraperitoneally starting at wk 3 of infection and in combination with 10 d of intralesional rIL-12 injection (Fig. 1 B). As previously reported (17), treatment at wk 3 with anti–IL-4 mAb alone only delayed disease progression without affecting final outcomes (data not shown). Similarly, delayed treatment of established leishmaniasis with anti–IL-2 mAb, which is normally curative when started early in infection (33), also failed to restore protective immunity when combined with rIL-12 (data not shown). These studies indicated that the neutralization of Th2-promoting cytokines alone was insufficient to restore the therapeutic effects of rIL-12 in *L. major*–susceptible mice with advanced disease. Because established BALB/c Th2-biased T cell responses proved highly resistant to cytokine-induced phenotypic reversal, we next tested whether transient $CD4^+$ T cell depletion might remove pathogenic immune responses, while also providing a newly emergent population of T cells susceptible to cytokine-directed differentiation towards the curative Th1 phenotype.

$CD4^+$ T Cell Depletion Combined with rIL-12 and anti–IL-4 mAb Immunotherapy Cures Progressive Leishmaniasis. Treatment of BALB/c mice with the cytolytic antibody GK1.5 given in two doses of 0.5 mg each on days 21 and 22 of *L. major* infection resulted in $CD4^+$ lymphopenia lasting ~2–3 wk (Table I). Although this dose of antibody is curative when given during wk 1 of infection, we confirmed that delayed therapy with CD4 cytolytic antibody GK1.5 alone had no effect on outcome (Fig. 1 B). Continued pro-

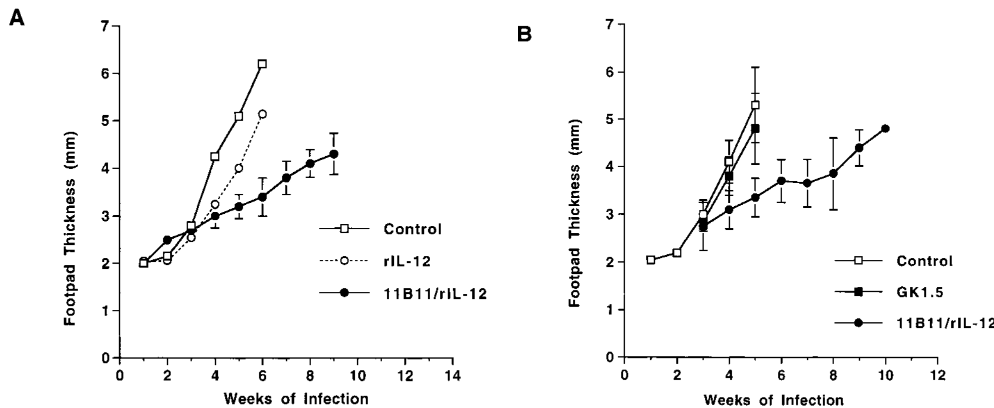

Figure 1. Treatment with Th1-deviating rIL-12 and anti–IL-4 antibodies or transient depletion of $CD4^+$ T cells fails to cure established murine leishmaniasis in susceptible BALB/c mice. (A) Combined treatment with neutralizing anti–IL-4 mAb (11B11) and rIL-12 does not reverse established infection. BALB/c mice (n = 6 per group) were infected with *L. major* and treated with (Control) 0.5 mg of rat IgG administered on days 7, 14, and 21 of infection (control) or 1.0 μm of rIL-12 (Hoffmann-LaRoche; 2.1 × 10^3 U/dose) injected intraperitoneally daily for 14 d starting on day 7 of infection. A separate group of mice (11B11/rIL-12) were injected intraperitoneally on days 7, 14, and 21 with 1.0 mg of neutralizing anti–IL-4 mAb 11B11 in combination with rIL-12 for 14 d. Shown are mean footpad thicknesses ± SEM. Although footpad swelling was significantly suppressed, all mice eventually progressed to develop ulceration and deep tissue necrosis. (B) Separate groups of BALB/c mice (n = 5 each) were infected with *L. major* for 3 wk and treated on days 21 and 22 with rat IgG (control) or with 0.33 mg of anti-CD4 mAb GK1.5 by intraperitoneal injection. Another group (11B11/rIL-12) was treated on day 21 with 1.0 mg of anti–IL-4 mAb 11B11 and then with 0.5 μg of rIL-12 given by intralesional injection into each footpad (Hoffmann-LaRoche; 1.1 × 10^3 U/dose) for 8 consecutive days. All mice progressed to develop ulceration and necrosis.

gression of disease was associated with the re-emergence of lymph node CD4+ T cells and IL-4 production in response to *L. major* antigen (data not shown). This suggested that the intrinsic bias of BALB/c mice towards Th2 development in this disease was not disrupted by transient CD4+ T cell depletion alone. Therefore, we examined the course of cutaneous leishmaniasis in 3 wk–infected BALB/c mice treated with GK1.5 in combination with anti–IL-4 11B11 mAb and intralesional rIL-12 administered during the 2-wk period leading up to CD4+ T cell recovery. In control BALB/c mice treated with nonspecific rat IgG, the cutaneous lesions progressed rapidly, as indicated by increasing footpad thickness and the development of ulceration and necrosis (Fig. 2). In contrast, mice treated with combined GK1.5, rIL-12, and anti–IL-4 mAb were able to limit and then reverse footpad swelling, with all five mice showing complete resolution of disease (Fig. 2).

