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Immunology

Production of interferon γ, interleukin 2, interleukin 4, and interleukin 10 by CD4+ lymphocytes *in vivo* during healing and progressive murine leishmaniasis

(T-cell subsets/interleukins/interferon γ)

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ABSTRACT **The expression of interleukin (IL) 2, IL-4, IL-10, and interferon γ (IFN-γ) by lymphocyte subsets was examined during infection of resistant C57BL/6 and susceptible BALB/c mice with the protozoan parasite *Leishmania major*. CD4+ and CD8+ T lymphocytes and B lymphocytes were isolated from the lymph nodes draining infectious lesions, and their RNA was examined for lymphokine transcripts. Distinct patterns of CD4+ cell cytokine expression were apparent: C57BL/6 CD4+ cells contained IFN-γ and IL-2 mRNA, whereas BALB/c CD4+ cells expressed IL-4 and IL-10 message. CD8+ cells contributed little lymphokine expression during disease, but B cells were a major source of IL-2 mRNA in both strains of mice. BALB/c mice made resistant by treatment with anti-CD4 antibody at the time of infection repopulated lymph nodes with CD4+ cells that expressed IL-2 and IFN-γ. Protective treatment with anti-IL-4 antibody *in vivo* also resulted in the appearance of CD4+ cells with increased IFN-γ and diminished IL-4 and IL-10 expression. These data establish CD4+ cells as the primary source of IFN-γ in healing mice and of IL-4 and IL-10 during progressive infection and confirm that the spectral extremes of this disease are characterized by the presence of CD4+ cells expressing Th1 or Th2 phenotypes *in vivo*.**

Leishmania cause a spectrum of infectious diseases in mammalian hosts ranging from self-healing cutaneous ulceration to progressive visceral dissemination (1). Strains of inbred mice experimentally infected with *Leishmania major* reproduce this spectrum of disease in association with distinct lymphokine responses. Notably, cells from lymph nodes draining the cutaneous lesions of resistant C57BL/6 mice produce interferon γ (IFN-γ), whereas cells from susceptible BALB/c mice generate interleukin 4 (IL-4) (2). Neutralization of IFN-γ *in vivo* abolishes resistance to progressive disease, consistent with the central role for this lymphokine in the activation of macrophages to kill the intracellular amastigotes (3). Conversely, neutralization of IL-4 *in vivo* allows otherwise susceptible BALB/c mice to heal (2). The detrimental role for IL-4 is not well understood; both direct inhibition of IFN-γ-mediated macrophage activation to kill leishmania (4, 5) and abrogation of IFN-γ production have been described (6).

These distinct patterns of lymphokine production during self-limited and progressive infection resemble the dichotomous secretory profile of cloned CD4+ subsets (7). Th1 cells produce IL-2 and IFN-γ in response to stimulation, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10. Additional cytokines are produced by both cells. These subsets are thought to arise from CD4+ precursor cells with less distinctive cytokine profiles; the mechanisms favoring the development of Th1 and Th2 cells remain incompletely understood. Antigen-specific CD4+ T-cell lines and clones that express either Th1 or Th2 phenotypes confer resistance or susceptibility, respectively, to Leishmania after transfer into naive hosts, suggesting that these cells can mediate polar responses to infection (8–10).

These observations have not demonstrated conclusively that reciprocal expansions of Th1 and Th2 CD4+ lymphocyte subsets occur *in vivo* during infection with *L. major*, however. The types of CD4+ cells obtained after *in vitro* growth and expansion may reflect subset biases introduced by the kind of antigen (8), the presence of exogenous cytokines (11), and the kind of antigen-presenting cell used (12). For instance, Th1 cells have been cloned from BALB/c mice with progressive disease and Th2 cells from BALB/c that have healed infection after sublethal irradiation (13). Further, IL-2, IL-4, and IFN-γ may be produced by non-CD4+ cells. Data supporting a protective role for CD8+ lymphocytes during murine leishmaniasis have been reported recently (14–16), and these cells are capable of IL-2 and IFN-γ production in other systems (17, 18). Similarly, natural killer (NK) cells are capable of generating IFN-γ (19). We assayed cytokine expression in isolated CD4+, CD8+, and surface immunoglobulin-positive (sIg+) cells from C57BL/6 and BALB/c mice infected with *L. major* to identify the cellular source of lymphokines associated with the Th1 and Th2 phenotypes.

