Regulatory Interactions between CD45RB<sup>high</sup> and CD45RB<sup>low</sup> CD4<sup>+</sup> T Cells Are Important for the Balance between Protective and Pathogenic Cell-mediated Immunity

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Summary

BALB/c mice infected with the intracellular protozoan Leishmania major mount a T helper cell 2 (Th2) response that fails to control growth of the parasite and results in the development of visceral leishmaniasis. Separation of CD4<sup>+</sup> T cells into CD45RB<sup>high</sup> and CD45RB<sup>low</sup> subsets showed that the L. major-specific Th2 cells were contained within the CD45RB<sup>low</sup> population as these cells produced high levels of antigen-specific interleukin 4 (IL-4) in vitro and transferred a nonhealing response to L. major-infected C.B-17 scid mice. In contrast, the CD45RB<sup>high</sup>CD4<sup>+</sup> population contained L. major-reactive cells that produced interferon γ (IFN-γ) in vitro and transferred a healing Th1 response to L. major–infected C.B-17 scid mice. Transfer of the Th1 response by the CD45RB<sup>high</sup> population was inhibited by the CD45RB<sup>low</sup> population by a mechanism that was dependent on IL-4. These data indicate that L. major-specific Th1 cells do develop in BALB/c mice, but their functional expression is actively inhibited by production of IL-4 by Th2 cells. In this response, the suppressed Th1 cells can be phenotypically distinguished from the suppressive Th2 cells by the level of expression of CD45RB. Although the CD45RB<sup>high</sup> population mediated a protective response to L. major, C.B-17 scid mice restored with this population developed a severe inflammatory response in the colon that was independent of L. major infection, and was prevented by cotransfer of the CD45RB<sup>low</sup> population. The colitis appeared to be due to a dysregulated Th1 response as anti-IFN-γ, but not anti-IL-4, prevented it. Taken together, the data show that the CD4<sup>+</sup> T cell population identified by high level expression of the CD45RB antigen contains cells that mediate both protective and pathogenic Th1 responses and that the reciprocal CD45RB<sup>low</sup> population can suppress both of these responses. Whether suppression of cell-mediated immunity is beneficial or not depends on the nature of the stimulus, being deleterious during L. major infection but crucial for control of potentially pathogenic inflammatory responses developing in the gut.

CD4<sup>+</sup> T cells play a central role in the induction and regulation of the immune response. The CD4<sup>+</sup> T cell population has been shown to be phenotypically and functionally heterogeneous. In the rat, CD4<sup>+</sup> T cells that produce IL-2 and transfer cell-mediated immune responses in vivo can be distinguished by their level of expression of the CD45RC antigen from those that provide B cell help (1). Such functional heterogeneity can be explained by differential cytokine repertoires. Murine CD4<sup>+</sup> T cell clones have been shown to fall into two major subsets: Th1 cells that produce IFN-γ, TNF-α and β, and IL-2, and that are efficient activators of macrophages and NK cells; and Th2 cells that are the principal stimulators of antibody production and that produce IL-4, IL-5, IL-6, and IL-10, but not IFN-γ or IL-2 (2). Differential development of Th1 or Th2 cells thus determines whether a cell-mediated or humoral immune response develops.

Control of Th1 and Th2 responses can be exerted at the level of Th cell development or on the expression of effector functions by mature, differentiated cells. Regarding the latter level, studies in vitro suggest that the products of Th1 and Th2 cells negatively regulate the functions of each other. Thus, IFN-γ inhibits the growth of Th2 clones (3), and IL-10 was first identified as a Th2-produced inhibitor of cytokine synthesis by Th1 cells (4). IL-4 has also been shown to inhibit IFN-γ production by mitogen-stimulated human PBMC (5, 6) or mouse CD4<sup>+</sup> T cells stimulated with soluble anti-CD3 (7), although it did not inhibit IFN-γ production by...
Th1 clones (4). More recently, we have shown that IL-4 and IL-10 synergize to inhibit secondary Th1 responses in vivo (8). However, most of the studies of crossregulation of Th1 and Th2 cells have been performed in vitro, and little information is available on how these populations interact during immune responses in vivo.

The murine model of L. major-induced cutaneous leishmaniasis has proven to be a particularly good model for studying Th1 and Th2 responses in vivo. Most mouse strains infected with L. major mount a strong Th1 response to the parasite and heal their infection. In contrast, BALB/c mice mount a Th2 response with the expansion of a CD4⁺ T cell population that produces high levels of IL-4 and low levels of IFN-γ and that is ineffective at controlling the growth of the parasite (9).

We have used the dominant Th2 response that occurs in BALB/c mice as a model to determine whether Th2-mediated suppression of Th1 responses occurs in vivo and how it is mediated. Here we present evidence that BALB/c mice contain L. major-reactive Th1 cells whose functions are actively suppressed by IL-4 produced by the dominant Th2 population. The "suppressed" Th1 cells can be distinguished from the "suppressive" Th2 cells by the level of CD45RB expression, which permits the study of the functional activities of these two populations in isolation and when recombined in vitro and in vivo.

Materials and Methods

**Animals.** Specific pathogen-free female BALB/c and C.B-17 scid mice were obtained from Simonsen Laboratories (Gilroy, CA) and maintained in the Animal Care Facility of the DNAX Research Institute. C.B-17 scid mice were kept in microisolator cages with filtered air. Mice were used at 8-12 wk of age.

