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Cytokines in the Differentiation of Th1/Th2 CD4+ Subsets in Leishmaniasis

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Abstract *Leishmania major* infect only macrophages in the host, where they reside in endolysosomal compartments into which MHC class II molecules co-localize. Experimental infection in mice has provided a useful model for the differentiation of Th1 CD4+ effector lymphocytes that are required for the generation of IFN-γ that activates the macrophage to a microbicidal state. Genetically susceptible BALB/c mice aberrantly activate Th2 CD4+ effector cells that are ineffective in arresting infection. Increasing evidence suggests that, rather than discrete parasite antigens or MHC molecules, cytokines mediate the critical decision in the developmental switch to either the Th1 or Th2 effector phenotype. © 1993 Wiley-Liss, Inc.

Key words: pathogenesis, protozoan, parasite, promastigote, kala-azar

Understanding the pathogenesis of infectious diseases that have varying penetrance within a population will be important for the development of effective vaccines and for determining the genetic basis that underlies susceptibility. This review focuses on experimental murine leishmaniasis as a model system for studying the genetic regulation of polarized immune responses that facilitate either the establishment of effective immunity or the failure to control replication of this intracellular protozoan parasite.

THE PARASITE AND THE DISEASE

Leishmania species are protozoan parasites transmitted to mammalian hosts by the bite of phlebotomine sandflies. The parasite exists as a flagellated promastigote within the gut of the sandfly vector. Upon appropriate maturation of a major outer membrane molecule, lipophosphoglycan (LPG), the organisms are released from the gut epithelium and move to the proboscis, where they are introduced at the time of a blood meal. Mature organisms are resistant to the hemolytic effects of complement and instead use deposited complement to target themselves to host macrophages through the CR1 (in the case of *L. major*) or CR3 (in the case of *L. donovani*) receptors [1]. The promastigotes rapidly convert to intracellular, aflagellate forms, or amastigotes, that replicate by binary fission, ultimately resulting in lysis of the cell, and spread to surrounding macrophages. Cure of disease requires activation of macrophages by T cell–derived lymphokines to a microbicidal state mediated by reactive nitrogen or oxygen products. Disease caused by Leishmania ranges from milder cutaneous forms through more severe disfiguring mucocutaneous ulceration to disseminated visceral disease (kala-azar) with parasitization of macrophages throughout the reticuloendethelial system. An estimated 16 million persons are infected with these parasites worldwide.

Within areas endemic for visceral leishmaniasis, some 10–20 cases of asymptomatic infection, as assessed by acquisition of skin test positivity to Leishmania antigens, occur for each case of disseminated disease [2]. Further, patients cured of disease using antimicrobial therapy gain solid immunity to reinfection, despite continuing to live in the endemic area. These observations suggest some underlying susceptibility trait(s) and the possibility of successful vaccination of naive hosts against disease.

IMMUNE RESPONSES

Among humans infected with Leishmania, resistance correlates with a positive delayed type

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hypersensitivity (DTH) response and the capacity of peripheral blood lymphocytes to generate interferon-gamma (IFN-γ) when stimulated with parasite antigens in vitro [3]. In contrast, patients with kala-azar have marked hypergammaglobulinemia and remain skin test negative, and their lymphocytes do not produce IFN-γ during stimulation in vitro. These patients have lymphocytes that produce interleukin 10 (IL-10) when stimulated in vitro [BJ Holaday and RM Locksley, unpublished observations], and about one-third have IL-4 detected in the serum [4,5]. These data support the concept that the development of cell-mediated immunity (CMI) capable of activating host macrophages underlies successful control of the infection, whereas humoral immunity represents aberrant and ineffective host responses.

These observations suggest that some genetic basis for susceptibility exists for these polarized outcomes, a hypothesis supported using experimental infection of inbred strains of mice. Most strains of mice, including C57BL/6 or C3H/HeN, control an inoculation of *L. major* into the footpad. These mice develop positive DTH responses, and CD4+ cells isolated from lymph nodes draining the lesion contain mRNA transcripts for IFN-γ [6] and produce IFN-γ following stimulation with parasite antigens in vitro [7]. In contrast, mice on a BALB background, best typified by BALB/c, develop progressive disease with ulceration at the site of parasite inoculation and dissemination of the parasite to the spleen and bone marrow. CD4+ lymphocytes from these mice contain transcripts for IL-4 and IL-10 [6] and are incapable of activating infected macrophages to a leishmanicidal state. The inability of BALB/c mice to control infection is not due to an inherent deletion of the requisite T cells, however, since a number of immunologic manipulations, including sublethal irradiation [8], or administration of antibodies to CD4 [9] or IL-4 [10] at the time of infection, allow these mice to heal and develop solid immunity. In each case, healing has been associated with the development of CD4+ T cells containing IFN-γ in the draining lymph nodes.

