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# Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 *scid* mice

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## Abstract

**CD4+ T cells in the mouse can be subdivided into two fractions based on the level of expression of the CD45RB determinant. Previous studies have shown that these subsets are functionally distinct. We have further characterized the properties of these subpopulations *in vivo* by injecting them into C. B-17 *scid* mice. The animals restored with the CD45RB$^{high}$CD4+ T cell population developed a lethal wasting disease with severe mononuclear cell infiltrates into the colon and elevated levels of IFN-γ mRNA. In contrast, animals restored with the reciprocal CD45RB$^{low}$ subset or with unfractionated CD4+ T cells did not develop the wasting or colitis. Importantly, the co-transfer of the CD45RB$^{low}$ population with the CD45RB$^{high}$ population prevented the wasting disease and colitis. These data indicate that important regulatory interactions occur between the CD45RB$^{high}$ and CD45RB$^{low}$CD4+ T cell subsets and that disruption of this mechanism has fatal consequences.**

## Introduction

CD4+ T helper ($T_h$) cells are central to the induction and regulation of immune responses. Inflammatory responses mediated by CD4+. T cells are crucial in effective immunity towards a variety of intracellular organism. In most cases, these responses are tightly regulated to ensure efficient destruction of the microorganism with minimal damage towards host tissues. However, in some circumstances immune regulation breaks down and dysregulated inflammatory responses result in severe pathology (1,2). Currently, little is known about the mechanisms by which cell-mediated inflammatory responses are controlled normally and the cell types involved.

A number of mAbs have been generated that subdivide peripheral CD4+ T cells. The most widely studied of these are those reactive with isoforms of the CD45 antigen. At least six isoforms of CD45 are generated by the differential usage of three exons, A, B, and C, which encode for protein sequence near to the N terminus of the molecule (3). Monoclonal antibodies that subdivide CD4+ T cells are reactive with the restricted (so-called CD45R) forms of CD45 and have been described in the rat (4), mouse (5 – 7), and human (8 – 11).

In all three species the phenotypic heterogeneity is accompanied by a functional one and it is the source of some debate whether this functional heterogeneity reflects stages of T cell differentiation and/or functional specialization of the memory T cell population (12 – 14). In the mouse antibodies reactive with CD45RB subdivide the CD4+ T cell population into CD45RB$^{high}$ and CD45RB$^{low}$ subpopulations (5,6,15). The CD45RB$^{high}$ cells produce high levels of IL-2 but little IL-4 in response to a variety of mitogenic stimuli, whereas the CD45RB$^{low}$ population produces both IL-4 and IL-2. Based on this finding, it has been suggested that the CD45RB$^{high}$ and CD45RB$^{low}$ populations contain the peripheral counterparts of the $T_h1$ and $T_h2$ populations of mouse CD4+ T cell clones (16). However, other data have suggested that a lineage relationship exists between the CD45RB$^{high}$ and CD45RB$^{low}$ subsets of CD4+ T cells. The CD45RB$^{high}$ subpopulation from naive donors provided help to B cells in assays of primary antibody responses *in vivo* whereas the CD45RB$^{low}$ population was active in memory responses. Further, injection of CD45RB$^{high}$ cells into nude mice led to the expansion of progeny with the CD45RB$^{low}$ phenotype (15).

The subsets of CD4+ T cells defined on the basis of the level of CD45RB expression in the mouse resemble those divided on

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the basis of CD45RC in the rat. The CD45RC$^{high}$ population contained the cells that provided help for primary antibody responses *in vivo* (17), that mediated alloreactivity *in vivo* (4), and that produced IL-2 and IFN-$\gamma$ but not IL-4 upon polyclonal stimulation *in vitro* (18,19). In contrast, the CD45RC$^{low}$ population mediated secondary B cell help *in vitro* and *in vivo*, and was shown to derive from the CD45RC$^{high}$ population *in vivo* (17,20). After mitogenic stimulation *in vitro* this population produced higher levels of IL-4 and lower levels of interferon (IFN-$\gamma$) and IL-2 (17–20). Based on these findings, it was suggested that CD45RC-defined subsets of CD4+ T cells in the rat were heterogeneous with the CD45RC$^{high}$ population containing naive and $T_h1$-like memory cells and the CD45RC$^{low}$ population containing activated cells and $T_h2$-like memory cells (21).

One of the striking functional differences between the CD45RC$^{high}$ and CD45RC$^{low}$ CD4+ T cell subsets in the rat was that the CD45RC$^{high}$ population induced a lethal wasting disease with multiple organ inflammation when injected into syngeneic nude rats which could be prevented by the CD45RC$^{low}$ population (22). To determine if a similar dysregulated inflammatory response could be revealed by separation of the CD45RB$^{high}$ subset from the CD45RB$^{low}$ subset in mice, we injected these subsets into C. B-17 *scid* (severe combined immunodeficient) mice, which lack B and T cells (23). Data contained herein show that the CD45RB$^{high}$CD4+ T cells induced a lethal wasting disease with severe colitis in the C. B-17 *scid* recipients, which was prevented by the CD45RB$^{low}$ population. The findings that injection of the CD45RB or C$^{high}$CD4+ T cell populations into immunodeficient hosts causes fatal diseases in different species and that in both cases this aggressive potential was suppressed by the corresponding CD45RB or C$^{low}$ population suggest that an important regulatory interaction occurs between these two subsets, which may have general significance for the regulation of inflammatory responses that occur in some infectious and autoimmune diseases.