**Parasite Infection and L. major Antigen Preparation.** L. major (WHO strain WHOM/—/173) were cultured in M199 (GIBCO BRL, Gaithersburg, MD) containing 30% FCS (J.R. Scientific, Woodland, CA), 2 mM l-glutamine, and 100 U/ml each of penicillin and streptomycin. Promastigotes were harvested from stationary phase cultures and washed in PBS. Animals were infected with 1.5 x 10⁶ promastigotes in the left hind footpad. Parasite infection was monitored with a metric caliper. L. major antigen (LmAg) was prepared by four cycles of freezing and thawing of the parasites followed by centrifugation.

**Antibodies.** The following mAbs were used for cell purification: biotinylated R.M.4-5 and RM-4-4, anti-mouse CD4 and AMS-32.1, anti-I-A stranded mAbs (PharMingen, San Diego, CA); 2-43, anti-mouse CD8 (American Type Culture Collection [ATCC] No. TIB210; Rockville, MD); M1/70, anti-mouse Mac-1 (ATCC No. TIB128); RA36B2, anti-mouse B220 (10); FITC-conjugated 16A, (anti-mouse CD45RB) (PharMingen); PE-conjugated anti-CD4 and PE-conjugated isotype control mAb (Becton Dickinson); and PE-conjugated isotype control mAb (PharMingen). The following mAbs were used for in vitro and in vivo assays: 11B11 (rat IgG1), a neutralizing anti-mouse IL-4 mAb (ATCC No. HB188); JESS-2A5 (rat IgG1), a neutralizing anti-mouse IL-10 mAb (11), and GL113 (rat IgG1), an isotype control mAb reactive with ß-galactosidase.

**Cell Purification and Flow Cytometry.** CD4⁺ T cell subsets were purified from the popliteal lymph nodes (draining lymph nodes [DLN]) 4-6 wk after L. major infection. Briefly, cells were depleted of B220⁺, MAC-1⁺, I-A⁺, and CD8⁺ cells by negative selection using sheep anti-rat coated Dynabeads (Robbins Scientific, Mountain View, CA), as previously described (12). In some cases, CD4⁺ T cells were further enriched from the resulting cell suspension, which was >95% CD4⁺, by positive selection using the magnetic activated cell sorter (MACS) according to the manufacturer's instructions (Miltenyi, Dusseldorf, Germany). The resulting cell population was >96% CD4⁺. For separation of CD4⁺ T cells into the CD45RBhigh and CD45RBlow populations, negatively selected enriched CD4⁺ T cells were labeled with FITC-conjugated anti-CD45RB and PE-conjugated anti-CD4 (Becton Dickinson) and fractionated into CD4⁺ CD45RBhigh and CD4⁺ CD45RBlow fractions by two-color sorting on a FACStar Plus (Becton Dickinson). The CD45RBhigh and CD45RBlow populations were defined as the brightest staining 40–50% and the dullest staining 15–20% of CD4⁺ T cells, respectively. Intermediate staining populations were discarded. In some cases, unseparated CD4⁺ T cells that had been labeled with FITC-anti-CD45RB were also sorted. All populations were >98% pure on reanalysis. Spleen cells from naïve BALB/c mice were depleted of CD4 and CD8 staining cells by negative selection with Dynabeads as described above. The resulting population was >98% CD4 and CD8 negative and was used as a source of APCs. Flow cytography was carried out on the spleen cells from C.B-17 scid mice restored with T cell subsets 4–6 wk earlier. Single cell spleen suspensions were depleted of erythrocytes by hypotonic lysis and labeled with PE-CD4 or PE-conjugated isotype control mAb. Labeled cells were analyzed on a FACScan (Becton Dickinson).

**Reconstitution of C.B-17 scid Mice with T Cell Subpopulations.** C.B-17 scid mice were injected intravenously with 100 μl of PBS containing sorted CD4⁺ T cell subpopulations or unseparated CD4⁺ T cells. Mice were infected with L. major 1 d after reconstitution.

**Induction of Delayed Type Hypersensitivity (DTH).** Mice were injected in the contralateral footpad with 50 μl of PBS containing the equivalent of 5 x 10⁶ organisms/ml. The protein concentration was 250 μg/ml. Footpad swelling was monitored with a spring-loaded metric caliper.

**Histology.** Tissues were removed from mice 8–12 wk after T cell reconstitution and fixed in PBS containing 10% formalin and 0.5% paraffin. 6-μm paraffin-embedded sections were cut and stained with hematoxylin and eosin. Photomicrographs were taken on an Axioshot Microscope (Zeiss, Obercohen, Germany).

**Recall Response to LmAg In Vitro.** CD45RBhigh and CD45RBlow CD4⁺ T cell subsets or unseparated CD4⁺ T cells were cultured in 96-well round-bottomed plates in 250 μl volumes of RPMI-1640 containing 5% FCS, 2 mM l-glutamine, 0.05 mM 2-ME, and 100 U/ml each of penicillin and streptomycin, together with 5 x 10⁴ APCs in the presence or absence of LmAg (equivalent, 2 x 10⁶ organisms/ml). APCs were pulsed with LmAg (equivalent, 2 x 10⁶ organisms/ml) or medium alone for 4 h and then exposed to 1,000 rad γ-irradiation. Supernatants from duplicate cultures were harvested after 72 h and pooled for detection of cytokine synthesis.