By wk 14 of infection, the hindlimb swelling of mice receiving GK1.5-based immunotherapy had diminished to an average thickness compatible with that of age-matched normal feet. To determine if successful immunotherapy provided long-lasting immunity against reinfection with *L. major*, these cured mice and a group of normal BALB/c mice were infected with 2×10^6 *L. major* promastigotes in each hind foot. The immunotherapy-cured mice rapidly contained reinfection by 4 wk, as indicated by the transience of footpad swelling, whereas control BALB/c mice developed progressive footpad swelling characteristic of nonhealing leishmaniasis (Fig. 2).

The protective effects of GK1.5-based immunotherapy were subsequently reproduced in four additional studies where advanced cutaneous lesions were durably cured in 20 out of 25 BALB/c mice (80%). None of the 27 control BALB/c mice recovered from infection (difference significant at $P < 0.01$, Fisher exact test). Decreased footpad swelling

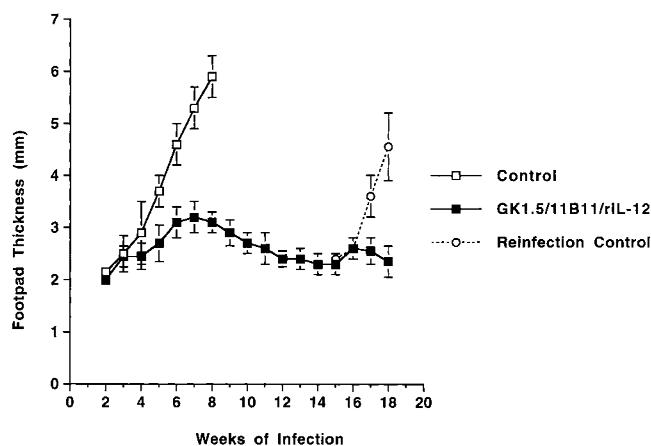

Figure 2. BALB/c mice infected for 3 wk with *L. major* can be cured by CD4+ depletion and Th1-deviating cytokine therapy. Groups of five BALB/c mice were treated with (GK1.5/11B11/rIL-12) 0.5 mg of GK1.5 mAb and 11B11 anti–IL-4 mAb given daily for 2 d, followed by a total of 10 intralesional injections of 0.5 μg of rIL-12 (Hoffmann-LaRoche; 1.1×10^3 U/dose) into each foot from days 21 through 32 or 0.5 mg of rat IgG and intralesional saline only (control). Data represent mean footpad thickness ± SEM measured weekly by micrometer. All control mice were killed at 8 wk because of progressive cutaneous ulceration and necrosis; five out of five mice receiving GK1.5 immunotherapy healed. At 14 wk of infection, the five BALB/c mice cured by immunotherapy and five normal BALB/c mice (reinfection control) were injected with 2×10^6 *L. major* promastigotes per footpad and footpad thicknesses recorded weekly.

was associated with reduced parasite load within the cutaneous lesions, with 42-fold decreases apparent at 3 wk after treatment ($10^{9.59 \pm 0.41}$ per gram of control tissue compared with $10^{7.96 \pm 0.24}$ for treated mice) and a further 10,000-fold reduction evident at 7 wk after therapy ($10^{3.1 \pm 0.40}$ per gram).

BALB/c Mice Cured of L. major Infection by GK1.5-based Immunotherapy Produce a Th1-polarized Cytokine Response. The draining lymph node cells from five cured and reinfected mice were harvested, and their antigen-specific cytokine responses were compared with infected, untreated BALB/c mice (Table II). Although the amounts of IFN-γ stimulated by antigen were not significantly different between the two groups, immunotherapy-cured mice produced concentrations of IL-4 that were ≥20-fold reduced relative to control BALB/c mice ($P < 0.05$). In a second experiment in which two out of five treated mice failed to heal, therapeutic success and failure correlated with the absence and presence of IL-4 production, respectively (Table II). There was evidence that the restored CD4+ T cell populations contributed to IFN-γ production, as synthesis of this cytokine in treated and control mice was 46 and 60% MHC II dependent, respectively. IL-4 production was reduced to undetectable levels in the presence of anti–MHC II mAb. These findings indicated that the immunotherapy-induced cure gave rise to antigen-specific CD4+ T cell populations producing IFN-γ without IL-4, consistent with protective immune deviation towards a unipolar Th1 phenotype.