## Methods

### *Animals*

Female BALB/c and C. B-17 *scid* mice were obtained from Simonsen Laboratories (Gilroy, CA). BALB/c mice were maintained in microisolator cages and C. B-17 *scid* mice were contained in lamina flow hoods under specific pathogen-free conditions in the Animal Care Facility of the DNAX Research Institute. Mice were used at 8–12 weeks of age.

### *Antibodies and cytokines*

The following mAbs were used for purification of cell populations and immunohistochemistry: GK1.5 anti-mouse CD4 (ATCC #TIB207); GL117, an isotope control mAb reactive with $\beta$-galactosidase; AMS-32.1, anti-I-A$^d$ (PharMingen, San Diego, CA); 2-43, anti-mouse-CD8 (ATCC #TIB210); M1/70, anti-mouse Mac-1 (ATCC #TIB128); RA36B2, anti-mouse B220 (24); RB6-8C5, specific for mouse granulocytes (R. L. Coffman, unpublished results); FITC-conjugated 16A, anti-mouse CD45RB (PharMingen); phycoerythrin (PE)-conjugated anti-mouse CD4 (Becton-Dickinson, Sunnyvale, CA); PE-conjugated isotype control mAb (PharMingen).

### *Cell purification and flow cytofluorography*

Spleen cells were removed from BALB/c mice and teased into single cell suspensions in PBS containing 0.25% BSA (PBS/BSA). Erythrocytes were removed by hypotonic lysis. For enrichment of CD4+ T cells all manipulations were carried out at 4°C. Spleen cells were then incubated with PBS/BSA containing 12 $\mu$g/ml each of anti-B220, anti-Mac-1, anti-CD8, and anti-I-A$^d$ for 30 min. The mAb labelled cell suspension, after washing in PBS/BSA, was then incubated on a rotating wheel for 30 min, with sheep anti-rat coated Dynabeads (Dynabeads; Robbins Scientific, Mountain View, CA) at a ratio of three beads/cell. Negative cells were obtained by magnetic separation. Enriched CD4+ T cells (85% pure) were labelled with FITC-conjugated CD45RB (20 $\mu$g/ml) and PE-conjugated anti-CD4 (1/100 dilution) for 30 min and fractionated into CD4+CD45RB$^{high}$ and CD4+CD45RB$^{low}$ fractions by two-colour sorting on a FACSTAR PLUS (Becton-Dickinson). The CD45RB$^{high}$ and CD45RB$^{low}$ 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 labelled with FITC–CD45RB were also sorted. All populations were >98% pure on reanalysis. Flow cytography was carried out on the spleen cells from mice restored with T cell subsets 10–14 weeks earlier. Single cell spleen suspensions were depleted of erythrocytes by hypotonic lysis and labelled with PBS/BSA, containing a 1/100 dilution of PE–CD4 or PE-conjugated isotope control mAb, for 20 min at 4°C. Labelled cells were analysed on a FACScan (Becton-Dickinson).

### *Reconstitution of C. B-17 scid mice with T cell subpopulations*

C. B-17 *scid* mice were injected i.v. with 100 $\mu$l of PBS containing sorted CD4+ T cell subpopulations or unseparated CD4+ T cells.

### *Histology and immunohistochemistry*

Tissues were removed from mice 8–14 weeks after T cell reconstitution and frozen or fixed in PBS containing 10% formaldehyde. Six-micrometre paraffin-embedded sections were cut and stained with haematoxylin and eosin. Photomicrographs were taken on a Zeiss Axiophot Photomicroscope (Germany). Five-micrometre frozen sections were cut on a cryostat and stored dry at 4°C. Immunohistochemical staining of sections was performed by a peroxidase technique, with the Vectorstain Elite ABC kit (Vectastain, Burlingame, CA) and DAB substrate kit (Vectastain), according to the manufacturer's instructions.

### *RNA and cDNA preparation*

Colons were removed and RNA was prepared from 40–200 mg pieces of the distal colon by a modification of the guanidium isothiocyanate method (25). Briefly, colons were lysed in 2.5 ml of 4 M guanidine thiocyanate, containing 0.25 M trisodium citrate, 0.5% sarkosyl, 0.1 M $\beta_2$-mercaptoethanol and 0.03% antifoam A, homogenized in a Polytron homogenizer (Brinkman, Switzerland), and layered over 5.7 M CsCl, 0.5 M EDTA. Tubes were spun overnight at 219,000 *g* and at 15°C in a Beckman SW55 rotor. The pellets were resuspended, subjected to phenol chloroform extraction, and precipitated overnight. RNA was resuspended in diethylpyrocarbonate-treated water and the RNA content determined by OD measurement. RNA for standard curves was prepared from the $T_h1$ clone, D1.1 (26), and the $T_h2$ clone, CDC35 (27), as described above. A 1:1 mixture of the RNA from

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these two clones was used as a standard ($T_h$ standard). cDNA was prepared from 2 – 3 μg of RNA from test samples and $T_h$ standard by reverse transcription using Superscriptase II RNase H reverse transcriptase (Gibco BRL, Gaithersberg, MD), according to the manufacturer's instructions except that 10× synthesis buffer with the following composition was used: 200 mM Tris – HCl (pH 8.4), 500 mM KCl, 25 mM $MgCl_2$, 1 mg/ml BSA.