**Detection of Cytokines.** Cytokine levels in supernatants were detected by two-site sandwich ELISA as previously described for IFN-γ (13), IL-4 and IL-10 (11), and IL-3 (14). Samples were assayed in duplicate and quantitated by comparison with standard curves of
Figure 1. IL-4 and IL-10 production by CD4+ T cells from mice with chronic leishmaniasis inhibits IFN-γ production in vitro. CD4+ T cells positively selected on the MACS from L. major-infected BALB/c mice were stimulated at 5 x 10^5 cells/well with LmAg and T cell-depleted splenocytes. Antibodies were added to cultures at 10 μg/ml. Supernatants were removed after 72 h and IFN-γ levels determined. Data are expressed as a percentage of the levels obtained with LmAg alone (1.6 ng/ml) and represent the mean of duplicate cultures ± SEM. CD4+ T cells cultured in the absence of antigen produced <0.3 ng/ml IFN-γ. Three further experiments gave similar results.

purified recombinant or natural cytokine. Results are presented as the mean ± SEM.

Results

IFN-γ Production by CD4+ T Cells Is Inhibited by IL-4 and IL-10 Production during a Th2 Response to L. major in BALB/c Mice

Infection of BALB/c mice with L. major results in the preferential expansion in the DLN of Th2 cells that contain high levels of IL-4 and IL-10 mRNA (15) and that produce these cytokines upon in vitro stimulation (16). Despite this dominant Th2 response, low levels of IFN-γ are also consistently observed (16, 17). It seemed possible that the potential to produce IFN-γ in response to L. major may be underestimated because of the concomitant production of IL-4 and IL-10 in the cultures, as both IL-4 and IL-10 have been shown to inhibit IFN-γ production, in vitro and in vivo. To test this, CD4+ T cells were purified from the DLN of L. major-infected BALB/c mice and restimulated in vitro with LmAg and T cell-depleted splenocytes from naive BALB/c mice as a source of APCs. Neutralization of IL-4 or IL-10 production in the cultures, by addition of anticytokine mAbs, led to twofold increases in the level of IFN-γ detected compared with medium or isotype control–treated cultures (Fig. 1), confirming active inhibition of IFN-γ production by both of these cytokines.

The Level of Expression of CD45RB Distinguishes IFN-γ-producing from IL-4-producing CD4+ T Cells

To address whether IFN-γ was being made by a separate subpopulation of CD4+ T cells from those that produced the IL-4 and IL-10 that regulated it, the CD4+ T cell population isolated from the DLN was further subdivided into two fractions based on the level of expression of the CD45RB antigen. FACS®-sorted CD45RBhigh and CD45RBlow CD4+ T cells were restimulated in vitro with LmAg and T cell-
Adoptive Transfer of L. major Reactivity to L. major-infected scid Mice. From a representative experiment is shown in Fig. 2. CD45RB\textsuperscript{high} and CD45RB\textsuperscript{low} CD4\textsuperscript{+} subpopulations produced equivalent amounts of IL-3 in response to L. major, indicating comparable activation by the antigen; however, IL-4 and IFN-\(\gamma\) levels varied depending on the phenotype of the responding CD4\textsuperscript{+} T cells. The CD45RB\textsuperscript{high} subset produced the highest levels of IFN-\(\gamma\), being approximately fourfold more potent in this regard than the CD45RB\textsuperscript{low} subset. In contrast, the CD45RB\textsuperscript{low} population was 10-fold more potent in IL-4 production than the CD45RB\textsuperscript{high} subset. In other experiments, IL-10 was measured and was also found to be produced preferentially by the CD45RB\textsuperscript{high} population (data not shown). Cytokine production was antigen specific as cytokine levels were below the limit of detection in cultures that did not contain LmAg. Further, the response to LmAg was dependent on priming of the T cells as CD4\textsuperscript{+} T cell subsets isolated from the spleen of naive recipients failed to synthesize detectable levels of IL-4, IL-3, IFN-\(\gamma\), or IL-10 in response to stimulation with LmAg (data not shown). These data show that primed L. major-reactive Th1 cells are present in BALB/c mice with nonhealing leishmaniasis, and that they can be distinguished from the Th2, IL-4 producers by the level of expression of the CD45RB antigen.

Adoptive Transfer of L. major Reactivity to C.B-17 scid Mice

CD4\textsuperscript{+} T Cells Transfer Nonhealing Th2-like Responses to L. major-infected scid Mice. To test whether the correlation between CD45RB phenotype and function evident in response to L. major in vitro was relevant in vivo, CD45RB\textsuperscript{high} and CD45RB\textsuperscript{low} CD4\textsuperscript{+} T cells were purified from the DLN of L. major-infected BALB/c mice and transferred to congenic C.B-17 scid mice. Recipients were infected with L. major at the time of T cell reconstitution, and disease progression was monitored by quantitation of footpad swelling at the site of initial infection and by histological analysis of the infected footpads 5–7 wk after infection. Dissemination of the parasite was documented by histological analysis of the liver and parasite culture from the spleen. As previously described, CD4\textsuperscript{+} T cells from L. major-infected BALB/c mice transferred a nonhealing response to parasite-infected scid mice (Fig. 3 A), very similar to that which occurs in the donor mice (Correa-Oliveira, P., and R. L. Coffman, unpublished data).