CD4+-specific Depletion Is Required for the rIL-12/Anti–IL-4–mediated Cure of Established Murine Leishmaniasis. Although

Table I. *Treatment with GK1.5 Anti-CD4 Antibody Transiently Depletes CD4+ T Cells in BALB/c Mice Infected for 3 wk with L. major*

Days after GK1.5	Percentage of CD4	Percentage of CD8
0	30.3 ± 1.9	12.6 ± 0.9
3	5.4* (4.1)	27.4* (22.5)
7	5.9 ± 1.9	21.2 ± 0.7
21	14.6 ± 1.1	20.4 ± 1.2
63	18.6	21.4

Groups of five BALB/c mice were infected with *L. major* for 3 wk and injected with 1.0 mg of GK1.5 mAb. Spleen cells were harvested at the indicated number of days after injection with GK1.5 and the percentage of total cells expressing surface CD4 or CD8 was determined by two-color FACS® analysis. Shown are mean percentage of cells positive ± SEM ($n = 4$).

*Cells were pooled in one group and the parentheses indicate the results from a separate experiment where data represent pooled lymph node cells from four mice.

Table II. *Cure of Leishmaniasis after GK1.5-based Immunotherapy Is Associated with Unipolar Th1-type Cytokine Responses*

Group	Antigen-induced cytokine levels	
	IFN-γ	IL-4*
	ng/ml ± SEM	
Experiment 1		
Control (n = 5)	1.42 ± 0.19	1.24 ± 0.35
Immunotherapy (n = 5)	0.88 ± 0.39	0.06 ± 0.02
Experiment 2		
Uninfected	0.11	≤0.05
Control (n = 5)	1.36 ± 0.07‡	1.28 ± 0.17
Immunotherapy (n = 3)	1.01 ± 0.12‡	0.05
Nonhealing (n = 2)§	2.08	1.53

BALB/c mice were treated with GK1.5 and 11B11 mAb, followed by intralesional rIL-12 as described in the text. Lymph node cells were harvested after disease recovery at wk 4 of reinfection (Experiment 1) or at wk 7 after immunotherapy (Experiment 2) and were cultured for 48 h in the presence of 10 μg/ml of soluble leishmania antigen. Cytokine concentrations were measured by specific ELISA. Control lymph node cells were from BALB/c mice infected for 4 wk with *L. major.*
*IL-4 was measured in antigen-stimulated cultures to which anti–IL-4 receptor mAb had been added (10 μg/ml). IL-4 levels in all cultures were reduced by >96% in the presence of anti–MHC II mAb.
‡Antigen-stimulated IFN-γ production was reduced 60.8 ± 5.4% for control mice and 46.3 ± 7.5% for immunotherapy mice after neutralization of MHC II by anti–I-E^d/I-A^d mAb added at 10 μg/ml to antigen-stimulated cultures.
§Two mice failed therapy, as indicated by chronic footpad swelling and cutaneous ulceration.

we hypothesized that the protective effects of GK1.5-based immunotherapy were dependent on the removal of IL-4–producing cells present in the CD4$^+$ T cell population, noncytolytic effects of anti-CD4 antibody on the function of MHC II–restricted T cells may have affected the outcome separately. For instance, in vivo and in vitro Th2 development requires that CD4 bind to MHC II during antigen presentation, suggesting that neutralization of CD4 function alone might effectively substitute for cellular depletion in the immunotherapy of established leishmaniasis (34, 35). Another possibility requiring experimental exclusion was that the process of in vivo cellular depletion, regardless of the population targeted, was contributing directly or indirectly to cure of leishmaniasis and reversal of Th2 dominance. To test these alternative hypotheses, separate groups of BALB/c mice infected for 3 wk with *L. major* were treated with anti–IL-4 antibody and intralesional rIL-12 after receiving 0.5 mg of either depleting or nondepleting anti-CD4 mAb (GK1.5 and YTS177, respectively) or after being treated with depleting anti-CD8 mAb 2.43. In pilot studies, YTS177 failed to reduce peripheral blood CD4$^+$ counts by >15%, as previously described (36). Only the immunotherapy regimen based on depleting GK1.5 antibody was capable of healing the cutaneous lesions of progressive leishmaniasis (four out of six mice) (Fig. 3). These experiments clearly indicated that specific depletion of CD4$^+$ T cells was required to mediate the cure by combined treatment with rIL-12 and anti–IL-4 and that depletion of an alternative T cell subset did not invoke nonspecific protective responses.

Effects of GK1.5-based Immunotherapy on IL-4 and IFN-γ Synthesis. We next determined how immunotherapy affected the in vitro and in vivo cytokine responses of infected mice at 7 and 21 d after treatment. The first time point corresponds to the inductive stage of immunotherapy, when CD4$^+$ T cells were freshly depleted and the mice were receiving intralesional therapy with rIL-12. The second time point corresponds to the subsequent repopulation of the lymph node by CD4$^+$ cells. At 1 and 3 wk after the start of immunotherapy, the popliteal lymph node cells of treated mice produced approximately three- and sevenfold less IL-4 in response to soluble leishmania antigen than did the lymph node cells of control mice (Fig. 4). Consistent with a predominantly CD4$^+$ T cell source for IL-4 in control mice, IL-4 levels were reduced by ~80% in the presence of anti–MHC II antibodies capable of blocking both I-A^d and I-E^d (data not shown). The small amounts of IL-4 produced by CD4$^+$-depleted mice also remained MHC II dependent, indicating that the residual IL-4 response was derived from CD4$^+$ T cells that had escaped depletion.

