

Regulatory Interactions between CD45RB^{high} and CD45RB^{low} CD4⁺ 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⁺ T cells into CD45RB^{high} and CD45RB^{low} subsets showed that the *L. major*-specific Th2 cells were contained within the CD45RB^{low} 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^{high}CD4⁺ 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^{high} population was inhibited by the CD45RB^{low} 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^{high} 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^{low} 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⁺ 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^{low} 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⁺ T cells play a central role in the induction and regulation of the immune response. The CD4⁺ T cell population has been shown to be phenotypically and functionally heterogeneous. In the rat, CD4⁺ 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⁺ 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⁺ 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 as promastigotes 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 1.5×10^7 promastigotes in the left hind footpad. Parasite infection was monitored with a metric caliper. *L. major* antigen (LmAg)¹ 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 RM-4-5 and RM-4-4, anti-mouse CD4 and AMS-32.1, anti-I-A^d (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-mouse CD4 (Becton Dickinson, Sunnyvale, CA); and PE-conjugated isotope 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), JES5-2A5 (rat IgG1),

a neutralizing anti-mouse IL-10 mAb (11), and GL113 (rat IgG1), an isotope control mAb reactive with β -galactosidase.

Cell Purification and Flow Cytofluorography. 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^d, 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 ~85% 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 CD45RB^{high} and CD45RB^{low} 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⁺ CD45RB^{high} and CD4⁺ CD45RB^{low} fractions by two-color sorting on a FACStar Plus[®] (Becton Dickinson). The CD45RB^{high} and CD45RB^{low} populations were defined as the brightest staining 40–50% and the dimmest 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 naive 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 isotope 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 μ l 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 μ l of PBS containing the equivalent of 5×10^5 organisms/ml. The protein concentration was 250 μ g/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% formaldehyde. 6- μ m paraffin-embedded sections were cut and stained with hematoxylin and eosin. Photomicrographs were taken on a Axiophot Photomicroscope (Zeiss, Obercohen, Germany).

Recall Response to LmAg In Vitro. CD45RB^{high} and CD45RB^{low} CD4⁺ T cell subsets or unseparated CD4⁺ T cells were cultured in 96-well round-bottomed plates in 250 μ l 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 5×10^5 APCs in the presence or absence of LmAg (equivalent, 2×10^6 organisms/ml). APCs were pulsed with LmAg (equivalent, 2×10^6 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

¹ Abbreviations used in this paper: DLN, draining lymph node; DTH, delayed type hypersensitivity; LmAg, *L. major* antigen; MACS, magnetic activated cell sorter.

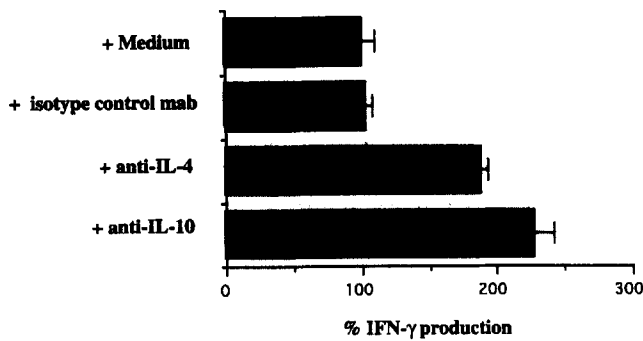


Figure 1. IL-4 and IL-10 production by CD4⁺ T cells from mice with chronic leishmaniasis inhibits IFN- γ production in vitro. CD4⁺ T cells positively selected on the MACS from *L. major*-infected BALB/c mice were stimulated at 5×10^5 cells/well with LmAg and T cell-depleted splenocytes. Antibodies were added to cultures at 10 μ g/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 plus 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 \pm 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*