This T cell–mediated nonhealing response, which is ultimately fatal, differed from the disease course of unreconstituted C.B-17 scid mice, which have no B or T cells (18). The latter were highly susceptible to L. major infection, developing a late footpad swelling that progressed rapidly (Fig. 3 A). By 4–5 wk, the parasite had disseminated, often causing death by 12 wk. Compared with unrestored control mice, CD4\textsuperscript{+} T cell–restored mice developed an earlier footpad swelling that increased in size more slowly (Fig. 3 A). Consistent with the footpad swelling, disease progression was slower and animals could survive for up to 5 mo with this chronic lesion (data not shown), indicating that even these “disease-promoting” CD4\textsuperscript{+} T cells from infected BALB/c mice did provide some protective immunity to the parasite.

Transfer of the CD45RB\textsuperscript{high} CD4\textsuperscript{+} T Cell Subset to L. major-infected C.B-17 scid Mice Leads to a Healing Th1 Response. Consistent with the different profiles of cytokine production by the CD45RB\textsuperscript{high} and CD45RB\textsuperscript{low} CD4\textsuperscript{+} subpopulations in response to L. major antigen in vitro, the outcome of L. major infection in scid mice restored with these subsets was very different. Transfer of 5 \times 10\textsuperscript{5} CD45RB\textsuperscript{high} CD4\textsuperscript{+} cells led to a healing pattern of disease characterized by an initial footpad swelling that by 6 wk had resolved (Fig. 3 B). Histological analysis of the footpads taken 6–8 wk after infection revealed an extensive leukocytic infiltrate with very few live parasites inside the macrophages (data not shown). The parasites appeared not to have disseminated to the viscera as they were not detectable upon histological analysis of spleen or liver.
nor could they be cultured from splenocyte preparations (data not shown). As few as $10^4$ CD45RB$^\text{high}$ CD4$^+$ T cells were capable of transferring a healing response (Fig. 3 B). Healing correlated with the induction of Th1 immunity as these mice gave large DTH responses (a function of Th1 and not Th2 cells) upon challenge with LmAg (Fig. 4). Further, the healing response was dependent on IFN-γ production, as administration of anti-IFN-γ mAb led to the abrogation of protective immunity. Compared with animals that received an isotype control mAb, anti-IFN-γ–treated mice developed a large footpad lesion (Fig. 5 A) and dissemination of the parasite to the spleen and liver (data not shown).

**CD45RB$^\text{low}$ CD4$^+$ T Cells Suppress the Protective Immunity Transferred to scid Mice by the CD45RB$^\text{high}$ Population via an IL-4–dependent Mechanism**

$5 \times 10^5$ CD45RB$^\text{high}$ CD4$^+$ T cells transferred a healing Th1 response to L. major–infected scid mice, however, $5 \times 10^5$ CD4$^+$ T cells, which contain 60–70% CD45RB$^\text{high}$ cells, transferred a nonhealing Th2 response, suggesting that the CD45RB$^\text{low}$ subset inhibited the transfer of protective immunity by the CD45RB$^\text{high}$ subset. To test this suppression directly and determine its mechanism of action, subsets of CD4$^+$ T cells were purified from the DLN of L. major–infected BALB/c mice and injected alone or in combination into L. major–infected scid mice. As described above (Fig. 3), transfer of $4 \times 10^5$ CD45RB$^\text{high}$ cells led to resolution of the lesion (Fig. 6 A) and a healing pattern of disease, whereas transfer of $4 \times 10^5$ CD45RB$^\text{low}$ cells led to a progressively increasing lesion size and nonhealing disease (Fig. 6 A). Cotransfer of both subsets together also led to a nonhealing response.

**Figure 4.** DTH responses in L. major–infected C.B-17 scid mice restored with CD4$^+$ T cell subsets. C.B-17 scid mice were reconstituted with CD4$^+$ T cell subsets and infected with L. major as described in Fig. 3. Five wk after infection, mice were challenged with LmAg in the contralateral footpad and the swelling measured after 48 h. Data represent the mean plus SEM for the group. Values represent the mean plus SEM for the group. Numbers in parentheses indicate the number of animals in each group.

**Figure 5.** Transfer of the healing response by the CD45RB$^\text{high}$ population or the nonhealing response by the CD45RB$^\text{low}$ population to L. major–infected scid mice is dependent on IFN-γ or IL-4 production, respectively. C.B-17 scid mice were reconstituted with CD4$^+$ T cell subsets and infected with L. major as described in Fig. 3, except that some animals were treated at the time of infection and 10 d later with anti-IFN-γ (2 mg/injection), anti-IL-4 (5 mg/injection), or isotype control mAb GL113 (5 mg/injection). Values represent the mean plus SEM for the group. Numbers in parentheses indicate the number of animals in each group.
pattern of disease (Fig. 6 A), indicating that the CD45RB^low population did indeed suppress the transfer of a Th1 response by the CD45RB^high population. Suppression of the CD45RB^high population by CD45RB^low cells was antigen specific and required priming as cotransfer of 4 x 10^5 CD45RB^low CD4^+ T cells from the spleen of naive BALB/c mice with the primed CD45RB^high population did not inhibit the healing Th1 response (Fig. 6 A).