The lymph node cells of treated mice produced twofold more IFN-γ than controls in the first week of therapy. Some of this response may have been mediated by ongoing rIL-12 therapy, as the majority of the IFN-γ response was spontaneous. By 3 wk after therapy, control and treated mice produced similar amounts of IFN-γ in response to antigen. Although production of IFN-γ in control lymph

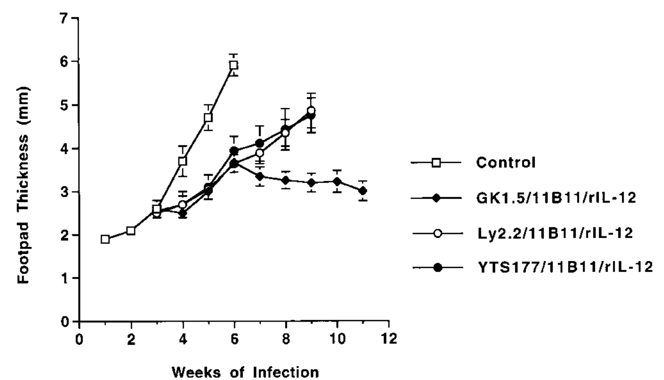

Figure 3. Specific depletion of CD4$^+$ T cells is required for cure of established leishmaniasis in response to treatment with rIL-12 and anti–IL-4 mAb. Groups of five BALB/c mice were infected with *L. major* for 3 wk and then injected on 2 consecutive d with 0.5 mg of rat IgG (control), depleting anti-CD4 mAb GK1.5 (GK1.5), depleting anti-CD8 mAb (Lyt2.2), or nondepleting anti-CD4 mAb YTS177.9 (YTS). All groups except for controls were subsequently treated intraperitoneally with 1.0 mg of anti–IL-4 mAb 11B11 on day 21 and 1.0 μg of rIL-12 (1.1 × 10^3 U/dose) injected into each footpad daily for 8 d. Data represent footpad thicknesses (in millimeters) measured weekly and displayed as mean ± SEM. With the exception of GK1.5-treated mice, all groups developed progressive ulceration and footpad necrosis.

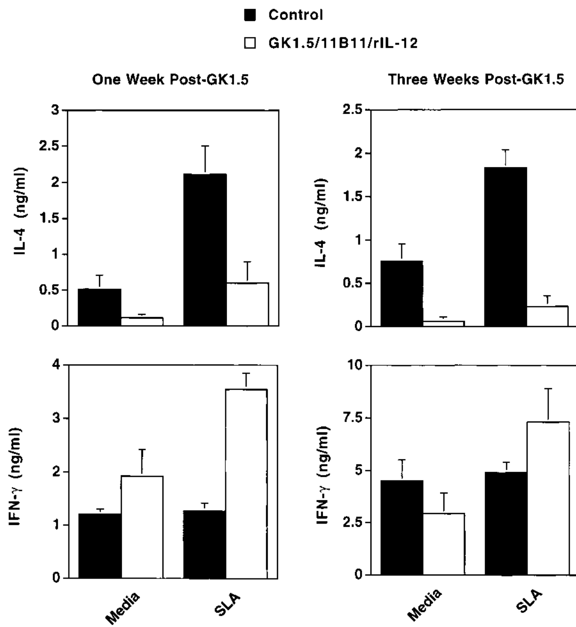

Figure 4. Early and delayed effects of GK1.5-based immunotherapy on lymph node cytokine responses. Groups of six BALB/c mice each were infected with *L. major* for 3 wk and treated with rat IgG alone (control) or GK1.5 and anti–IL-4 mAbs combined with intralesional rIL-12 as described in Fig. 2 (GK1.5/11B11/rIL-12). Lymph node cells were harvested at 7 and 21 d after the start of therapy and cultured in DMEM/10% FBS alone or in the presence of 10 μg/ml of soluble leishmania antigen. Anti–IL-4 receptor mAb (M-1) was added at 10 μg/ml in duplicate cultures to measure total production of IL-4. After 48 h of culture, supernatants were assayed by ELISA. Data represent mean cytokine concentrations (ng/ml) ± SEM for IL-4 (top) and IFN-γ (bottom). IL-4 production was decreased significantly ($P < 0.05$) for immunotherapy mice compared with control mice for all times and conditions. IFN-γ production increased significantly ($P < 0.05$) for immunotherapy mice only during wk 1.