TH1 AND TH2 SUBSETS

In the mid 1980s, Mosmann and Coffman described a functional and phenotypic dichotomy among long-term antigen specific, murine CD4+ T helper clones [11]. Th1 clones produced high levels of IFN-γ, lymphotoxin, and IL-2 and mediated DTH and IgG2a isotype switching. In contrast, Th2 clones produced high levels of IL-4, IL-5, IL-6, and IL-10 and promoted B cell growth and differentiation, eosinophil and mast cell growth and activation, and IgE and IgG1 isotype switching. The actions of each subset tended to inhibit the function of the other: IFN-γ downregulated proliferation of Th2 clones and the release of IL-10 while IL-4 and IL-10 markedly abrogated the target effects and release of IFN-γ.

These established Th1 and Th2 effector populations are now known to mediate the polarized outcomes of experimental murine leishmaniasis due to *L. major*. As noted above, there is reciprocal expansion of these cell types during infection and, importantly, Leishmania-specific Th1 or Th2 cell lines or clones mediate resistance or disease exacerbation, respectively, following adoptive transfer into irradiated or congenitally immunodeficient mice [12–14]. Interventions that reverse these disease phenotypes have helped to decipher some of the cross-regulatory signals between these subsets. Murine leishmaniasis has become an excellent model for studying Th1 and Th2 regulation, and this paradigm has been extended to a number of infectious, allergic, and autoimmune disorders, including those that afflict humans.

DIFFERENTIATION: CONVERGENT OR PARALLEL?

Naive T cells characteristically produce only IL-2 following stimulation with cognate antigen. After several rounds of antigenic stimulation, however, T helper cells begin to acquire patterns of cytokine production that allow their segregation into Th1 or Th2 subsets. Frequently an intermediate, less differentiated stage in which an overlapping cytokine pattern, including IL-2, IL-4, and IFN-γ, termed Th0, may be encountered during differentiation, although this Th0 may also represent a stable effector phenotype. Of importance for questions of genetic susceptibility and vaccine development is whether these cell types arise from common or distinct precursors.

Earlier studies in the *L. major* system demonstrated that distinct antigens could induce the selective maturation of distinct T helper subsets [12,15,16], suggesting that different precursor T cells might explain the polarized immune response in this disease. However, more recent