As the concomitant production of IL-4 and IL-10 was shown to inhibit IFN-γ production by CD4^+ T cells in response to L. major in vitro (Fig. 1), the role of these cytokines in the suppression of the CD45RB^high cells by the CD45RB^low population in vivo was tested. Mice given the combination of CD45RB^high and CD45RB^low CD4^+ T cells were treated at the time of L. major infection and 10 d later with anti-IL-4 or anti-IL-10 mAbs. Treatment with anti-IL-4 prevented the inhibition of the Th1 response by the CD45RB^low population and led to resolution of the L. major infection with a pattern of lesion development similar to mice reconstituted with only CD45RB^high cells (Fig. 6 A). In contrast, anti-IL-10 treatment had no effect on the suppression of the CD45RB^high population. These animals developed a footpad swelling indistinguishable from mice given both CD45RB^high and CD45RB^low cells (Fig. 6 A). When 10^6 unseparated CD4^+ T cells were transferred, results were obtained identical to those of the mixture of sorted CD45RB^high and CD45RB^low cells. Treatment with anti-IL-4, but not anti-IL-10 or an isotype control mAb, prevented the suppression and revealed protective immunity (Fig. 6 B).

**Injection of CD45RB^high CD4^+ T Cells Led to Greater T Cell Reconstitution than the Reciprocal CD45RB^low Subset**

Transfer of Th1 or Th2 immunity did not appear to correlate with the total reconstitution potential of the donor cell population. Equivalent numbers of CD4^+ T cells were detectable in the spleens of mice 4–6 wk after injection of 5 x 10^5 unseparated CD4^+ T cells (0.88 x 10^6 ± 0.29 x 10^6, n = 4), which transferred a Th2 response to the parasite (Fig. 3 A), or 5 x 10^5 CD45RB^high cells (0.83 x 10^6 ± 0.2 x 10^6, n = 4) which transferred a healing response (Fig. 3 B). However, mice restored with the CD45RB^low CD4^+ population contained threefold fewer (0.29 x 10^6 ± 0.08 x 10^6, n = 3) CD4^+ T cells in the spleen. The level of T cell reconstitution produced by the two subsets correlated with the potential of these populations to transfer a healing response to L. major infection at low cell numbers, as the ability to transfer a healing response to L. major began to titrate out at a dose of 10^4 CD45RB^low cells, whereas this point was not reached until 10-fold fewer (10^5) CD45RB^high cells were transferred (data not shown).

**Scid Mice Restored with CD45RB^high CD4^+ T Cells Developed a Severe Inflammatory Response in the Colon which Was Prevented by Anti-IFN-γ mAb**

During the course of these experiments, it was noticed that despite healing their L. major infection, animals that received 1–5 x 10^5 CD45RB^high CD4^+ T cells developed a wasting disease 5–8 wk after infection (data not shown). The wasting disease was accompanied by severe pathology in the colon, characterized by extensive mononuclear cell infiltrates, ulceration, and pronounced epithelial cell hyperplasia (Fig. 7, E–F; Table 1). Induction of colitis in C.B-17 scid mice by transfer of CD45RB^high CD4^+ T cells was not dependent on L. major infection, as an identical lesion occurred in uninfected C.B-17 scid mice restored with CD45RB^high CD4^+ T cells isolated from naive C.B-17 (19) or BALB/c mice (12). Concurrent infection with L. major did not affect the kinetics of the wasting disease or the incidence or severity of the colitis (data not shown). We have previously shown that the pathology in the colon was accompanied by significant increases in IFN-γ and TNF-α mRNA transcripts, but not in IL-4 or IL-10 mRNA levels, suggesting that it may be Th1 mediated (12). Indeed, IFN-γ was involved in the pathogenesis, as scid mice that received CD45RB^high CD4^+ T cells together with anti-IFN-γ had a reduced incidence of severe colitis (0/4, Table 1), compared with animals that received CD45RB^high cells alone (10/12, Table 1), or together with anti-IL-4 mAb (4/4, Table 1), or GL113, an isotype control mAb (4/4, Table 1).
Animals injected with the CD45RB<sup>low</sup> subset of CD4<sup>+</sup> T cells did not develop the colitis (Table 1); colons from these mice were indistinguishable from those of normal BALB/c mice (Fig. 7, A and B). This population also prevented induction of colitis by the CD45RB<sup>high</sup> population as animals that received unseparated CD4<sup>+</sup> T cells or purified CD45RB<sup>high</sup> cells with CD45RB<sup>low</sup> cells at either a 2:1 or 1:1 ratio developed a significantly reduced incidence of severe colonic pathology (pooling these groups 3/17, Table 1) than animals that received CD45RB<sup>high</sup> cells either with or without isotype control mAb (14/16, Table 1). Mild pathology was evident in 7/17 mice restored with CD45RB<sup>high</sup> and CD45RB<sup>low</sup> CD4<sup>+</sup> T cells, which consisted of a small leukocytic infiltrate and limited hyperplasia (Fig. 7, C and D).

Inhibition of the Th1 response to <i>L. major</i> was mediated by IL-4-producing Th2 cells (Fig. 6), however in these experiments, in which anti-IL-4 or anti-IL-10 was administered, no significant increase in the incidence of severe colitis was observed (Table 1), suggesting that the CD45RB<sup>low</sup> population may regulate the development of colitis by a mechanism other than IL-4 and IL-10 production.