*Polymerase chain reaction (PCR) and quantitation of cytokine RNA*

Semi-quantitative analysis of cytokine RNA was carried out by a method described by O'Garra (28). PCR reactions contained sample cDNA, 0.2 mM dNTP, 0.01 OD sense and antisense primers, and 1.2 U Taq DNA polymerase (Boehringer Mannheim Biochemica, Indianapolis, IN) and were amplified in a Perkin Elmer Cetus 9600 GeneAmp. The following conditions were used: hypoxanthine phosphoribosyltransferase (HPRT), 92°C for 2 min, then 30 consecutive cycles of 91°C for 10 s, 59°C for 25 s, and 72°C for 25 s followed by 72°C for 10 min; IL-4, IL-10, tumour necrosis factor (TFN)-α, and IFN-γ, 92°C for 2 min, then 30 consecutive cycles of 88°C for 1 min, 61°C for 1 min, and 72°C for 1 min, followed by 72°C for 10 min. Hybridization of amplified cDNA was performed by the dot blot method described by Murphy *et al.* (28), modified from that of Dallman *et al.* (29). Briefly, the entire PCR reaction was denatured and transferred to a nylon membrane by vacuum blotting. The membrane was allowed to dry and then UV cross-linked. Specific cDNA was detected by hybridization of filters with [$\gamma$-$P^{32}$]end-labelled oligonucleotide probes and washed exactly as described (28). Radioactivity was detected by exposure to film or quantitated on a Molecular Devices Phosphoimager (Sunnyvale, CA). The primers and probes for HPRT, IL-4, TNF-α, IFN-γ, and IL-10 have all been described previously (28). An aliquot from the single pool of $T_h$ standard RNA (a mixture of D1.1 and CDC35, $T_h1$ and $T_h2$, respectively) was reverse transcribed along with each group of samples and used to construct all standard curves for those samples. Relative quantitation of samples was carried out as previously described (30) using quantitation of the housekeeping enzyme HPRT as an internal standard to normalize for variations in mRNA recovery and reverse transcription. HPRT levels in the samples were determined by comparison with a standard curve of HPRT constructed with 10 – 0.087 ng of $T_h$ RNA. Sample cDNA was run at several dilutions and those falling within the linear portion of the HPRT standard were used to determine the amount of HPRT in each sample (1 unit of HPRT is defined as the amount in 1 ng $T_h$ standard cDNA). Equivalent amounts of HPRT (100 units of HPRT) from the test samples were then analysed for cytokine cDNA. For each cytokine the standard $T_h$ cDNA was run and specific cytokine levels determined (IL-10, IL-4, and IFN-γ, 156 – 1.2 pg; TNF-α, 10 – 0.019 ng). Units of specific cytokine were determined for each sample by comparison with the standard curve (1 unit of cytokine is defined as the amount in 1 ng $T_h$ standard cDNA). Values were expressed as units of cytokine per $10^5$ units of HPRT.

## Results

*C. B-17 scid mice reconstituted with CD45RB$^{high}$ CD4+ T cells developed a wasting disease*

To analyse the functions of CD4+ T cell subsets *in vivo*, C. B-17 *scid* mice were reconstituted with unfractionated CD4+ T cells or the CD45RB$^{high}$ and CD45RB$^{low}$ subfractions, isolated from the spleens of naive BALB/c mice. BALB/c and CB-17 mice are congenic with different alleles of the *Igh* chain locus. The phenotype of the donor cell inoculum had a drastic effect on the subsequent health of the recipients. Animals restored with the CD45RB$^{high}$ subset developed a wasting disease which became evident 3 – 5 weeks post-reconstitution (Fig. 1). By 12 weeks post-reconstitution animals in these groups (given 1 or 4 × $10^5$ CD45RB$^{high}$CD4+ cells) had lost 15 – 20% of their body weight and in this experiment 1/9 had died (Fig. 1). The wasting induced by the CD45RB$^{high}$CD4+ T cell subset was T cell dose dependent; animals given doses of 1 or 4 × $10^5$ cells both developed wasting and were indistinguishable from each other, whereas animals given 1 × $10^4$ cells did not develop the wasting disease over the 12 week period of the study (Fig. 1). However, by 16 weeks these animals had started to lose weight, indicating that very low numbers of the CD45RB$^{high}$ subset could induce the wasting disease, albeit with a slower onset (data not shown). The wasting disease was dependent upon the injection of CD45RB$^{high}$ cells since unreconstituted C. B-17 *scid* mice gained weight over the 12 week period [at 12 weeks mice in this group ($n$ = 9), weighed 113 ± 3.5% of their weight at the time of T cell reconstitution].

In contrast to the CD45RB$^{high}$ restored mice, animals given the CD45RB$^{low}$CD4+ T cell subset gained weight (18 – 20% over what they were at the beginning of the experiment) and continued to thrive throughout the course of the experiment (Fig. 1). Indeed, animals restored with CD45RB$^{low}$ cells showed no wasting disease when followed up to 9 months after T cell reconstitution (data not shown). The CD45RB$^{low}$ population not only did not cause the wasting but also inhibited the potential of the CD45RB$^{high}$ population to do so, as animals given