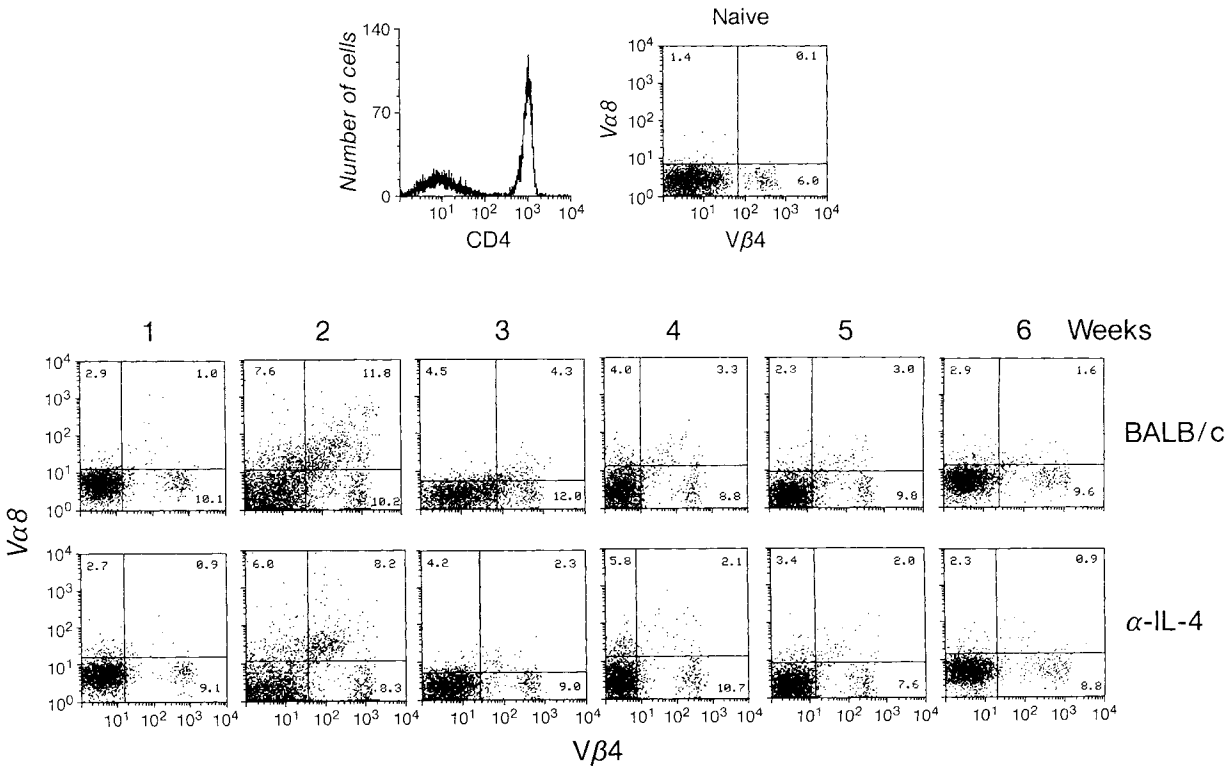

Fig. 1. Time course of Vα8-Vβ4 expression on CD4+ cells during experimental leishmaniasis. Popliteal lymph node cells from nohealer (BALB/c) and healer (anti–IL-4–treated BALB/c) mice were analyzed at designated time points following infection with three-color flow cytometry using monoclonal antibodies against CD4, Vα8, and Vβ4. For V region analysis cells were pre-selected by gating on the CD4+ peak **(upper left panel).** For the two channel dot-plots, cells expressing CD4 only are in the left lower quadrant, cells expressing CD4 and Vα8 are in the left upper quadrant, cells expressing CD4 and Vβ4 are in the right lower quadrant, and cells expressing CD4, Vα8, and Vβ4 are in the right upper quadrant. Uninfected ("naive") mice use the Vα8-Vβ4 heterodimer on 0.1% of CD4+ cells **(upper right panel).** Although maximal expansion was evident among Th1 and Th2 responses at 2 weeks, cells of this clonotype were still over-represented at 6 weeks following infection **(lower panels).**

investigations using conventional antigens and cells from T cell receptor (TCR) transgenic mice documented that a CD4+ T cell expressing an identical TCR could differentiate into either phenotype in a manner dependent upon external culture conditions [17,18]. In particular, inclusion of IL-4 in the culture media was absolutely required for the development of Th2 cells. Similar results were obtained in various in vitro manipulations using bulk cultures [19].

Analysis of the response in vivo to a complex antigenic mixture remains difficult because the implicated parasite antigens remain unknown. To circumvent this difficulty, we indirectly characterized the antigen-specific response through an analysis of TCR usage by CD4+ T cells used by resistant or susceptible strains of mice during naturally progressive or controlled infection [20]. Since TCR usage by a population of T cells reflects underlying clonal expansion, responses to the same antigens would be predicted to share TCR types, whereas responses to distinct antigens would be predicted to have diverse TCR types. Surprisingly, characterization of the TCR response following *L. major* infection revealed an oligoclonal expansion of a single TCR type that expressed the Vα8-Vβ4 heterodimer among the CD4+ population which occurred in both progressive and healing infection (Fig. 1) and across several MHC haplotypes [20]. This TCR appears to delineate an immunodominant antigen by virtue of its relatively unique expansion compared to other TCR types. A Th1 clone using this TCR phenotype was capable of adoptively transferring resistance in the murine system [14], but a Th2 clone with the same TCR was cloned from a line that was capable of adoptively transferring exacerbation of disease [13]. Such studies emphasize that the lymphokines produced by these cells, rather than their antigenic specificity, mediate the effector functions that ultimately mediate the immune response.