**Discussion**

The host response to invading microorganisms is tightly regulated to ensure activation of appropriate immune effector functions to eliminate the infectious agent without destroying “self”. Our data indicate that interactions between CD45RB<sup>low</sup> and CD45RB<sup>high</sup> subsets of CD4<sup>+</sup> T cells play a critical role in this process. CD45RB<sup>high</sup> CD4<sup>+</sup> T cells,
### Table 1. Colitis in L. major-infected C.B-17 scid Mice Restored with CD4+ T Cell Subsets

| Phenotype of CD4+ T cell inoculum | mAb treatment | Colitis (12-14 wk) |
|-----------------------------------|---------------|--------------------|
|                                   |               | Minimal | Mild | Severe |
| 1-5 × 10⁵ CD45RB<sup>high</sup>   | GL113         | 0/12    | 2/12 | 10/12  |
|                                   | anti-IL-4     | 0/4     | 0/4  | 4/4    |
|                                   | anti-IFN-γ    | 0/4     | 0/4  | 4/4    |
| 2-5 × 10⁵ CD45RB<sup>low</sup>    |               | 1/4     | 3/4  | 0/4    |
| 4 × 10⁵ CD45RB<sup>high</sup>     |               | 6/6     | 0/6  | 0/6    |
| + 2 × 10⁵ CD45RB<sup>low</sup>    |               | 0/3     | 2/3  | 1/3    |
| 4 × 10⁵ CD45RB<sup>high</sup>     |               | 1/5     | 3/5  | 1/5    |
| + 4 × 10⁵ CD45RB<sup>low</sup>    | anti-IL-10    | 3/4     | 1/4  | 0/4    |
|                                   | anti-IL-4     | 3/5     | 1/5  | 1/5    |
| 6-10 × 10⁵ CD4+                   | GL113         | 6/9     | 2/9  | 1/9    |
|                                   | anti-IL-10    | 3/5     | 0/5  | 2/5    |
|                                   | anti-IL-4     | 3/4     | 1/4  | 0/4    |

C.B-17 scid mice were reconstituted with CD4+ T cell subsets and infected with L. major and treated with anticytokine or isotype control mAb as described in Fig. 5. Mice were killed 8-10 wk after infection and colons scored for pathology. Minimal, indistinguishable from control BALB/c mice (Fig. 7, A and B); mild, limited mononuclear infiltrates and slight epithelial cell hyperplasia (Fig. 7, C and D); severe, extensive mononuclear cell infiltrate and marked epithelial cell hyperplasia (Fig. 7, E and F). Data were pooled from three independent experiments.

Isolated from L. major-infected BALB/c mice transferred a protective Th1 response to L. major-infected scid mice; however, a lethal inflammatory response developed in the colon of these mice. The CD45RB<sup>low</sup> CD4+ population prevented the development of colitis when transferred together with the CD45RB<sup>high</sup> population. However, the protective antileishmanial response was also inhibited and the mice developed visceral leishmaniasis. Based on these data we propose that the deleterious suppression of cell-mediated immunity, which in some instances occurs in response to infectious microorganisms, may be the price to be paid for the immunoregulation, which under normal circumstances, prevents the development of inflammatory responses to immunological self-antigens, including the intestinal flora.

4 wk after infection, BALB/c mice infected with L. major show few signs of a functional Th1 response in vivo. Such mice have no DTH reactivity to parasite antigens and exert little if any control of the growth or spread of the pathogen (9). However, we present evidence herein that these mice do contain a significant population of primed Th1 cells whose function was actively suppressed by the dominant Th2 cells. In the CD4<sup>+</sup> T cell response to LmAg in vitro, IFN-γ levels were significantly increased by neutralization of IL-4 and IL-10 produced in the cultures. Separation of the CD4<sup>+</sup> T cells into CD45RB<sup>high</sup> or CD45RB<sup>low</sup> fractions revealed that the LmAg-specific IFN-γ-producing cells were contained within the CD45RB<sup>high</sup> population, whereas the specific IL-4-producing cells were contained within the reciprocal CD45RB<sup>low</sup> population. Thus, the minority Th1 population was phenotypically distinguishable from the dominant Th2 population.

The regulatory interaction between L. major-reactive Th1 and Th2 populations and the correlation with CD45RB phenotype was confirmed in vivo. 5 × 10⁵ CD4<sup>+</sup> or CD45RB<sup>low</sup> CD4<sup>+</sup> cells from mice with chronic leishmaniasis adoptively transferred a nonhealing Th2 response to L. major-infected C.B-17 scid mice. However, 4 × 10⁵ CD45RB<sup>high</sup> cells (which comprise ∼70% of CD4<sup>+</sup> T cells) transferred a healing Th1 response, suggesting that in the transfers of CD4<sup>+</sup> T cells, the protective potential of this population was inhibited by CD45RB<sup>low</sup> cells. The dominant suppressive effects of the CD45RB<sup>low</sup> population were illustrated by the fact that cotransfer of 4 × 10⁵ CD45RB<sup>low</sup> cells with 4 × 10⁵ CD45RB<sup>high</sup> cells led to a nonhealing Th2 response. Identical results were obtained when CD45RB<sup>high</sup> and CD45RB<sup>low</sup> CD4<sup>+</sup> T cells were mixed at their physiological ratio of 2:1 (data not shown). Suppression of the Th1 response to L. major required specific antigen recognition as it could be mediated by CD45RB<sup>low</sup> cells from L. major–primed but not naive recipients. The most direct interpretation of these data is that primed Th1 cells exist in the DLN of mice making a dominant Th2 response to L. major, but...
their expression is actively suppressed by the dominant Th2 cells.