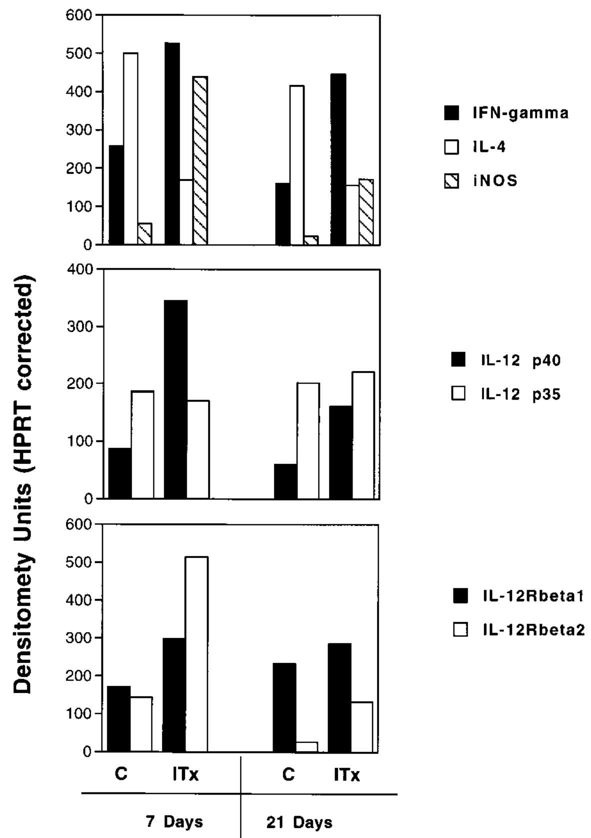

Figure 5. $CD4^+$ depletion and cytokine therapy restores Th1 type mRNA responses in vivo while suppressing IL-4 mRNA expression. RNA was harvested from lymph node tissues taken from control (C) and GK1.5/11B11/rIL-12-treated (ITx) mice at 7 and 21 d after the start of immunotherapy. Reverse transcriptase PCR was used to compare expression of selected immune response genes. Mice had been infected previously for 3 wk with *L. major.* Data represent the expression of the indicated mRNA species as determined by densitometric analysis of gel products obtained by semiquantitative reverse transcriptase PCR. Data are normalized for expression of HPRT in the same sample. Expression of IL-4, IFN-γ, IL-12 receptor β2, and iNOS mRNA was not detectable in naive lymph node tissue at the level of amplification used in these analyses. Primers used for amplification of IL-12 receptor β2 cDNA were (5′ to 3′): GCACAGACTGTTAGAGAATGC (sense) and CCTTCCTGGACACATGATATG (antisense). Primers for murine iNOS cDNA amplification were: TCACGCTTGGGTCTTGTTCACT (sense) and TTGTCTCTGGGTCCTCTGGTCA (antisense).

node cultures was reduced by 60% in the presence of anti–MHC II antibodies (difference significant at $P < 0.05$), IFN-γ declined by only 18% ($P > 0.10$) when lymph node cells from mice were cultured under the same conditions at 1 wk after treatment (data not shown). These findings indicate that starting in the first week of immunotherapy treated mice were markedly less able to produce MHC II–dependent IL-4, but maintained antigen-specific IFN-γ synthesis by MHC II–unrestricted mechanisms before $CD4^+$ T cell recovery. In contrast, IFN-γ responses were reduced 46.3 ± 7.5% in the presence of anti–MHC II at 7–8 wk after immunotherapy (Table II), indicating recovery of $CD4^+$ T cell populations responsive to leishmania antigen and biased towards the production of IFN-γ without IL-4.

Immunotherapy Leads to Sustained Increases in the Expression of mRNA Encoding Inducible Nitric Oxide Synthase, IL-12 p40, and the IL-12 Receptor β2 Subunit. Compared with control mice, expression of IFN-γ mRNA increased twofold and IL-4 mRNA expression decreased approximately threefold in the lymph nodes of treated mice at 7 and 21 d after the start of immunotherapy (Fig. 5). A separate group of 3 wk–infected BALB/c mice treated with GK1.5 anti-CD4 alone also demonstrated threefold decreases in IL-4 mRNA after 3 d (data not shown), consistent with reduced IL-4 production as a result of $CD4^+$ depletion. Although the extent to which IL-4 mRNA was decreased in vivo was less than that measured for IL-4 protein in antigen-stimulated lymph node cultures, treated mice also expressed ninefold more inducible nitric oxide synthase (iNOS) mRNA relative to control nodes. Because iNOS production is stimulated by IFN-γ, reciprocally inhibited by IL-4, and necessary for curing of infection, increased iNOS mRNA expression in treated mice confirms both the overall Th1 predominance of the in vivo cytokine response and its significance with regards to the development of leishmanicidal activity.

The induction and expansion of protective Th1 responses in leishmaniasis is dependent on IL-12 synthesis by accessory cells and functional IL-12 receptor expression by responder T cells. In this regard, the lymph nodes of treated

mice expressed threefold more IL-12 p40 mRNA than did those of control mice at 7 and 21 d after immunotherapy. This correlated with increased spontaneous secretion of IL-12 p40 protein in lymph node cultures at 21 d after immunotherapy (0.37 ± 0.11 ng/ml for treated compared with 0.19 ± 0.01 ng/ml for control mice; $P < 0.05$). Finally, IL-12 receptor β2 subunit mRNA was increased threefold in the lymph node tissue of treated compared with control mice. The IL-12Rβ2 subunit is required for transduction of IL-12–dependent signals in $CD4^+$ T cells and its rapid downregulation in BALB/c mice under neutral conditions is thought to be essential to the preferential development of Th2 cells when mice of this strain are infected with *L. major* (18, 37). Therefore, these in vivo–based findings confirm that the shift towards Th1-polarized cytokine responses after GK1.5 based immunotherapy is accompanied by enhanced IL-12 production and IL-12 responsiveness necessary for the induction and maintenance of protective immunity.