REGULATING MATURATION OF CD4 SUBSETS

With a more unified view of the lineage relationship between Th1 and Th2 subsets now in consensus, factors that regulate the maturation of these functional CD4 subsets constitute the focus of current research. Not surprisingly, the first factors implicated in the differentiation of T helper subsets were the cytokines themselves. In vitro experiments had identified the critical role for IL-4 in Th2 cell development [17,19], a finding confirmed in the L. major system in vivo. Administration of neutralizing antibodies to IL-4 to susceptible BALB/c mice at the time of infection enabled these mice to cure and results in the maturation of Th1 effector cells in the draining lymph nodes [10]. Further, administration of neutralizing antibodies to IFN-γ to resistant C3H/HeN [21] or C57BL/6 [10] mice at the time of infection abrogated the establishment of protective Th1 immunity in these animals. Of interest was the finding that both interventions had to be given within the first week of infection; later administration had no effect on the outcome of disease, suggesting that factors modulating the development of effector Th1 and Th2 cell populations are delivered soon following infection with the parasite. Further, the recombinant cytokines themselves did not alter the outcome of infection. Thus IFN-γ was not sufficient to promote healing in susceptible mice [10] nor was recombinant IL-4 sufficient to promote disease in resistant mice [22]. Experiments with IL-4 transgenic mice, however, indicated that they may develop slowly progressive infection and did not develop protective immunity [23], although these mice have additional abnormalities besides elevated expression of IL-4 [24]. Targeting IFN-γ directly into host macrophages using transfected Leishmania that expressed and secreted biologically active cytokine was not sufficient to activate host macrophages to a leishmanicidal state nor direct development of a Th1 effector population [25]. Clearly, although IL-4 and IFN-γ are necessary for the development of Th2 and Th1 maturation, respectively, neither is sufficient.

The role of additional cytokines in the development of effector Th1 or Th2 cells has been further elucidated using the *L. major* model. IL-2, although identified as a Th1 cytokine, is required for the development of Th2 effectors [19,26]. In experimental leishmaniasis, depletion of IL-2 using anti-cytokine or anti-cytokine receptor antibodies for the first 4 weeks following inoculation of the parasite successfully protected BALB/c mice and was associated with the development of Th1 cells [27]. Why longer administration of the antibody (as compared to the single dose required for anti–IL-4) remains unclear. Transforming growth factor beta (TGF-β), which is expressed by a wide range of both lymphoid and nonlymphoid cells, has also been implicated in the developmental regulation of Th1/Th2 cells in murine leishmaniasis [28]. Infection of macrophages from both resistant and susceptible mice in vitro was associated with release of TGF-β, and neutralization of TGF-β in vivo for 3 weeks after infection of susceptible mice resulted in the control of disease and the development of Th1 responses in the draining lymph nodes. Further, intermittent administration of the recombinant molecule to resistant mice worsened the course of disease and was able to render mice susceptible to a normally avirulent species of the parasite. IL-12 is a heterodimeric cytokine released by populations of antigen presenting cells, including macrophages and B cells, that stimulates the production of IFN-γ from CD4+, CD8+, and NK cells [29]. When administered to susceptible BALB/c mice for 1 week after infection, recombinant IL-12 was capable of generating protective immunity in almost 75% of animals [30]. Additional cytokines that have been implicated in Th1/Th2 development, such as IFN-α, which mediates development of Th1 responses [31], or IL-10, which mediates development of Th2 responses in some systems [18], either have not been studied or have been reported to have little effect in the *L. major* system. A summary of these various interventions is depicted in Table I.

TABLE I. Interventions That Alter the Disease Phenotype in Experimental Leishmaniasis

Healing	Sublethal irradiation [8]
	CD4+ cell depletion [9]
	Anti–IL-4 [10]
	Anti–IL-2 [27]
	Anti–TGF-β [28]
	Recombinant IL-12 [30]
Nonhealing	Anti–IFN-γ [10,21]
	Recombinant TGF-β [28]