MATERIALS AND METHODS

Mice. Female BALB/c and C57BL/6 mice (6-week-old) were purchased from Charles River Breeding Laboratories and kept in the University of California, San Francisco Animal Care Facility. Animals were infected in the hind footpads with 4×10^6 stationary-phase *L. major* promastigotes, as described (2). In selected experiments, BALB/c mice received 0.5 mg of GK1.5 monoclonal antibody (mAb) i.p. the day before infection or 1 mg of 11B11 mAb on the day of infection (20, 21).

mAbs and Magnetic Beads. Ascites-derived mAb GK1.5 (anti-murine CD4; IgG2b, American Type Culture Collection), mAb 2.43 (anti-murine CD8; IgG2b, ATCC), and mAb 11B11 (anti-murine IL-4; IgG1, ATCC) were produced and purified as described (20). Antibodies were biotinylated in designated experiments (22). Fluorescein isothiocyanate (FITC)-linked or biotinylated anti-B220 (Caltag, South San Francisco, CA), anti-rat IgG (mouse serum adsorbed), and anti-mouse IgG/IgM (Kirkegaard and Perry Laboratories,

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Abbreviations: IL, interleukin; IFN-γ, interferon γ; NK, natural killer; sIg+, surface immunoglobulin-positive; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; HBSS/FCS, Hanks' balanced salt solution/fetal calf serum.

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Gaithersburg, MD) were purchased. FITC-conjugated PK136 (anti-NK1.1, IgG_{2b}) was the gift of W. Seaman (San Francisco VA Medical Center, San Francisco, CA). Tosyl chloride-activated ferrous beads (Dynabeads; Robbins Scientific, Mountain View, CA) were coated with avidin by overnight incubation in buffered boric acid (0.5 M, pH 9.5) containing avidin at 75 μg/ml (Extravidin; Sigma) followed by extensive washing in phosphate-buffered saline with 0.1% bovine serum albumin.

Nucleic Acid Probes. Nucleic acid probes specific for murine IL-2, IL-4, and IFN-γ have been described (2). Subclones of murine CD4 (1.5 kb) and murine CD8 (0.6 kb) cDNA in pSP64 (Promega) were gifts from D. Littman [University of California, San Francisco (23)]. IL-10 cDNA was a gift from K. Moore [DNAX, Palo Alto, CA (24)].

Isolation of Lymphocyte Subsets. All procedures were done at 4°C. Lymph nodes were minced and passed through a 200-mesh stainless-steel screen. The cell suspension was washed twice in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS)/1% fetal calf serum (FCS). Approximately 4×10^8 cells in 2 ml of HBSS/FCS were incubated with biotinylated anti-CD4 or anti-CD8 antibody at 20 μg/ml for 30 min while gently mixed on a rotary platform. Cells were washed three times with HBSS/FCS and gently mixed for 15 min with avidin-coated ferrous beads (five beads per target cell). The bead–cell conjugates were harvested by using a magnetic separator and washed twice in HBSS/FCS. sIg^+ B cells were incubated with anti-mouse IgG-coated ferrous beads (Robbins Scientific) using three beads per cell and separated magnetically. After isolation, cells were lysed with 6 M guanidine hydrochloride for RNA extraction. Actinomycin D (2 μg/ml) was added in selected cases to prevent *de novo* transcription. Surface Ig^+, $CD4^+$, and $CD8^+$ cells were isolated sequentially in designated experiments, and the remaining cells were saved for study. To confirm the efficacy of isolation, aliquots of bead-bound cells were incubated in RPMI medium/10% FCS overnight at 37°C in 5% CO_2 to allow release of cells into the medium. The beads were removed in a magnetic field, and the cells were analyzed by flow cytometry (FACScan, Becton Dickinson) with fresh anti-CD4, anti-CD8, and FITC-conjugated anti-rat antiserum (mouse serum adsorbed). B cells were labeled with FITC-linked anti-murine IgG/IgM or anti-B220 antibodies. C57BL/6 NK cells were quantitated by flow cytometry by using FITC-linked anti-NK1.1. Mast cells in frozen-tissue sections or in cytocentrifuge preparations were identified by their characteristic staining after incubation with Wright–Giemsa (DiffQuick, American Scientific Products, Stone Mountain, GA).