$CD4^+$ T Cell Depletion and Anti–IL-4 mAb Constitute the Minimum Effective Immunotherapy. Although these studies were initially designed to reconstitute the therapeutic effect of rIL-12 in the setting of progressive leishmaniasis, we also addressed the possibility that $CD4^+$ depletion followed by neutralization of IL-4 alone was sufficient to achieve a cure. Groups of BALB/c mice infected for 3 wk with *L. major* were treated with GK1.5 and 11B11 with or without a 10-d course of intralesional injection with rIL-12 (Fig. 6). Surprisingly, mice treated with GK1.5 and anti–IL-4 mAb healed at the same rate regardless of the presence or absence of intralesional rIL-12 in the immunotherapeutic protocol. The only notable difference between the two groups was the transient suppression of footpad swelling during the 2-wk period in which mice were actively treated with rIL-12. Furthermore, rIL-12 could not substitute for anti–IL-4 in the restoration of curative immunity in GK1.5-treated mice. In a separate experiment (data not shown), the combination of GK1.5 and rIL-12 cured only one out of six mice previously infected for 3 wk, whereas a combination of GK1.5, anti–IL-4 11B11 mAb, and rIL-12 cured four out of six mice. These data indicated that suppression of IL-4 bioactivity alone was sufficient to permit the establishment of unipolar Th1 cytokine responses after GK1.5 treatment of BALB/c mice with advanced infection with *L. major.*

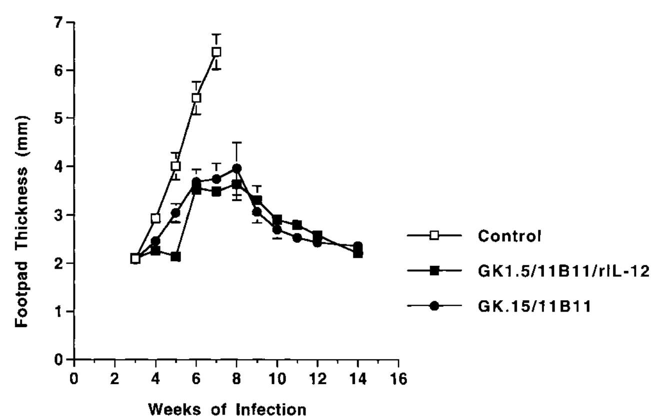

Figure 6. The combination of transient $CD4^+$ T cell depletion and anti–IL-4 mAb therapy alone is sufficient to cure *L. major*–infected BALB/c mice. Groups of five BALB/c mice each were infected with *L. major* for 3 wk and treated with saline alone (control) or with 0.5 mg of anti-CD4 mAb GK1.5 on days 21 and 22 of infection and 1.0 mg of anti–IL-4 mAb 11B11 on day 22 combined with 10 d of intralesional rIL-12 administered at 1.0 μg per footpad (1.1×10^3 U/dose) (GK1.5/11B11/rIL-12) or GK1.5 and 11B11 given without rIL-12 (GK1.5/11B11). Data represent mean footpad thicknesses (mm) ± SEM at weekly intervals.

Discussion

In these studies, we demonstrate that transient depletion of Th2-biased $CD4^+$ T cell responses in 3-wk *L. major*–infected BALB/c mice restores the therapeutic Th1-differentiating effects of anti–IL-4 mAb, with or without the addition of rIL-12. The mechanisms mediating protection included a requirement for specific depletion of $CD4^+$ T cells, as merely blocking CD4 function with nondepleting anti-CD4 antibody did not change the course of disease. Similarly, selective depletion of CD8 cells also failed to restore the cure in response to cytokine therapy and it is unlikely that the process of cellular depletion itself nonspecifically contributed to parasite clearance. Therefore, GK1.5-based immunotherapy appears to mediate protection by durably ablating Th2 $CD4^+$ T cells responsible for the progression of leishmaniasis, while permitting the eventual recovery of curative, MHC II-dependent IFN-γ responses—effectively "resetting" the $CD4^+$ T cell immune phenotype at the populational level. The protective effects of GK1.5-based immunotherapy were reproduced in five groups of mice and resulted in the cure of advanced cutaneous lesions in 20 out of 25 animals (80%). Once cured, these cutaneous lesions did not recur during a 14-wk period of observation and mice that had recovered after immunotherapy were fully resistant to reinfection with a large inoculum of *L. major.* These findings confirm the stable reversal of antigen-specific Th2 responses by a combination of $CD4^+$ elimination and therapy-directed Th1 expansion. Surprisingly, BALB/c mice with advanced infection and genetic predispositions towards Th2 development were fully capable of supporting unipolar Th1 responses provided only that IL-4 activity was neutralized at the time of $CD4^+$ T cell recovery.