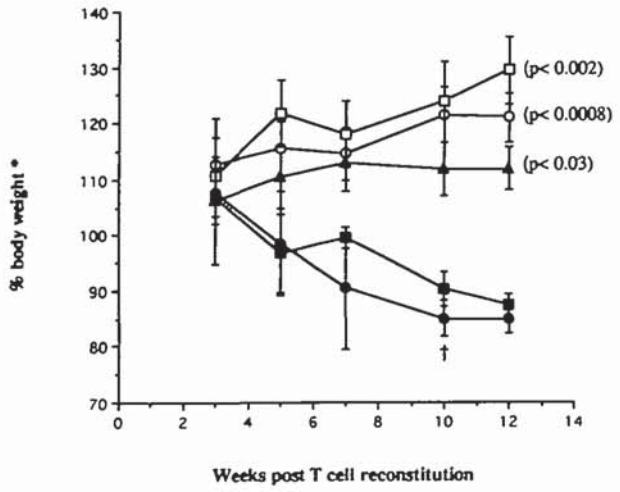


**Fig. 1.** CD45RB$^{high}$CD4+ T cells induce a wasting disease when injected into C. B-17 *scid* mice. C. B-17 *scid* mice were reconstituted with (□) 4 × $10^5$ CD4+ T cells, (○) 2 × $10^5$ CD45RB$^{low}$CD4+ T cells, or (■) 4 × $10^5$, (●), 1 × $10^5$, or (▲) 1 × $10^4$ CD45RB$^{high}$CD4+ T cells. *The change in weight over time is expressed as a percentage of the weight at the beginning of the experiment. Data are the mean of 4 – 5 mice per group plus SE. †Animal was moribund and had to be killed. Statistical analysis was performed using a two-tailed Student's *t*-test on the indicated groups versus animals restored with 4 × $10^5$ CD45RB$^{high}$CD4+ T cells.

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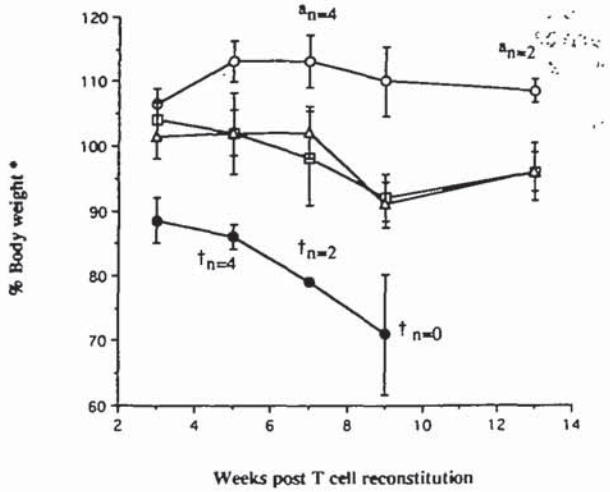


**Fig. 2.** CD45RB$^{low}$CD4$^+$ T cells prevent the wasting disease induced by CD45RB$^{high}$CD4$^+$ T cells. C. B-17 *scid* mice were restored with CD4$^+$ T cell subsets and their weight monitored as described in Fig. 1. (●) 2.5 × 10$^5$ CD45RB$^{high}$, (○) 2.5 × 10$^5$ CD45RB$^{high}$ + 1 25 × 10$^5$ CD45RB$^{low}$, (□) 2.5 × 10$^5$ CD45RB$^{high}$ + 0.6 × 10$^5$ CD45RB$^{low}$, and (△) 2.5 × 10$^5$ CD45RB$^{high}$ + 0.3 × 10$^5$ CD45RB$^{low}$ cells. *The change in weight over time is expressed as a percentage of the weight at the beginning of the experiment. Data represent the mean of 4 – 5 animals per group plus SE. †Animal was moribund and had to be killed. [a]Animals were killed for histopathological analysis; the number of animals remaining in the group (*n*) is indicated.

**Table 1.** Survival and pathology in C. B-17 *scid* mice restored with CD4$^+$ T cell subsets

| Phenotype of CD4$^+$ T cell inoculum | % survival >12 weeks | *n* | Colitis (12 – 14 weeks) | | |
|---|---|---|---|---|---|
| | | | – | + | + + + |
| 1 – 5 × 10$^5$ CD45RB$^{high}$ | 70 | 23 | 1/20 | 2/20 | 17/20 |
| 2 – 5 × 10$^5$ CD45RB$^{low}$ | 100 | 10 | 4/5 | 1/5 | 0/5 |
| 2 – 5 × 10$^5$ CD4$^+$ | 100 | 9 | 3/9 | 5/9 | 1/9 |
| 2 × 10$^5$ CD45RB$^{high}$ + 2 × 10$^5$ CD45RB$^{low}$ | 100 | 5 | 5/5 | 0/5 | 0/5 |
| 2 × 10$^5$ CD45RB$^{high}$ + 1 × 10$^5$ CD45RB$^{low}$ | 100 | 2 | 0/2 | 2/2 | 0/2 |

Data were pooled from three experiments.

4 × 10$^5$ CD4$^+$ T cells, which contain approximately two-thirds CD45RB$^{high}$ cells and one-third CD45RB$^{low}$ cells, did not develop the wasting disease and survived significantly better ($P < 0.002$) than animals given an equal number or 4-fold fewer CD45RB$^{high}$ cells alone (Fig. 1).

To determine how effectively the CD45RB$^{low}$ subpopulation could regulate the wasting disease induced by the CD45RB$^{high}$ subset, CD4$^+$ T cells were fractionated into the CD45RB$^{high}$ and CD45RB$^{low}$ subsets and injected into C. B-17 *scid* mice at different ratios. Figure 2 shows that optimal inhibition of the wasting, equivalent to that seen with unseparated CD4$^+$ T cells (Fig. 1), required combination of the subsets at their physiological ratio of 2:1 (Fig. 2). However, the inclusion of lower numbers of CD45RB$^{low}$ cells in the inoculum (as few as one-eighth the number of CD45RB$^{high}$ cells) still provided significant protection compared with animals given the CD45RB$^{high}$ population alone (Fig. 2). Animals in this group showed no clinical signs of the wasting disease until 9 weeks post-reconstitution and had lost only 5 – 10% of their body weight by 12 weeks. In contrast, the CD45RB$^{high}$ restored animals had developed the wasting disease by 3 weeks and had lost 20 – 30% of their body weight by 9 weeks, at which point they were killed (Fig. 2). These data indicate that the regulatory cell present in unseparated CD4$^+$ T cells responsible for inhibiting the CD45RB$^{high}$-induced wasting disease is contained within the CD45RB$^{low}$ population and is not lost in the overlapping population discarded during sorting.