In a series of pioneering studies in the early 1980s, Liew (20) showed that CD4+ T cells existed in BALB/c mice infected with L. major which could suppress DTH responses to LmAgs, although it was not possible at that time to phenotype these cells or determine their mechanism of action. Several studies support our conclusion that Th1 cells exist in mice with chronic leishmaniasis. The frequency of L. major–specific T cells capable of mediating delayed type hypersensitivity responses was found to be substantially higher in the DLN of nonhealing BALB/c mice than in healing CBA mice (21), and that both Th1 and Th2 L. major reactive clones could be generated from BALB/c mice with chronic leishmaniasis (22). Further, in a study of a number of inbred mouse strains infected with L. major, the level of expression of IL-4 mRNA was found to correlate with nonhealing disease, whereas IFN-γ mRNA levels were similar between healing and nonhealing strains (23).

A lack of cell-mediated immunity is a feature of a number of infectious diseases and it has been suggested that Th2–mediated suppression of Th1 responses may in part account for this (24). Apart from the studies described here, Th2–specific T cells capable of mediating delayed type hypersensitivity responses was found to be substantially higher in the DLN of nonhealing BALB/c mice than in healing CBA mice, and that both Th1 and Th2 L. major reactive clones could be generated from BALB/c mice with chronic leishmaniasis (22). Further, in a study of a number of inbred mouse strains infected with L. major, the level of expression of IL-4 mRNA was found to correlate with nonhealing disease, whereas IFN-γ mRNA levels were similar between healing and nonhealing strains (23).

A lack of cell-mediated immunity is a feature of a number of infectious diseases and it has been suggested that Th2–mediated suppression of Th1 responses may in part account for this (24). Apart from the studies described here, Th2–mediated suppression has been studied largely in vitro and has focused on the role of IL-10. Thus, it has been shown that addition of anti-IL-10 to cultures of spleen cells from mice infected with Schistosoma mansoni enhanced IFN-γ production in response to parasite antigens (25). A similar effect was seen when anti-IL-10 was added during Con A stimulation of spleen cells from mice infected with the helminth Nippostrongylus brasiliensis (26). The effects of IL-4 were not analyzed in these studies. In studies with mouse T cell clones, IL-10 but not IL-4 inhibited Th1 cytokine synthesis (4). However, suppression of Th1 responses by both IL-4 and IL-10 has been described in a number of parasite diseases in humans. IL-4 was shown to be one of the activities produced by CD8+ T cell clones isolated from patients with lepomatosus leprosy that inhibited the proliferation of Th1 clones (27), and neutralization of IL-10 and, to a lesser extent IL-4, led to enhanced IFN-γ synthesis by PBL from patients with visceral leishmaniasis (Carvalho, E.M., personal communication). Our studies indicate that, in the dominant Th2 response to L. major in vitro, both IL-4 and IL-10 inhibit IFN-γ production. It may be that in some situations IL-4 will be more effective than IL-10 in suppression of Th1 functions and vice versa.

Differences in the role of IL-4 and IL-10 in suppression of Th1 responses to L. major were evident in the in vivo studies. Administration of anti-IL-4, but not anti-IL-10, to L. major–infected scid mice restored with a mixture of CD45RBlow and CD45RBhigh cells led to the development of protective Th1 responses, suggesting that the CD45RBlow population inhibited the Th1 response to L. major in vivo by an IL-4–dependent mechanism. It is not clear why anti-IL-10 was effective in vitro but not in vivo, however these two forms of analysis are very different as the former consists of an acute 3-d restimulation, whereas the latter requires expansion of the donor cell population. The finding that anti-IL-10 failed to modulate the adoptive transfer of the Th2 response to L. major is consistent with the finding that anti-IL-10 had no effect on the course of L. major infection in BALB/c mice (16).

CD45RBlow and CD45RBhigh subsets of mouse CD4+ T cells are widely thought to represent naive and memory T cells, respectively. The CD45RB antigen is lost on T cell activation in vitro (28) and in vivo (29). CD45RBhigh CD4+ cells from naive donors provided help to B cells in assays of primary antibody responses, whereas the CD45RBlow population was active in memory responses (29); the recall response to KLH for both Th1 and Th2 cytokines was shown to be contained within the CD45RBlow population (30). However, after infection with L. major, primed L. major–reactive CD4+ T cells can be found in both the CD45RBlow and CD45RBhigh CD4+ populations. On a cell/cell basis, antigen-stimulated CD45RBhigh and CD45RBlow populations produced equivalent amounts of IL-3 in vitro, suggesting approximately equivalent levels of activation. However, the CD45RBlow population produced predominantly IFN-γ, whereas the CD45RBlow subset was the more potent producer of antigen-stimulated IL-4. As the recall response to LmAgs requires infection or immunization with L. major in vivo, these results indicate that the CD45RBlow population contains most of the L. major–reactive Th1 cells, whereas the CD45RBhigh population contains most of the L. major–reactive Th2 cells. Although these results differ from previous studies with a defined protein antigen, the two studies are very different as infection with L. major leads to chronic stimulation and a relatively polarized Th2 response, whereas immunization with KLH together with Bordetella pertussis appeared to activate a more acute and mixed Th1 and Th2 response (30).