Recovery of leishmanial skin lesions was notably delayed and coincided with the onset of lymphoid tissue repopulation by $CD4^+$ T cells expressing a Th1 cytokine phenotype. Footpad thickening initially continued for up to 4 wk after treatment with GK1.5, anti–IL-4 mAb, and rIL-12, with ~25% of the cutaneous lesions progressing to shallow ulceration before healing began. The parasite load within the cutaneous lesions also declined after a significant delay, with relatively minor 42-fold decreases apparent at 3 wk after treatment and a more substantial 10,000-fold reduction apparent at 7 wk after therapy. Although T cell populations of untreated animals produced both IL-4 and IFN-γ in re-

sponse to *L. major* antigen, lymph node cells from BALB/c mice treated 3 wk previously with GK1.5, 11B11, and rIL-12 produced IFN-γ in similar quantities, but with sevenfold reduced synthesis of IL-4. IL-4 production was undetectable in BALB/c mice that had been cured for 19 wk and that demonstrated resistance to infection. This was observed not only in comparison to control BALB/c mice infected with *L. major*, but also in comparison to the few mice that failed to heal after immunotherapy and that produced amounts of IL-4 no different than untreated controls. IL-4 productive capacity was similarly unchanged after ineffective treatment with anti–IL-4 and rIL-12 alone or with GK1.5 alone (data not shown). Treatment also increased expression of IL-12 p40, IL-12 receptor, and iNOS mRNA in the draining lymph nodes, indicating the biologically significant restoration of IL-12–dependent cytokine cascades and Th1-dependent leishmanicidal defenses. Because IFN-γ synthesis was greater than 50% MHC II-dependent in healing mice, these findings are most consistent with the delayed recovery of advanced lesions in response to repletion of a CD4$^+$ T cell population expressing a unipolar Th1 phenotype capable of activating nitric oxide–dependent parasite killing.

We first confirmed that monotherapy with rIL-12, anti–IL-4 mAb, or GK1.5 at wk 2–3 of infection had no lasting effect on the progression of disease, although they are curative when given in wk 1 of infection. Because rIL-12, GK1.5, and anti–IL-4 have been reported to alter the course of leishmaniasis when given as late as 14 d after infection in other studies (16, 17, 38), we studied the effects of immunotherapy at wk 3 to further ensure that monotherapy would be ineffective. Under these stringent conditions, none out of 18 mice treated with rIL-12, anti–IL-4 mAb, or GK1.5 alone were able to cure disease. Combinations of anti–IL-4 and rIL-12 therapies were also ineffective when started as early as day 7 of infection. Although IL-4 is necessary for Th2 development in *L. major*–infected BALB/c mice, the well-characterized inhibitory effects of this cytokine on IL-12 receptor expression and rIL-12–directed Th1 cell differentiation (18, 30, 31) do not appear to be necessary for maintaining the in vivo resistance of established Th2 CD4$^+$ T cells to rIL-12 therapy. Once developed, the Th2 CD4$^+$ T cells of BALB/c mice either do not require IL-4 to maintain numbers and function, or else impractically large doses of anti–IL-4 antibody are necessary to adequately neutralize IL-4 bioactivity in vivo.

However, relatively low doses of anti–IL-4 mAb were both sufficient and essential for establishing curative immunity after GK1.5 treatment of *L. major*–infected BALB/c mice. In this regard, the recovering CD4$^+$ T cells of GK1.5-treated mice appear to be functionally similar to those present in wk 1 of infection, when anti–IL-4 mAb prevents the expansion of Th2 cells by preserving IL-12 responsiveness (30). We observed residual IL-4 in GK1.5-treated animals. This probably derived from cytolysis-resistant CD4$^+$ T cells, as antigen-induced IL-4 production remained MHC II dependent in these mice. The failure to eradicate >85% of CD4$^+$ T cells in infected mice, compared with >95% in uninfected mice (data not shown), is compatible with previous demonstrations of the relative resistance to GK1.5-mediated depletion of T cells with a memory/effector phenotype (39). The residual IL-4 production by CD4$^+$ T cell–depleted mice was not immediately affected by additional treatments with rIL-12 and anti–IL-4 mAb (data not shown), but declined gradually over a 3-wk period. This suggests that anti–IL-4 mAb limited re-expansion of Th2-committed T cells in infected mice without directly inhibiting residual IL-4 synthesis, consistent with recent demonstrations of cytokine-autonomous production of IL-4 in differentiated Th2 cells (40, 41). These data otherwise do not determine whether the GK1.5-resistant cellular sources of IL-4 eventually disappear due to attrition, suppression, or phenotypic reversal at the cellular level. Instead, the recovering CD4$^+$ T cell population was biased towards production of IFN-γ. Although Th1-biasing cytokine therapy may have preferentially affected undifferentiated precursor CD4$^+$ T cells that were recent thymic emigrants (42), a subset of peripheral memory/effector cells susceptible to differentiating cytokines may have provided an alternative source for the reconstituted Th1 response (43). Further investigations are needed to distinguish between these mechanisms.