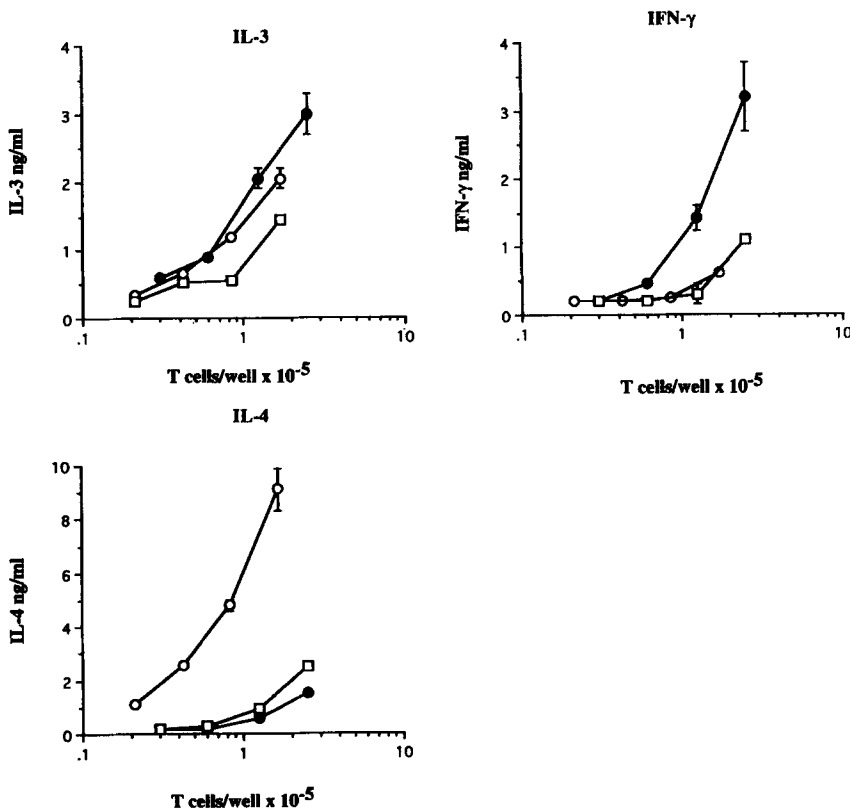


Figure 2. In the recall response to *L. major*, the IFN- γ producers can be distinguished from the IL-4 producers based on the level of expression of the CD45RB antigen. CD4⁺ T cell subsets from *L. major*-infected BALB/c mice were stimulated with LmAg and T cell-depleted splenocytes. (□) CD4⁺ T cells; (○) CD45RB^{low} CD4⁺ T cells; (●) CD45RB^{high} CD4⁺ T cells. After 72 h of culture, supernatants were collected and assayed for cytokine levels. Data represent the mean value of duplicate cultures plus SEM. Cytokine levels were undetectable when the cells were cultured in the absence of LmAg; <0.3 ng/ml IFN- γ , <0.15 ng/ml IL-4, <0.15 ng/ml IL-3. Two further experiments gave similar results.

depleted spleen APCs, and supernatants were harvested after 3 d of culture and assayed for cytokine content. The result from a representative experiment is shown in Fig. 2. CD45RB^{high} and CD45RB^{low} CD4⁺ subpopulations produced equivalent amounts of IL-3 in response to *L. major*, indicating comparable activation by the antigen; however, IL-4 and IFN- γ levels varied depending on the phenotype of the responding CD4⁺ T cells. The CD45RB^{high} subset produced the highest levels of IFN- γ , being approximately fourfold more potent in this regard than the CD45RB^{low} subset. In contrast, the CD45RB^{low} population was 10-fold more potent in IL-4 production than the CD45RB^{high} subset. In other experiments, IL-10 was measured and was also found to be produced preferentially by the CD45RB^{low} 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⁺ T cell subsets isolated from the spleen of naive recipients failed to synthesize detectable levels of IL-4, IL-3, IFN- γ , 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⁺ 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^{high} and CD45RB^{low} CD4⁺ 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⁺ 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, R., 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⁺ 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⁺ T cells from infected BALB/c mice did provide some protective immunity to the parasite.