*Severe colitis in C. B-17 scid mice given CD45RB$^{high}$CD4$^+$ T cells in the absence of the CD45RB$^{low}$ subpopulation*

After injection of CD45RB$^{high}$CD4$^+$ T cells only 70% of the mice survived past 12 weeks (Table 1) and the majority of remaining mice were unwell. Severe colonic pathology was immediately obvious in mice from this group killed 9 – 14 weeks post-reconstitution. Macroscopically, colons were significantly enlarged and weighed 717 ± 36 mg ($n$ = 6). This weight was >2-fold greater than the colonic weights observed in animals given both CD45RB$^{low}$ and CD45RB$^{high}$ cells, either as unseparated CD4$^+$ T cells or as a 2:1 mixture of CD45RB$^{high}$ and CD45RB$^{low}$ cells. Colons in these groups weighed 296 ± 12.5 mg ($n$ = 6), in the range of those obtained from unrestored C. B-17 *scid* mice (259 ± 28 mg, $n$ = 5) or normal BALB/c mice (242 ± 12.3 mg, $n$ = 5).

Microscopically, colitis was graded as severe (+ + +), moderate (+ +), minimal (+), or absent (–). Seventeen of 20 CD45RB$^{high}$ restored mice had severe colitis (Table 1), characterized by prominent epithelial hyperplasia with glandular elongation and loss of goblet cells (Fig. 3B and D), Extensive leukocytic infiltrates were seen in the lamina propria and submucosa and, to a lesser degree, in the muscularis, serosa, and mesentery (Fig. 3B, D, and E). Inflammatory infiltrates consisted of mainly mononuclear cells with smaller numbers of neutrophils. Ulceration, often overlying sparsely populated lymphoid aggregates, was sometimes seen (Fig. 3E). Intestinal glands were variably dilated and contained sloughed epithelial cells and neutrophils (Fig. 3F). Histopathologic changes were most severe in the distal colon and the rectum, but were also present in the proximal colon and in some cases the caecum. Immunohistochemical analysis of the infiltrate revealed large numbers of CD4$^+$ cells (Fig. 3G), macrophages (MAC-1$^+$, data not shown), and granulocytes (RB6-8C5$^+$, data not shown). Interestingly, severe pathology was restricted to the large intestine with little involvement of the small intestine (data not shown).

Consistent with the incidence of the wasting disease, 4/5 animals restored with the CD45RB$^{low}$CD4$^+$ T cell subset showed little colonic pathology (Table 1), and were microscopically indistinguishable from normal BALB/c mice. One of five animals had minimal infiltrates, described as + in Table 1. Alterations in these animals consisted of scattered macrophages and lymphocytes restricted to the lamina propria. This infiltrate when present was most evident in the caecum and proximal colon. Animals given unseparated CD4$^+$ T cells or a mixture of the CD45RB$^{high}$ and CD45RB$^{low}$ cells also had a significantly lower incidence of severe colitis (1/9 and 0/7, respectively; Table 1) than animals given CD45RB$^{high}$ cells alone. These findings indicate that the CD45RB$^{low}$ subset inhibited the development

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of colonic lesions induced by the CD45RB$^{high}$ population. Minimal pathological changes were evident in 5/9 CD4+ T cell restored mice and 2/2 animals given a 2:1 ratio of CD45RB$^{high}$:CD45RB$^{low}$ cells (Table 1). Colons from mice (5/5) given a 1:1 mixture of CD45RB$^{high}$:CD45RB$^{low}$ cells and 3/9 CD4+ T cell restored mice were microscopically indistinguishable from normal BALB/c mice (Table 1, Fig. 3A and C).

Other organs from CD45RB$^{high}$CD4+ T cell restored mice, including liver, lungs, stomach, thyroids, salivary glands, heart, kidney, female reproductive tract, lung, tongue, and pancreas, were examined. Minor microscopic lesions were found in several of these organs. Extramedullary haematopoiesis was evident in the liver, which appeared to comprise mainly myeloid precursors with some megakaryocytes. Compared with controls, there was a slight increase in mononuclear cells in the lamina propria and submucosa of the stomach, and in the periportal areas of the liver (data not shown). One of 10 CD45RB$^{high}$CD4+ T cell-restored mice examined had severe thyroiditis.