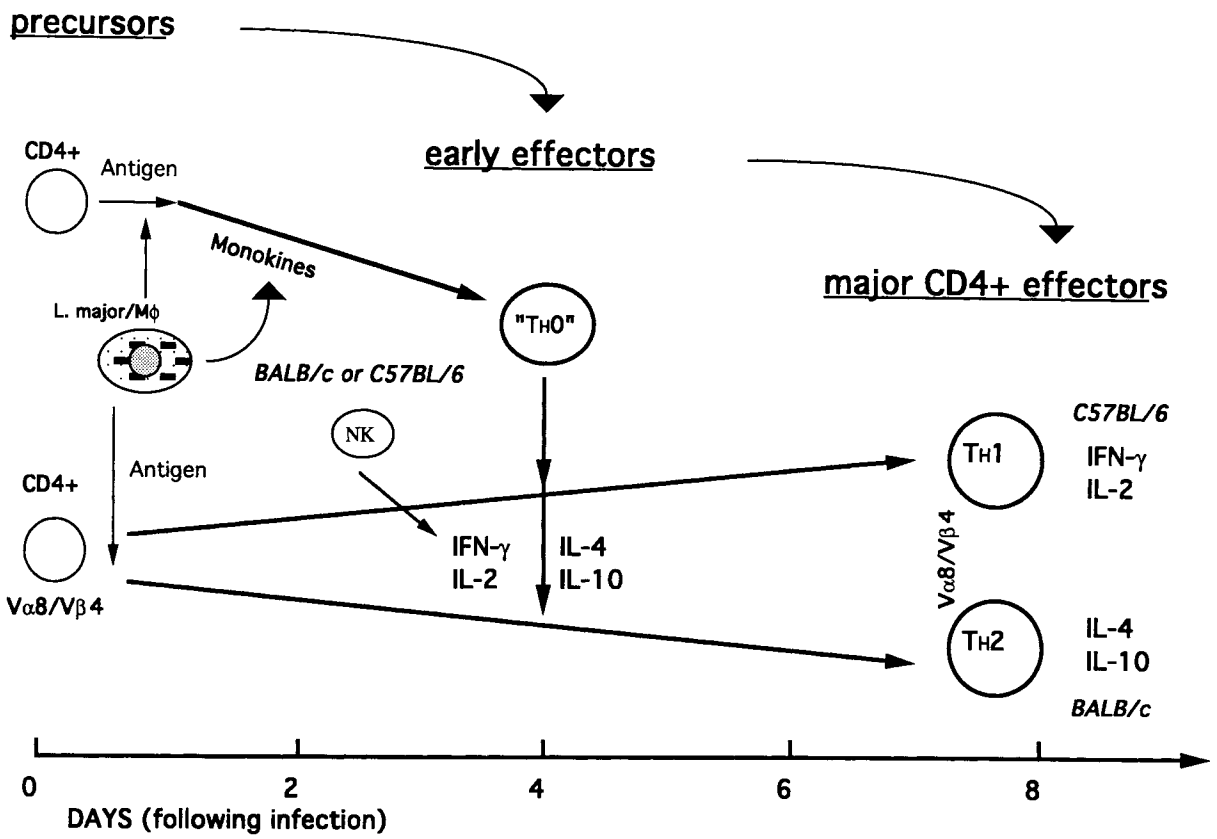

Fig. 2. CD4+ subset maturation during leishmaniasis. After infection macrophages serve as a source of antigen and soluble factors such as monokines that induce T helper expansion. Early T helper cell responses may proceed through a Th0 stage. Some segregating event(s) occurs during the first week of infection to bias the major CD4+ effectors into a fixed Th1 or Th2 phenotype. The immunodominant Th1 and Th2 effectors first seen at 1–2 weeks post-infection are typified by the Vα8/Vβ4 T-cell receptor clonotype.

MAKING THE SWITCH: APC AND T CELLS

Leishmania are parasites only of macrophages, suggesting that critical signals induced by the organism, such as TGF-β [28] or TNF-α [32], may be important in subserving its intracellular survival and in modulating the subsequent CD4+ response. The parasite lives in a late endolysosomal compartment into which MHC class II molecules have been shown to co-localize [33], thus explaining the dominant function of CD4+ cells in the host response against this pathogen. The CD4+ response will be modulated by the monokines released by infected cells, or possibly by bystander cells in the skin, including keratinocytes, DEC cells, Langerhans cells, and tissue mast cells and basophils. The interaction of the infected macrophage with circulating CD4+ cells will occur in a milieu of cytokines that will critically affect the development of the functional capacity of the CD4+ cell. Several studies have suggested that maturation of the CD4+ response in the murine L. major system may proceed through a Th0-like intermediate [34,35], suggesting that immunologic interventions at the time of antigen presentation may affect subsequent outcomes. As shown in Figure 2, current studies will need to focus on early events that shape this response in order to understand the genetic basis underlying the susceptibility phenotype. Perhaps appropriately transfected organisms can be used to deliver these critical signals themselves [25] in a manner that will trigger the appropriate Th1 responses that will enable the establishment of solid immunity. Understanding the basis of the "switch" will be critical not only for the development of effective vaccines against Leishmania, but for the understanding of the most fundamental of decisions made by the developing CD4+ effector population against all antigens.

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