RNA Preparation and RNA Hybridization. RNA was purified, subjected to electrophoresis, and transferred to nylon membranes as described (2). IFN-γ and IL-4 antisense probes were generated by using [^{32}P]CTP (3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) and SP6 RNA polymerase (Promega). CD4, CD8, IL-10, and IL-2 cDNA were labeled in the presence of [^{32}P]dCTP (800 Ci/mmol, New England Nuclear) by using random hexamer priming and Klenow polymerase (Amersham). Membranes were hybridized with labeled probes and exposed to x-ray film.

RESULTS

Purification of Cells by Magnetic Beads. Rapid purification of lymphocytes expressing CD4, CD8, and surface immunoglobulin could be established from heterogeneous lymph node populations by incubation with the designated antibodies and magnetic bead ligands and subsequent selection in a magnetic field. A typical enrichment as assessed by flow cytometry analysis was 93% for $CD4^+$ cells, 85% for $CD8^+$ cells, and 96% for sIg^+ cells (Fig. 1). Cells remaining after

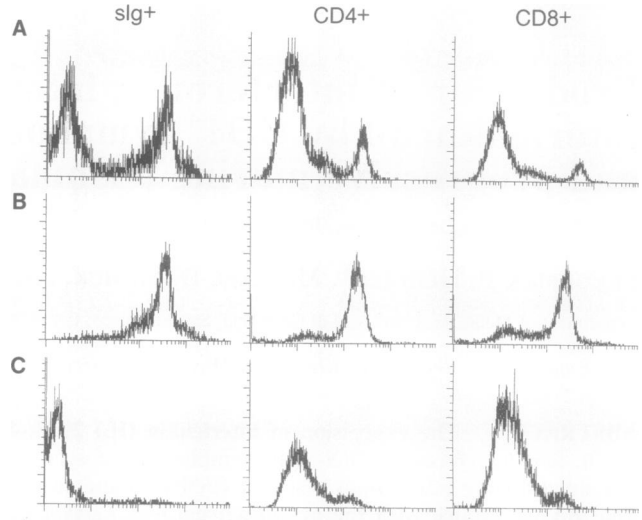

FIG. 1. Immunofluorescence profiles of lymphocyte populations expressing the designated surface antigens before and after magnetic-bead selection. Cellular suspensions obtained from the draining lymph nodes of C57BL/6 mice infected with *L. major* were aliquoted for separate isolations of sIg^+, $CD4^+$, and $CD8^+$ cells, as indicated by each column. Rows represent immunofluorescence observed for cells before bead selection (*A*), cells positively selected by adherence to bead ligands (*B*), and cells remaining after bead selection (*C*). $CD4^+$ and $CD8^+$ cells were labeled by using rat anti-CD4 or anti-CD8 mAb and FITC-linked anti-rat IgG. B cells were labeled by using FITC anti-mouse IgG; identical results were obtained by using FITC anti-B220. *x* axis, logarithm of fluorescence intensity; *y* axis, number of cells counted.

sequential selections for these three populations consisted of 10% $CD4^+$, 6% $CD8^+$, 10% sIg^+ cells, and 34% $NK1.1^+$ cells. The remaining cells were composed of neutrophils, macrophages, and eosinophils. Although mast cells were present in tissue sections of infected lymph nodes, <1% of the cells in the depleted cell population were mast cells as assessed by Wright–Giemsa staining.