*Detection of cytokine mRNA levels by semi-quantitative PCR analysis*

To determine whether the inflammatory infiltrate in the colons of the CD45RB$^{high}$CD4+ T cell-restored C. B-17 *scid* mice reflected activation of $T_h1$ or $T_h2$ type cytokines, RNA was extracted from the colons of these mice and the levels of IL-4, IL-10, TNF-$\alpha$, and IFN-$\gamma$ mRNA were determined by semi-quantitative PCR analysis and compared with colons from normal BALB/c mice, CD45RB$^{low}$-restored mice, or animals given a 1:1 mixture of CD45RB$^{high}$ and CD45RB$^{low}$CD4+ cells (Fig. 4, Table 2). Samples of cDNA containing equivalent amounts of HPRT were amplified, blotted, and probed for the presence of the various cytokines. The results of the dot blot for IFN-$\gamma$ are shown in Fig. 4. Units of IFN-$\gamma$ were determined from the IFN-$\gamma$ $T_h$ standard curve (Fig. 4) and expressed as units of IFN-$\gamma$/$10^5$ units of HPRT for each sample. Values for IL-4, IL-10, and TNF-$\alpha$ mRNA levels were likewise determined by reference to standard curves for these cytokines and expressed as units/$10^5$ units of HPRT (Table 2). The results clearly show that IFN-$\gamma$ mRNA levels were substantially higher (46-fold) in mice which had been inoculated with CD45RB$^{high}$CD4+ T cells (Fig. 4, Table 2) than in animals from the other groups, in which IFN-$\gamma$ mRNA levels were below the sensitivity of the assay (1.3 units of IFN-$\gamma$/$10^5$ units of HPRT). TNF-$\alpha$ mRNA levels were also enhanced in CD45RB$^{high}$-restored C. B-17 *scid* mice compared with normal BALB/c mice (7-fold) and animals given a 1:1 mixture of CD45RB$^{high}$ and CD45RB$^{low}$ CD4+ T cells (14-fold). However, similar levels of TNF-$\alpha$ mRNA were obtained from the CD45RB$^{low}$CD4+ T cell-restored mice (Table 2). IL-10 mRNA levels were not elevated in the mice with colitis, when compared with controls, indicating that the colitis was not associated with a general activation of cytokine genes but rather a differential one. IL-4 mRNA was below the sensitivity of the assay in all samples. The inability to detect IL-4 mRNA is not due to an inappropriate range of sensitivity of the assay, as under similar conditions IL-4 mRNA was readily detectable in lymph node cells making strong $T_h2$ responses to *Leishmania major* (L. Barcomb Caddle, unpublished data). The finding that $T_h2$ cytokine transcripts were not enhanced in the CD45RB$^{high}$CD4+ T cell-restored mice compared with the control groups but that the $T_h1$ cytokine IFN-$\gamma$ was indicated a preferential enhancement of $T_h1$ cytokine mRNA in these mice.

*Engraftment of CD4+ T cell subsets into the spleen of C. B-17 scid mice*

Previous studies have shown that CD4+ T cells can repopulate the spleen of C. B-17 *scid* mice (31,32), and our study confirms this finding. Both the CD45RB$^{high}$ and CD45RB$^{low}$ subpopulations of CD4+ T cells led to engraftment of CD4+ T cells compared with the unrestored C. B-17 *scid* mouse (Table 3). Analysis of the total number of splenic CD4+ T cells showed that mice restored with the CD45RB$^{high}$ subpopulation of CD4+ T cells contained 2-fold higher numbers of CD4+ T cells than mice restored with the reciprocal CD45RB$^{low}$ subset (Table 3), indicating an enhanced expansion potential of the former population, which is in accord with previous studies in the rat (14,22). However, in the rat studies the CD45RC$^{high}$CD4+ T cells exhibited extensive proliferative capacity *in vivo*. In our studies this proliferation was much more restricted, as the total number of CD4+ T cells obtained in these animals was 20-fold lower than that found in normal BALB/c mice (Table 3). The limited expansion of unseparated CD4+ T cells in C. B-17 *scid* mice has been noted before (31), where it was suggested that this may be caused by the lack of B cells. In spite of having a higher number of total CD4+ T cells, spleens from CD45RB$^{high}$-restored mice had a reduced frequency of CD4+ T cells compared with T cell-restored mice which did not develop the wasting (Table 3). This was because of significant increase in cellularity of these spleens. Two populations which clearly had expanded compared with spleens in the other groups were macrophages and granulocytes, detected by MAC-1 and RB6-8C5 staining respectively (data not shown). The relationship between colitis and expansion of these cell populations is presently unknown. However, $5 \times 10^6$ spleen cells from C. B-17 *scid* mice restored with CD45RB$^{high}$CD4+ T cells 10 weeks earlier were able to transfer the colitis to C. B-17 *scid* recipients (data not shown), indicating that the cells able to induce the colitis were present in the spleen.

## Discussion

Injection of the CD45RB$^{high}$ subset of CD4+ T cells from naive donors into C. B-17 *scid* mice resulted in the development of a lethal wasting disease in the recipients with severe leukocyte infiltration in the colon, particularly macrophages and CD4+ T cells accompanied by marked epithelial hyperplasia. In clear contrast, C. B-17 *scid* mice restored with the reciprocal CD45RB$^{low}$ subset showed no signs of the wasting disease, no significant colon pathology, and no mortality when followed up to 9 months after T cell reconstitution. The differences between the CD45RB$^{high}$ and CD45RB$^{low}$ cells in their potential to cause wasting disease and colitis did not appear to be due simply to a lack of reactivity within the CD45RB$^{low}$ population, as co-transfer of this population with CD45RB$^{high}$ cells prevented the wasting disease and destructive inflammatory response in the colon, and co-transferred animals continued to thrive throughout the course of the experiment.