Transfer of the CD45RB^{high} CD4⁺ 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^{high} and CD45RB^{low} CD4⁺ 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 5×10^5 CD45RB^{high} CD4⁺ 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,

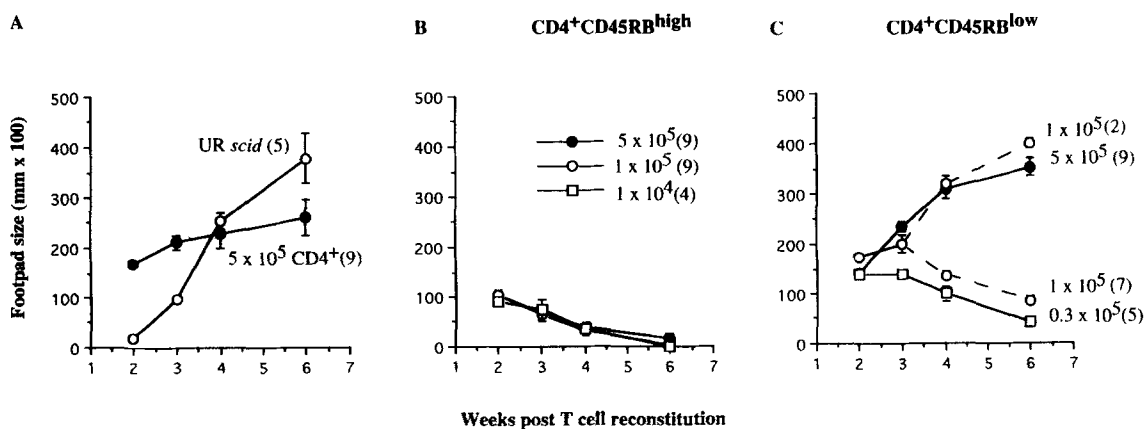


Figure 3. Adoptive transfer of *L. major* responses to C.B-17 *scid* mice with CD4⁺ T cell subsets. CD4⁺ T cell subsets from *L. major*-infected BALB/c mice were injected intravenously at different doses into C.B-17 *scid* mice that were infected with *L. major* 1 d later. Data represent the mean footpad swelling plus SEM. Data were pooled from two independent experiments. Numbers in parentheses indicate the number of animals in each group. Two further experiments gave similar results.

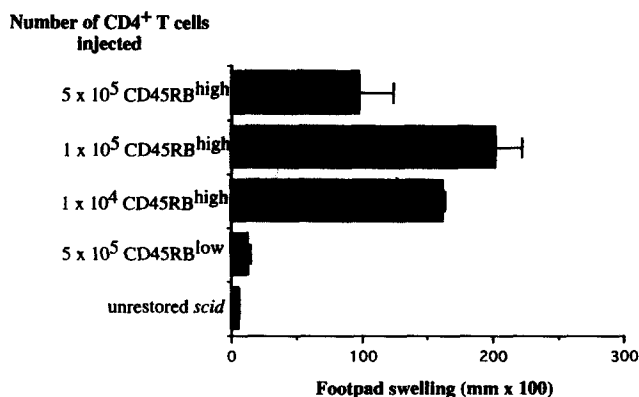


Figure 4. DTH responses in *L. major*-infected C.B-17 *scid* mice restored with CD4⁺ T cell subsets. C.B-17 *scid* mice were reconstituted with CD4⁺ T cell subsets and infected with *L. major* as described in Fig. 3. 5 wk after infection, mice were challenged with LmAg in the contralateral footpad and the swelling measured after 48 h. Data represent the mean value of two animals per group plus SEM. A further experiment gave a similar result.

nor could they be cultured from splenocyte preparations (data not shown). As few as 10⁴ CD45RB^{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^{low} CD4⁺ T Cells Transfer a Nonhealing Th2 Response to *L. major*-infected C.B-17 *scid* Mice. In contrast to the result above, *scid* mice restored with 5 × 10⁵ CD45RB^{low} CD4⁺ T cells exhibited a nonhealing pattern of disease, similar to mice restored with the same number of unseparated CD4⁺ T cells (Fig. 3 C). Large numbers of parasite-laden macrophages were evident in the footpads of these mice