$CD4^+$ Cells Are the Principal Source of IL-4, IL-10, and IFN-γ Expression *in Vivo* During Murine Leishmaniasis. $CD4^+$, $CD8^+$, and sIg^+ lymphocytes were isolated from the draining lymph nodes of resistant C57BL/6 mice and susceptible BALB/c mice at 5 weeks of infection, when the course of disease is divergent in the two hosts and lymphokine expression is maximal (25). Hybridization with labeled IFN-γ antisense RNA showed that $CD4^+$ cells were the major source of this lymphokine in C57BL/6 mice (Fig. 2). Prolonged autoradiographic exposures showed only minimal amounts of IFN-γ mRNA in the $CD8^+$ cells from C57BL/6 and in the $CD4^+$ and $CD8^+$ cells from BALB/c mice. IL-2 was also expressed by $CD4^+$ cells from healing mice but not by $CD4^+$ cells from nonhealing BALB/c mice. Unexpectedly, B cells produced IL-2 in infected C57BL/6 mice and were the only source of this factor in infected BALB/c mice. Splenic B cells from uninfected BALB/c mice similarly contained IL-2 mRNA (Fig. 3). Continuous exposure to actinomycin D during isolation did not alter these results, making it unlikely that cross-linking of surface immunoglobulin by the beads activated IL-2 synthesis *ex vivo*. Selection of cells using the B-cell-specific B220 antigen as ligand gave comparable results (data not shown). Hybridization with CD4- and CD8-specific probes (Fig. 3) verified that the sIg^+ cell population was free of contamination by $CD4^+$ or $CD8^+$ T cells.

$CD4^+$ cells from BALB/c mice with progressive infection contained abundant mRNA for the Th2 lymphokines, IL-4 and IL-10 (Fig. 2). There was no apparent mRNA for IL-4 and minimal mRNA for IL-10 in the $CD4^+$-enriched population

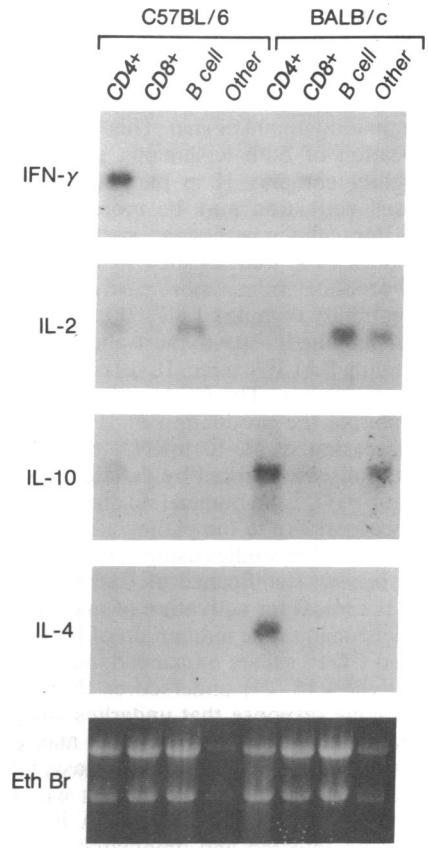

FIG. 2. RNA blot analysis (20 μg of total RNA per lane) extracted from $CD4^+$, $CD8^+$, and sIg^+ lymph node cells from resistant C57BL/6 (left lanes) and susceptible BALB/c mice (right lanes) infected 5 weeks previously with *L. major*. Columns indicate RNA isolated from the designated lymphocyte subsets. Lanes designated Other contain RNA from cells remaining after sequential selection for $CD4^+$, $CD8^+$, and sIg^+ lymphocytes. These samples were successively hybridized with the indicated ^{32}P-labeled nucleic acid probes. The agarose gel was stained with ethidium bromide (Eth Br) before transfer to assess uniformity of RNA loading. The lymph nodes from five infected mice of each group were pooled before magnetic-bead selections.

from healing C57BL/6 mice. Although BALB/c $CD4^+$ cells were the only source for IL-4, IL-10 was present in both $CD4^+$ cells and the cell population previously depleted of sIg^+, $CD4^+$, and $CD8^+$ cells (Fig. 2, Other).