Our data taken together with those of others, suggest that the CD45RBlow CD4+ population is heterogeneous and made up of naive cells together with, in some circumstances, primed Th1 cells. A similar heterogeneity has been postulated to occur within the CD45RBlow CD4+ T cell population in humans which was shown to be comprised of CD45RA high and CD45RA low cells (31). The finding that the CD45RBlow population contained the L. major–responsive Th2 cells is consistent with previous reports that this population produced high levels of IL-4 in response to mitogens and specific antigen (29, 30, 32). However, the adoptive transfer of Th2 responses to L. major–infected C.B-17 scid mice by the CD45RBlow population was complicated by the fact that Th1 responses could be revealed by treatment of the recipients with anti-IL-4 or by transfer of low numbers (3 × 104–1 × 105) of CD45RBlow cells. It is not clear whether this finding represents the preferential outgrowth of a small number of contaminating CD45RBhigh Th1 cells that are suppressed by the IL-4 producers at high cell number, or reflects a true functional heterogeneity of the CD45RBlow population. Experiments with genetically marked cell populations will be required to distinguish between these two possibilities. A shift to Th1 responses with the transfer of low
numbers of cells occurs with naive T cells (33) and unfracti-
nated CD4+ T cells from L. major-infected donors (Correa-
Olivera, R., and R. L. Coffman, unpublished data) and ap-
pears to be due in part to the dominance of scid-derived
IFN-γ. Combining data from a number of studies, including
this one, it is clear that the regulation of expression of the
CD45RB antigen is complex and caution should be exer-
cised when assigning a population a particular function based
on the level of expression of this antigen alone. Use of mAbs
that further subdivide the CD45RB subsets of CD4+ T
cells may clarify the situation.

Scid mice restored with CD45RBhigh CD4+ T cells from
L. major-infected BALB/c mice healed their L. major infec-
tion but developed a progressive inflammatory response in
the colon. Colitis was, however, not mediated by L. major-
specific T cells as it has been shown to occur equally well
in C.B-17 scid mice restored with the CD45RBhigh popu-
lation isolated from naive BALB/c (12) or C.B-17 mice (19).
The colitis appeared to be a manifestation of a dysregulated
Th1 response with the expression of high levels of IFN-γ
and TNF-α mRNA in diseased colons (12). Further, the co-
litis in mice reconstituted with CD45RBhigh CD4+ T cells
was significantly diminished by treatment with anti-IFN-γ,
but these mice developed instead a nonhealing Th2 response
to L. major infection. Thus, Th1 immunity is a double-edged
sword, capable of generating both protective and pathogenic
responses in the host at the same time.

The nature of the antigenic stimulation that leads to the
development of colitis in the CD45RBhigh-restored mice is
presently unknown. It may be an inflammatory response
against components of the gut flora, as has been suggested
to occur in inflammatory bowel disease in humans (34).
Relevant to this, colitis similar to that described here developed
in mice that lacked a functional IL-2 gene when housed under
conventional conditions, but not when maintained in a germ-
free environment (35). Whatever the nature of the antigenic
drive, it is clear that the development of the Th1-mediated
pathology in the colon of mice given the CD45RBhigh subset
is inhibited under normal circumstances by cells con-
tained within the CD45RBlow population. Mice restored
with unseparated CD4+ T cells, or with a mixture of
CD45RBhigh and CD45RBlow cells did not develop colitis.
Thus, the CD45RBlow population suppressed both protective
and pathogenic Th1 responses mediated by the CD45RBhigh
population. This illustrates the importance of appropriate ac-
tivation of this subset, which mediates beneficial regulation
of immune responses in the gut but inhibits protective re-
sponses to intracellular pathogens like L. major.

Suppression of the Th1 response to L. major was depen-
dent on IL-4 production, however, anti-IL-4 treatment failed
to block the protective effect of the CD45RBlow cells on co-
litis induction or to induce colitis in mice that received un-
separated CD4+ T cells. This suggests that the cells that
regulated the colitis acted via a different mechanism to those
that inhibited Th1 immunity to L. major. There is evidence
that IL-10 plays an important role in regulation of inflamma-
atory responses in the intestine as mice with a targeted dis-
ruption of the IL-10 gene developed a wasting disease and
severe enterocolitis (36). However anti-IL-10 mAbs also failed
to inhibit the protection by the CD45RBlow cells, sug-
suggesting that IL-10 was not the key regulatory molecule in
this model of colitis. It may be that other suppressive cytokines
produced by Th2 cells are more important in the colitis that
develops in the CD45RBhigh CD4+ T cell–restored scid mice.
Alternatively, the regulators of colitis may have a cytokine
profile distinct from Th2 cells. In this regard, multiple inflam-
atory lesions, including colitis, developed in mice with tar-
geted disruption of the TGF-β1 gene (37, 38), and IL-2–
deficient mice developed colitis (35).

Multiple organ inflammation developed in nude rats recon-
stituted with CD45RChigh CD4+ T cells which was pre-
vented by the CD45RClow population (39). Further, transfer
of CD45RClow population from normal rats into adult thymectomized, sublethally irradiated recipients prevented the
development of spontaneous diabetes in the recipients (40).
The finding that similar regulatory interactions exist between
the subsets of CD4+ T cells identified on the basis of
CD45RC in the rat and CD45RB in the mouse emphasizes
the importance of this regulatory pathway.

In summary, separation of the CD4+ T cell population
into the CD45RBhigh and CD45RBlow subfractions has re-
vealed two populations that mediate distinct immunological
functions. Regulatory interactions were shown to occur be-
tween these subsets which were important in the response
to L. major and in a model of colitis. Study of these subsets
may therefore provide a useful model for defining the regula-

ty interactions that occur within the CD4+ T cell popu-
lation and that are important in the host response to infec-
tious agents and the maintenance of peripheral tolerance.
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