5–6-wk after infection (data not shown), indicating that the increase in footpad swelling reflected the inability of the transferred T cells to control the growth of the parasite. By 6 wk, parasites had disseminated to the viscera and were visible upon histological examination of spleen and liver sections and could be cultured in vitro from spleen cell preparations (data not shown). Compared with healing CD45RB^{high}-restored mice, the CD45RB^{low}-restored mice made barely detectable DTH responses to soluble *L. major* antigens (Fig. 4), indicating impaired cell-mediated (Th1) immunity towards the parasite. The response transferred by the CD45RB^{low} population was not simply due to a lack of immunity towards *L. major*, but rather to transfer of disease-promoting Th2 cells, as a healing response to the parasite occurred in mice treated with anti-IL-4 mAb (Fig. 5). A healing response also developed when lower numbers of CD45RB^{low} CD4⁺ T cells were transferred. Thus, when 10⁵ cells were transferred, only two of nine mice developed nonhealing progressive disease (Fig. 3 C), whereas the remaining seven mice developed a small footpad lesion that was resolved. The situation was even clearer when 0.3 × 10⁵ cells were transferred, as all of the mice in this group exhibited a healing disease pattern (Fig. 3 C).

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

5 × 10⁵ CD45RB^{high} CD4⁺ T cells transferred a healing Th1 response to *L. major*-infected *scid* mice, however, 5 × 10⁵ CD4⁺ T cells, which contain 60–70% CD45RB^{high} cells, transferred a nonhealing Th2 response, suggesting that the CD45RB^{low} subset inhibited the transfer of protective immunity by the CD45RB^{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 × 10⁵ CD45RB^{high} cells led to resolution of the lesion (Fig. 6 A) and a healing pattern of disease, whereas transfer of 4 × 10⁵ CD45RB^{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

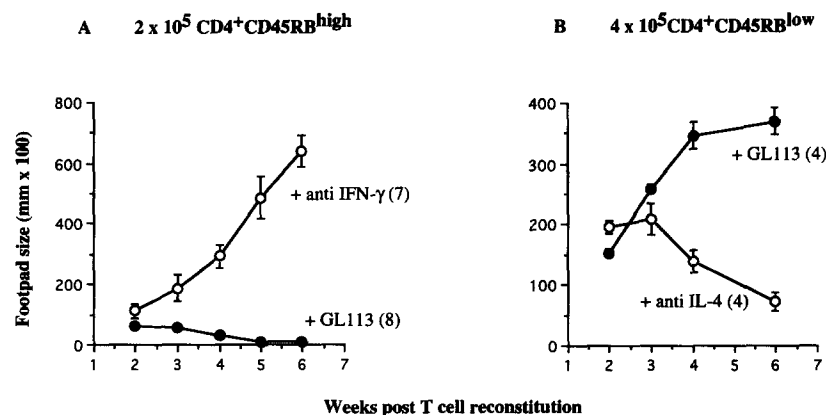


Figure 5. Transfer of the healing response by the CD45RB^{high} population or the nonhealing response by the CD45RB^{low} population to *L. major*-infected *scid* mice is dependent on IFN-γ or IL-4 production, respectively. C.B-17 *scid* mice were reconstituted with CD4⁺ T cell subsets and infected with *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.