Transient Depletion of $CD4^+$ Cells or Treatment with Anti-IL-4 Antibody Promotes Th1 Responses in BALB/c Mice During Subsequent Infection. Lymphocyte subsets were purified from the lymph nodes of resistant C57BL/6 and of BALB/c mice that control infection after transient depletion of $CD4^+$ cells (mAb GK1.5-pretreated). At the time of harvest (4 weeks), $CD4^+$ cells had repopulated the nodes of mAb GK1.5-pretreated mice and constituted 16% of the cell population. This reconstituted $CD4^+$ population showed a Th1 pattern of lymphokine expression comparable to that in C57BL/6 mice (Fig. 3). There was no evident shift of lymphokine production from $CD4^+$ to $CD8^+$ cells at this time. None of the cell populations from these healing mice produced detectable IL-4 mRNA (data not shown). RNA from these isolated subsets showed distinct segregation of CD4 and CD8 message by Northern analysis, confirming the efficacy of the purification procedure.

A single 1-mg injection of mAb 11B11 at the time of infection also protects BALB/c mice against progressive disease and was demonstrated in prior studies to augment IFN-γ and diminish IL-4 expression in unselected lymph

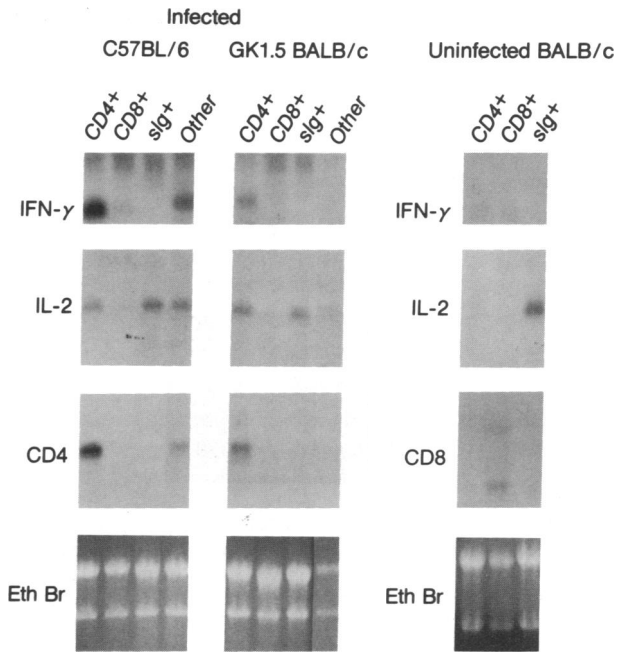

FIG. 3. Abundance of IL-2, IFN-γ, CD4, and CD8 mRNA in lymphocyte subsets isolated from the lymph nodes of resistant C57BL/6 and mAb GK1.5-pretreated BALB/c mice infected 4 weeks previously with *L. major* (*Left*) and the spleens of uninfected BALB/c mice (*Right*). Autoradiographic exposures were approximately twice as long for analysis of normal spleen subsets as for the infected animals. Each lane contains 20 μg of total RNA as confirmed by ethidium bromide (Eth Br) staining. Organs from five animals in each group were pooled before selection.

node populations (20). When examined 5 weeks after infection, the $CD4^+$ cells obtained from draining lymph nodes of anti-IL-4-treated mice expressed more IFN-γ and less IL-4 than did the $CD4^+$ cells of untreated BALB/c mice (Fig. 4), consistent with a shift from a Th2 to a Th1 phenotype in this population. IL-10 mRNA was most abundant in $CD4^+$ cells