Other mechanisms may have contributed to the GK1.5-induced cure of BALB/c leishmaniasis. Our studies suggest that CD8$^+$ T cells and/or NK cells were interim sources of IFN-γ in the first week after CD4$^+$ depletion and cytokine therapy, when IFN-γ synthesis was maintained and was transiently MHC II independent. Although resistant strains of mice deficient in either β2-microglobulin or CD8$^+$ T cells remain capable of curing primary infection with *L. major* (44, 45), MHC I–restricted responses have been implicated in the late recovery and subsequent resistance to reinfection (38). Detailed studies beyond the scope of the current report will be required to determine if the CD8$^+$ T cells of disease-susceptible BALB/c mice mediate beneficial functions that are otherwise silenced in the face of functionally dominant Th2-type CD4$^+$ T cell responses. These studies also do not exclude selective tolerization of parasite-specific Th2 cells as an alternative curative mechanism for GK1.5-based immunotherapy. Treatment with cytolytic anti-CD4 mAb establishes antigen-specific immunologic tolerance and protects against Th1-mediated autoimmune pathologies in mice (46). The preservation of MHC II–dependent, *L. major*–specific IFN-γ production in GK1.5/11B11/rIL-12–treated BALB/c mice indicates that any tolerizing effects were limited to Th2-inducing antigens, which are known to protect against disease when induced by other methods (47). However, noncytolytic anti-CD4 mAb YTS177 that tolerizes developing immune responses in other disease models (36) failed to cure leishmaniasis in combination with anti–IL-4 mAb and rIL-12. These findings therefore favor depletion as the relevant protective mechanism.

The cure of established murine leishmaniasis by depletion-induced phenotypic "resetting" of the CD4$^+$ T cell population is not the first form of successful immunotherapy in this model of T cell–dependent pathology, but the mechanisms involved appear to be distinct. For instance,

cure by combined treatment with rIL-12 and sodium stibogluconate was associated with a Th2 to Th1 shift only after parasite burden was reduced (21). Although the central role of Th2 cytokines in the initiation of progressive leishmaniasis is well characterized, increased parasite burdens may subsequently contribute to sustaining the susceptible state by independently biasing towards Th2 differentiation and function. For instance, leishmania-infected accessory cells are less able to produce IL-12 and to respond to IFN-γ (19, 48). Increased antigenic stimulation of BALB/c T cell receptors also independently favors the differentiation of T cells with Th2 phenotypes (20). Finally, CD28-directed costimulatory signals are necessary for Th2, but not Th1 immune responses in murine leishmaniasis and further implicate accessory cell function in regulating $CD4^+$ cytokine polarity (49, 50). However, mice cured by "CD4 reset" showed their greatest declines in parasite burden after the recovery of Th1 $CD4^+$ T cell responses at wk 3 of therapy. This is most consistent with parasite killing as a result of Th1 immune deviation within the $CD4^+$ T cell population, rather than immune deviation in response to reduced infectious burden. Therefore, these findings suggest that increased parasite load, increased antigen presentation, or altered accessory cell costimulatory function are not sufficient by themselves to reconstitute a Th2-biased response after $CD4^+$ T cell depletion. We conclude that the perpetuation of cytokine-resistant Th2 T cell responses in progressive leishmaniasis is dependent on properties of the $CD4^+$ T cell and is not independently determined by infection-induced changes in the non-$CD4^+$ T cell immune environment.

These studies are significant more generally in that they demonstrate the potential to reverse established, pathologic $CD4^+$ T cell responses by combined antibody-directed cytolysis and subsequent treatment with differentiating cytokines or anticytokine antibodies. Our ability to modulate Th2 responses is especially remarkable because of the well-recognized stability of this phenotype to immunotherapeutic reversal (16, 32, 51, 52) and indicates the potential for use as immune-deviating therapy in other Th2-dependent experimental and clinical diseases. A similar approach to the reversal of severe allergic disorders is technically feasible, as cytolytic anti–human CD4 antibodies are already in experimental use for autoimmunity, and Th1-deviating agents, such as soluble IL-4 receptor and rIL-12, are in various stages of clinical investigation. By analogy, Th1-dependent autoimmune diseases might also be reversible if stable deviation towards a Th2 phenotype results in disease suppression (53, 54). The pathogenic Th1 responses of murine autoimmune encephalomyelitis, lupus nephritis, and collagen-induced arthritis can be prevented by pretreatment with anti-CD4 antibodies that tolerize against the inducing antigen. However, as in leishmaniasis, established and progressive forms of disease only briefly remit after short-term treatment with anti-CD4 mAb (10, 36). We speculate that GK1.5 treatment of advanced autoimmunity might restore $CD4^+$ T cell populations with naive-like susceptibility to the Th2-deviating effects of anti–IL-12 antibodies or rIL-4 that otherwise only prevent disease expression when given at the time of sensitization to self-antigen (55, 56). In conclusion, these studies show that curative and unipolar Th1 cellular responses are recovered in heavily parasitized, disease-susceptible mice merely by transient depletion of $CD4^+$ T cells and neutralization of residual IL-4 bioactivity. Because $CD4^+$ T cell depletion provides a unique window of opportunity to durably reprogram the cytokine polarity of an established and pathogenic T cell response, these findings suggest a novel immunotherapeutic strategy potentially applicable to the clinical amelioration of progressive allergic and autoimmune disorders.

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