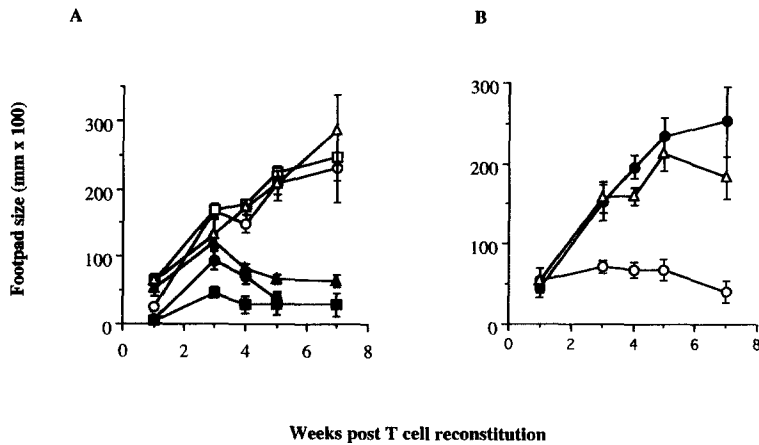


Figure 6. CD45RB^{low} CD4⁺ T cells inhibit the Th1 response transferred by the CD45RB^{high} population to *L. major*-infected *scid* mice via an IL-4-dependent mechanism. C.B-17 *scid* mice were reconstituted with CD4⁺ T cell subsets and infected with *L. major* as described in Fig. 3 and treated with anticytokine mAb or control mAb as described in Fig. 5, except that 2 mg/injection of anti-IL-10 was given. (A) Animals received 4×10^5 CD45RB^{high} (●), 4×10^5 CD45RB^{high} plus 4×10^5 CD45RB^{low} (□), 4×10^5 CD45RB^{high} plus 4×10^5 CD45RB^{low} plus anti-IL-4 (▲), 4×10^5 CD45RB^{high} plus 4×10^5 CD45RB^{low} plus anti-IL-10 (○), 4×10^5 CD45RB^{high} plus 4×10^5 CD45RB^{low} plus anti-IL-10 isolated from naive donors (■), 4×10^5 CD45RB^{low} (Δ). (B) 10^6 CD4⁺ T cells plus GL113 (●), anti-IL-4 (○), anti-IL-10 (Δ). Data are expressed as the mean value plus SEM for four to five animals per 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 4×10^5 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 5×10^5 unseparated CD4⁺ T cells ($0.88 \times 10^6 \pm 0.29 \times 10^6$, $n = 4$), which transferred a Th2 response to the parasite (Fig. 3 A), or 5×10^5 CD45RB^{high} cells ($0.83 \times 10^6 \pm 0.2 \times$

10^6 , $n = 4$) which transferred a healing response (Fig. 3 B). However, mice restored with the CD45RB^{low} CD4⁺ population contained threefold fewer ($0.29 \times 10^6 \pm 0.08 \times 10^6$, $n = 3$) 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 (10^3) 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 $1-5 \times 10^5$ 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).

CD45RB^{low} T Cells Suppressed Induction of Colitis by the CD45RB^{high} Population

Animals injected with the CD45RB^{low} subset of CD4⁺ 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^{high} population as animals that received unseparated CD4⁺ T cells or purified CD45RB^{high} cells with CD45RB^{low} 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^{high} cells either with or without isotype control mAb (14/16, Table 1). Mild pathology was evident in 7/17 mice restored with CD45RB^{high} and CD45RB^{low} CD4⁺ T cells, which consisted of a small leukocytic infiltrate and limited hyperplasia (Fig. 7, C and D).

Inhibition of the Th1 response to *L. major* 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^{low} 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^{high} and CD45RB^{low} subsets of CD4⁺ T cells play a critical role in this process. CD45RB^{high} CD4⁺ T cells,