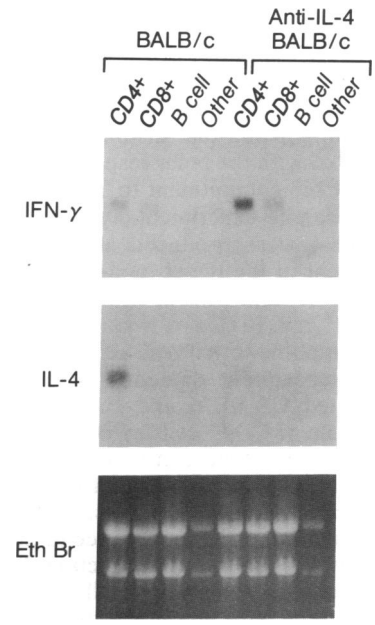

FIG. 4. Abundance of IL-4 and IFN-γ mRNA in lymphocyte subsets derived from the draining lymph nodes of susceptible BALB/c mice (*Left*) and BALB/c mice treated with anti-IL-4 antibody (*Right*). Each lane contains 20 μg of total RNA. The lymph nodes from six animals were pooled before selection.

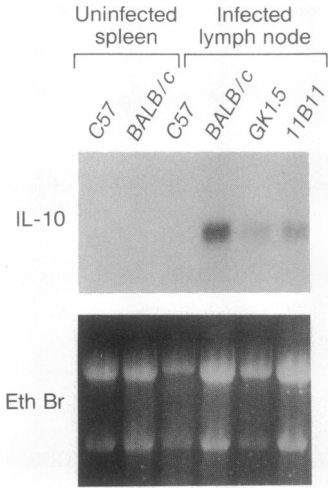

FIG. 5. Abundance of IL-10 mRNA in CD4$^+$ cells from uninfected spleen and infected lymph nodes from the designated animals. BALB/c mice were treated with mAb GK1.5 or mAb 11B11 (anti-IL-4) before infection, as described in text. Organs from four animals in each group were pooled. Each lane contains 20 μg of total RNA. Eth Br, ethidium bromide.

from BALB/c lymph node during infection and was diminished in cells from mice protected by mAb GK1.5 or anti-IL-4 therapy (Fig. 5). IL-10 mRNA was not present in CD4$^+$ cells obtained from infected C57BL/6 lymph nodes or from uninfected C57BL/6 or BALB/c spleen.

DISCUSSION

Prior studies have demonstrated that IFN-γ and IL-4 are reciprocally produced during the extremes of leishmaniasis (2, 20), although direct evidence for the existence of Th1 and Th2 cells during chronic infection *in vivo* has not been shown. These studies clearly delineate CD4$^+$ cells as the major source of IFN-γ produced in the draining lymph nodes of mice healing infection, as well as the principal source of IL-4 and IL-10 in mice with progressive leishmaniasis. Furthermore, BALB/c mice rendered resistant by pretreatment with anti-CD4 or anti-IL-4 antibody showed diminished Th2 lymphokine expression and corresponding increases in Th1 expression in the CD4$^+$ cell population. These data strengthen the supposition that distinct subsets of CD4$^+$ T cells are associated with the polar responses to *L. major* and demonstrate that the commitment to Th1 or Th2 responses during infection can be experimentally manipulated *in vivo*.