The results obtained here with transfer of CD45RB$^{high}$ and CD45RB$^{low}$ subsets of CD4+ T cells are similar to those previously reported with CD45RC subsets in the rat (22). In those studies CD4+CD45RC$^{high}$ T cells caused a lethal wasting disease with multiple organ pathology when injected into

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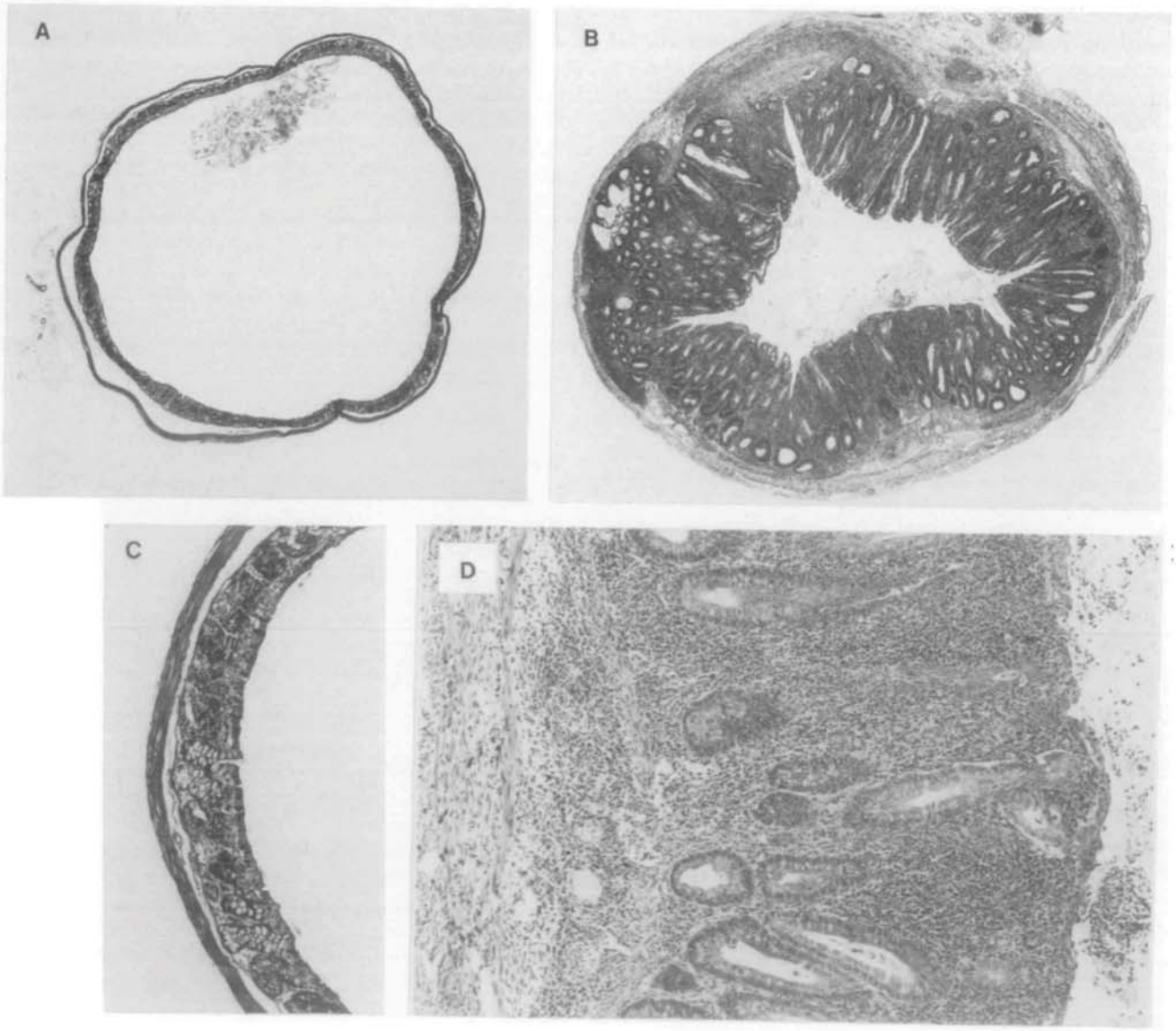
syngeneic nude rats. The disease was absent from CD4+ T cell-restored rats, indicating that the CD45RB$^{low}$ population inhibited the development of wasting disease and organ pathology induced by the CD45RC$^{high}$ population. The similarities between these findings and those seen in our mice suggest that the subpopulations of CD4+ T cells identified by antibodies reactive with CD45RC in the rat are functionally equivalent to those identified by antibodies reactive with CD45RB in the mouse. Furthermore, the ability of normal CD4+ T cell subpopulations to induce wasting disease and multiorgan inflammation is conserved in the rat and mouse. Consistent with this, comparison of CD45RB and CD45RC expression on murine CD4+ T cells has shown them to be quantitatively correlated (7).

The triggering event for the development of the wasting disease and colitis was the injection of CD45RB$^{high}$CD4+ T cells. It has been suggested that the wasting disease in nude rats restored with CD45RC$^{high}$CD4+ T cells may be attributable to an immunodeficiency amongst the CD45RC$^{high}$ population (14). This did not appear to be the case in our study, as unreconstituted C. B-17 *scid* mice showed no signs of wasting disease or colitis, indicating that the injected T cells triggered an immune-mediated pathogenesis. Further, the severe inflammatory infiltrates in the colon, accompanied by high levels of IFN-$\gamma$ mRNA detectable on PCR analysis, are not characteristic of immunodeficiency but rather immune reactivity.

The obvious question is reactivity to what? A pathogenic inflammatory response to an infectious agent cannot be ruled out, but several points argue against this: (i) an extensive necropsy examination of three animals with disease failed to identify any organisms other than the normal intestinal flora; (ii) special stains used to reveal bacterial and fungal organism did not reveal any of these agents in the colons of affected animals; and (iii) the pathologic changes did not resemble lesions typical of infection by known pathogens. These results do not exclude low numbers of slow growing organisms such as mycobacterium, as has been suggested in human inflammatory bowel disease (33).