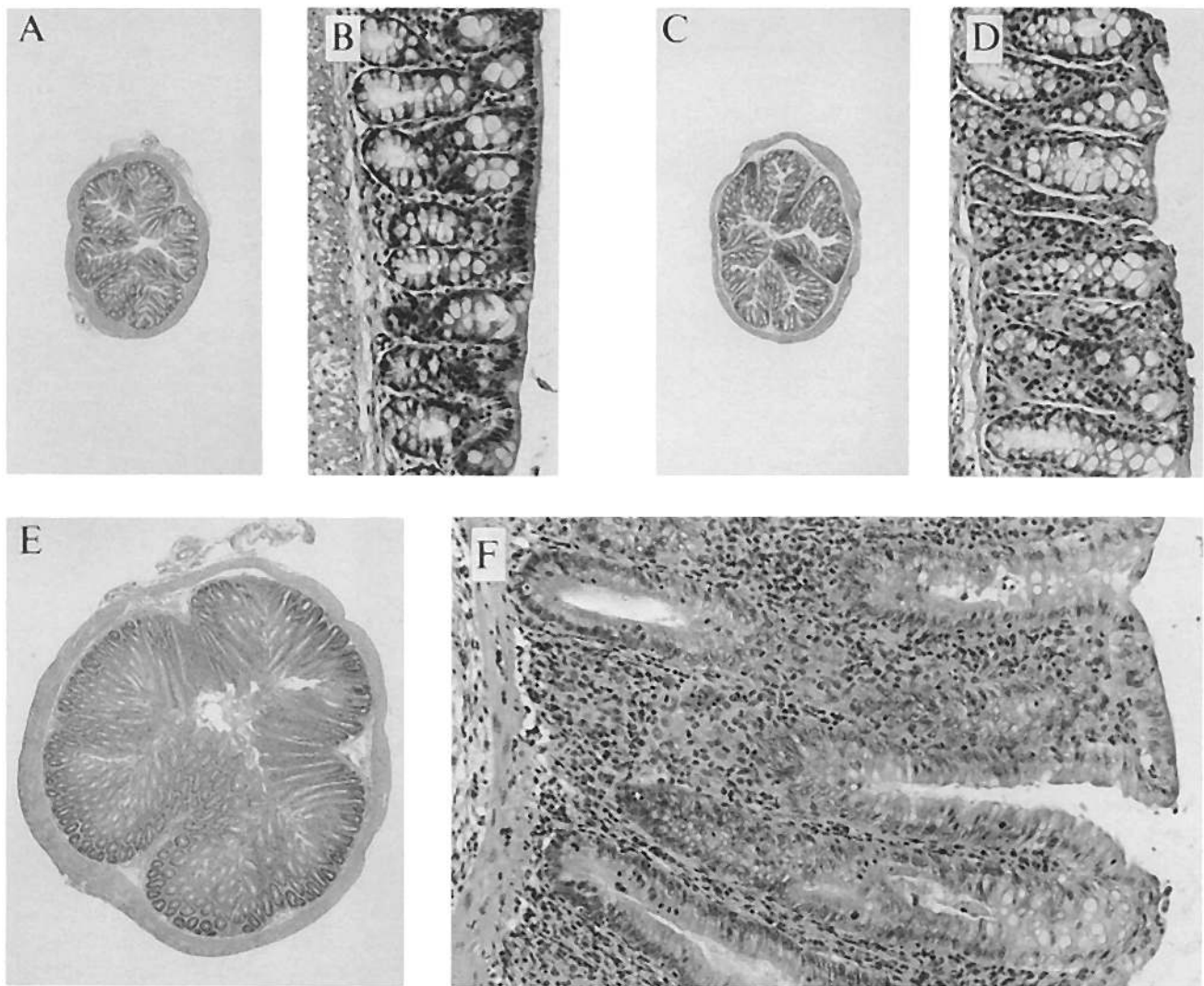


Figure 7. CD45RB^{high} CD4⁺ T cells induce colitis in C.B-17 *scid* mice. C.B-17 *scid* mice were reconstituted with CD4⁺ T cell subsets and infected with *L. major* as described in Fig. 3. Mice were killed 8 wk after reconstitution and paraffin-embedded sections of colon stained with hematoxylin and eosin. Distal colon from: normal BALB/c mouse (A and B), C.B-17 *scid* that received 4×10^5 CD45RB^{high} plus 2×10^5 CD45RB^{low} cells (C and D), C.B-17 *scid* that received 4×10^5 CD45RB^{high} cells (E and F). (A, C, E) $\times 17$; (B, D, F) $\times 162$.

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 × 10 ⁵ CD45RB ^{high}	–	0/12	2/12	10/12
	GL113	0/4	0/4	4/4
	anti-IL-4	0/4	0/4	4/4
	anti-IFN- γ	1/4	3/4	0/4
2–5 × 10 ⁵ CD45RB ^{low}	–	6/6	0/6	0/6
4 × 10 ⁵ CD45RB ^{high} + 2 × 10 ⁵ CD45 ^{low}	–	0/3	2/3	1/3
4 × 10 ⁵ CD45RB ^{high} + 4 × 10 ⁵ CD45RB ^{low}	–	1/5	3/5	1/5
	anti-IL-10	3/4	1/4	0/4
	anti-IL-4	3/5	1/5	1/5
6–10 × 10 ⁵ 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^{low} CD4⁺ population prevented the development of colitis when transferred together with the CD45RB^{high} 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⁺ 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⁺ T cells into CD4RB^{high} or CD45RB^{low} fractions revealed that the LmAg-specific IFN- γ -producing cells were contained within