IFN-γ and IL-4 were produced almost exclusively by CD4$^+$ cells present in the lymph nodes draining cutaneous lesions caused by *L. major*. In experiments not shown, acute depletion of CD4$^+$ cells *in vivo* by treatment with mAb GK1.5 a day before lymph-node harvest at 5 weeks of infection abrogated the subsequent detection of IFN-γ and IL-4 mRNA in infected C57BL/6 and BALB/c mice, further supporting the role of CD4$^+$ cells as the primary sources of these lymphokines. The failure of IFN-γ message to become enriched in cells greatly depleted of CD4$^+$, CD8$^+$, and sIg$^+$ cells and enriched in NK1.1$^+$ cells (Figs. 2–4, Other) suggests that NK cells are not a significant source of this lymphokine in the draining lymph nodes during infection. Mast cells were not released from tissue into single cell suspension, and we could not assess the contribution of these cells to the generation of IL-4 in intact tissues. We were unable to document the presence of significant amounts of IL-2 and IFN-γ message in CD8$^+$ lymphocytes *in vivo* despite prior evidence suggesting a protective role for these cells against *L. major* (14–16) and the ability of CD8$^+$ cells to produce Th1-like lymphokines under selected conditions (18, 26). The preferential activation of CD4$^+$ cells in leishmaniasis, as assessed by lymphokine expression, suggests that parasite antigens expressed in association with major histocompatibility complex II are immunodominant *in vivo*. This fact may reflect the lysosomal location of both leishmania and nascent major histocompatibility complex II in parasitized macrophages (27). CD8$^+$ cell activation may be more characteristic of infection with intracellular pathogens, such as *Listeria monocytogenes* and viruses, that localize to the cytoplasm and consequently produce antigen more easily accessible to major histocompatibility complex I (17, 28).

The Th2 lymphokine IL-10 was more abundant in the CD4$^+$ cells of nonhealing BALB/c mice. IL-10 is a newly described lymphokine produced by Th2 CD4$^+$ cells that is capable of markedly inhibiting the production of IFN-γ (24, 29). The enhanced expression of IL-10 mRNA during progressive leishmaniasis, a disease marked by deficient IFN-γ production in both murine (25) and human (30) disease, suggests that Th2 cells may contribute to the pathogenesis of infection by releasing IL-10. Further studies using neutralizing mAbs may establish the *in vivo* significance of IL-10 production. Because IFN-γ is critical for activation of macrophages to kill intracellular leishmania, the diminution of IFN-γ production by both biased CD4$^+$ subset expansion and IL-4 and IL-10 inhibitory activities (6, 24) underscores the profound dysfunctional immune response that underlies susceptibility to this organism. Similar immune responses may characterize the spectrum of human visceral leishmaniasis (30, 31).

An unexpected finding of these studies was that B cells were an abundant source of IL-2 mRNA in the lymphoid tissues from both infected and uninfected mice. Splenic B cells have been reported to produce IL-2 when cultured with mitogens (32), but a similar function *in vivo* has not been described. This observation may explain why IL-2 and IFN-γ mRNA expression did not cosegregate during murine leishmaniasis in earlier studies using total, rather than fractionated, lymph node cell populations (2). The relevance of B-cell-derived IL-2 in the immunology of murine leishmaniasis is currently unclear. B-cell depletion from birth in BALB/c mice has a significant protective effect, despite the absence of a demonstrated role for antibody in the outcome of disease (33). The possible involvement of B-cell-derived IL-2 in the promotion of IL-4 production (34) and Th2 differentiation *in vivo* (35, 36) may account for the benefits of B-cell depletion before infection with *L. major*.

Both transient depletion of CD4$^+$ cells using mAb GK1.5 and *in vivo* neutralization of IL-4 with mAb 11B11 promote subsequent healing of leishmaniasis in otherwise susceptible BALB/c mice (2, 21, 25). Our data suggest that these manipulations favor CD4$^+$ differentiation along a Th1 pathway, a response likely to contribute to the observed protection. The mechanism by which transient depletion of CD4$^+$ cells alters subset commitment is unclear; possibly CD4$^+$ cells newly emerged from the thymus more readily differentiate to Th1 cells. Because IL-4 is required for the maturation of CD4$^+$ precursors to the Th2 phenotype (36), neutralization of this factor *in vivo* would be expected to block both Th2 expansion and the subsequent formation of Th2-derived inhibitory factors, such as IL-4 and IL-10, that limit protective Th1 responses (29). Neutralization of IL-4 may additionally benefit the infected host by removing the deleterious effects of IL-4 on macrophage activation and killing of intracellular leishmania (4, 5). The ability to manipulate Th1 and Th2 cell maturation in murine leishmaniasis provides a valuable model for further dissection of signals modulating disparate CD4$^+$ cell effector pathways.

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