the CD45RB^{high} population, whereas the specific IL-4-producing cells were contained within the reciprocal CD45RB^{low} 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⁺ or CD45RB^{low} CD4⁺ T 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^{high} cells (which comprise ~70% of CD4⁺ T cells) transferred a healing Th1 response, suggesting that in the transfers of CD4⁺ T cells, the protective potential of this population was inhibited by CD45RB^{low} cells. The dominant suppressive effects of the CD45RB^{low} population were illustrated by the fact that cotransfer of 4 × 10⁵ CD45RB^{low} cells with 4 × 10⁵ CD45RB^{high} cells led to a nonhealing Th2 response. Identical results were obtained when CD45RB^{high} and CD45RB^{low} CD4⁺ 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^{low} 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 LmAg, 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-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 lepromatous 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 CD45RB^{high} and CD45RB^{low} cells led to the development of protective Th1 responses, suggesting that the CD45RB^{low} 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).

CD45RB^{high} and CD45RB^{low} 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). CD45RB^{high} CD4⁺ cells from naive donors provided help to B cells in assays of primary antibody responses, whereas the CD45RB^{low} 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 CD45RB^{low} population (30). However, after infection with *L. major*, primed *L. major*-reactive CD4⁺ T cells can be found in both the CD45RB^{high} and CD45RB^{low} CD4⁺ populations. On a cell/cell basis, antigen-stimulated CD45RB^{high} and CD45RB^{low} populations produced equivalent amounts of IL-3 in vitro, suggesting approximately equivalent levels of activation. However, the CD45RB^{high} population produced predominantly IFN- γ , whereas the CD45RB^{low} subset was the more potent producer of antigen-stimulated IL-4. As the recall response to LmAg requires infection or immunization with *L. major* in vivo, these results indicate that the CD45RB^{high} population contains most of the *L. major*-reactive Th1 cells, whereas the CD45RB^{low} 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 CD45RB^{high} 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 CD45RB^{high} CD4⁺ T cell population in humans which was shown to be comprised of CD45RA⁺ and CD45RA⁻ cells (31). The finding that the CD45RB^{low} 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 CD45RB^{low} 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 (3×10^4 – 1×10^5) of CD45RB^{low} cells. It is not clear whether this finding represents the preferential outgrowth of a small number of contaminating CD45RB^{high} Th1 cells that are suppressed by the IL-4 producers at high cell number, or reflects a true functional heterogeneity of the CD45RB^{low} 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 unfractionated CD4⁺ T cells from *L. major*-infected donors (Correa-Olivera, R., and R. L. Coffman, unpublished data) and appears 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 exercised 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 CD45RB^{high} CD4⁺ T cells from *L. major*-infected BALB/c mice healed their *L. major* infection 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 CD45RB^{high} population 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 colitis in mice reconstituted with CD45RB^{high} 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 CD45RB^{high}-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 CD45RB^{high} subset is inhibited under normal circumstances by cells contained within the CD45RB^{low} population. Mice restored with unseparated CD4⁺ T cells, or with a mixture of CD45RB^{high} and CD45RB^{low} cells did not develop colitis. Thus the CD45RB^{low} population suppressed both protective and pathogenic Th1 responses mediated by the CD45RB^{high} 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 responses to intracellular pathogens like *L. major*.

Suppression of the Th1 response to *L. major* was dependent on IL-4 production, however, anti-IL-4 treatment failed to block the protective effect of the CD45RB^{low} cells on colitis induction or to induce colitis in mice that received unseparated 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 inflammatory responses in the intestine as mice with a targeted disruption 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 CD45RB^{low} cells, 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 CD45RB^{high} CD4⁺ T cell-restored *scid* mice. Alternatively, the regulators of colitis may have a cytokine profile distinct from Th2 cells. In this regard, multiple inflammatory lesions, including colitis, developed in mice with targeted disruption of the TGF- β 1 gene (37, 38), and IL-2-deficient mice developed colitis (35).

Multiple organ inflammation developed in nude rats reconstituted with CD45RC^{high} CD4⁺ T cells which was prevented by the CD45RC^{low} population (39). Further, transfer of CD45RC^{low} 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 CD45RB^{high} and CD45RB^{low} subfractions has revealed two populations that mediate distinct immunological functions. Regulatory interactions were shown to occur between 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 regulatory interactions that occur within the CD4⁺ T cell population and that are important in the host response to infectious agents and the maintenance of peripheral tolerance.

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