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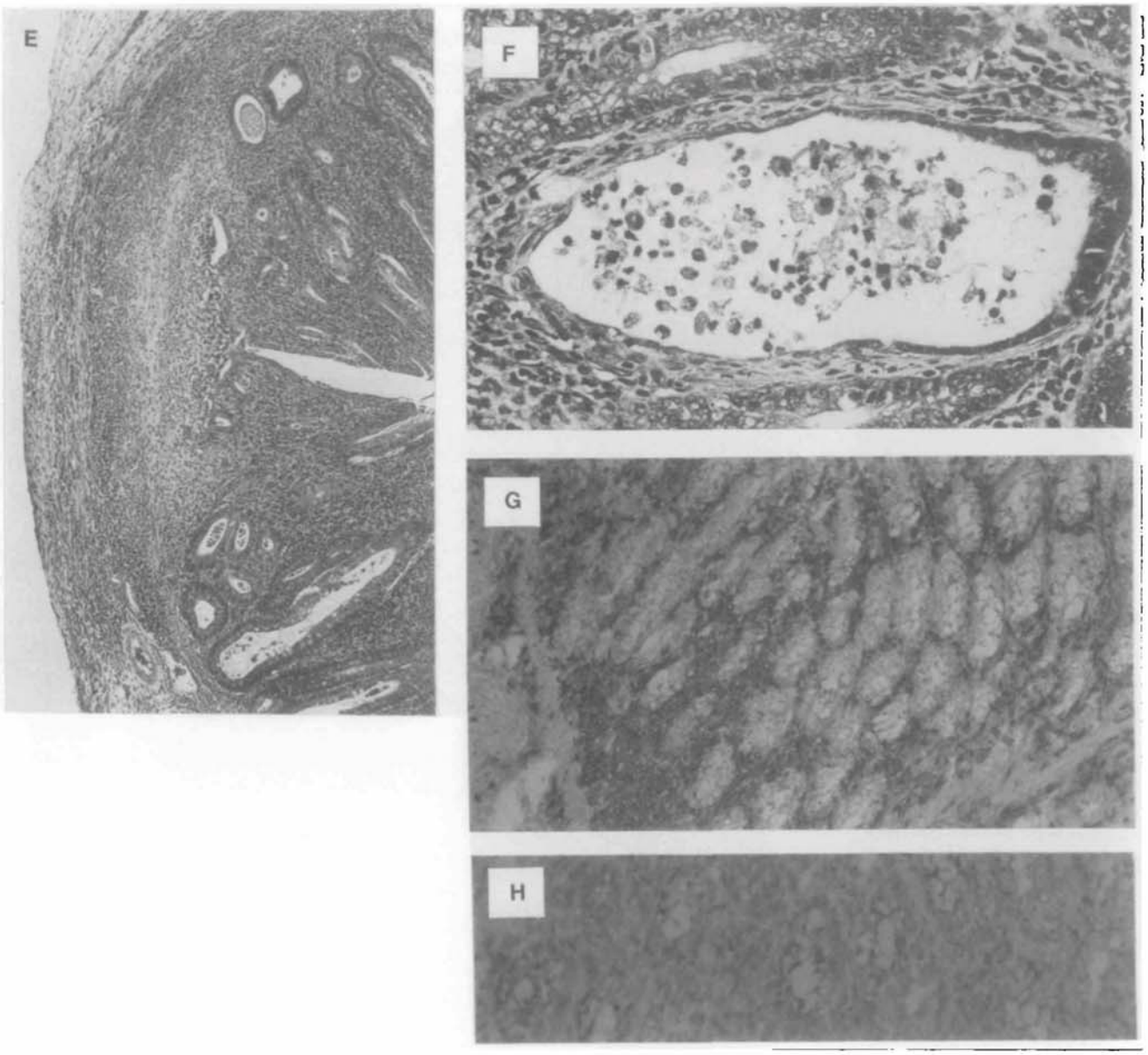


**Fig. 3.** Severe colitis in C. B-17 *scid* mice restored with $CD45RB^{high}CD4^+$ T cells. Comparison of distal colon from mice injected with $2 \times 10^5$ $CD45RB^{high}$ + $2 \times 10^5$ $CD45RB^{low}CD4^+$ T cells (A, ×22; C, ×92) or $4 \times 10^5$ $CD45RB^{high}CD4^+$ T cells (B, ×22; D, ×92). (E – H) Animals restored with $4 \times 10^5$ $CD45RB^{high}CD4^+$ T cells: (E) ulcer over lymphoid aggregate in distal colon (magnification ×70); (F) dilated intestinal gland in the rectum with infiltrating leukocytes (×350); (G) infiltrating $CD4^+$ T cells in the colon detected by immunochemical staining with anti-CD4 (×200); and (H) a negative control mAb (×200).

The finding that significant bowel pathology is limited to the large intestine, and is generally more severe distally, correlates with increasing loads of bacterial flora, making it tempting to speculate that the $CD45RB^{high}CD4^+$ T cells are mounting a dysregulated immune response to components of the normal gut flora. However, autoimmune reactivity to colonic epithelial cells could also be involved. Intestinal inflammation similar to that described here is found in rats made transgenic for the human class I allele HLA-B27 (34) and in cyclosporin-induced syngeneic graft-versus-host disease (SGVHD) in rats and mice (35). In both these models the aetiology of the autoimmune disease, which also affects the skin and liver, is unknown, but involvement of the intestinal flora has been suggested. Responses to the intestinal flora and the development of autoimmunity may be linked since the incidence of thyroiditis in immunologically manipulated rats was lower in animals reared under SPF conditions than those reared under conventional housing, but could be increased by transfer of intestinal material from the latter (36). Recently direct evidence for a causal link between the intestinal flora and colitis has come from studies with mice which lack a functional IL-2 gene. Mice with this mutation housed under conventional conditions developed a lethal wasting and colitis which was absent

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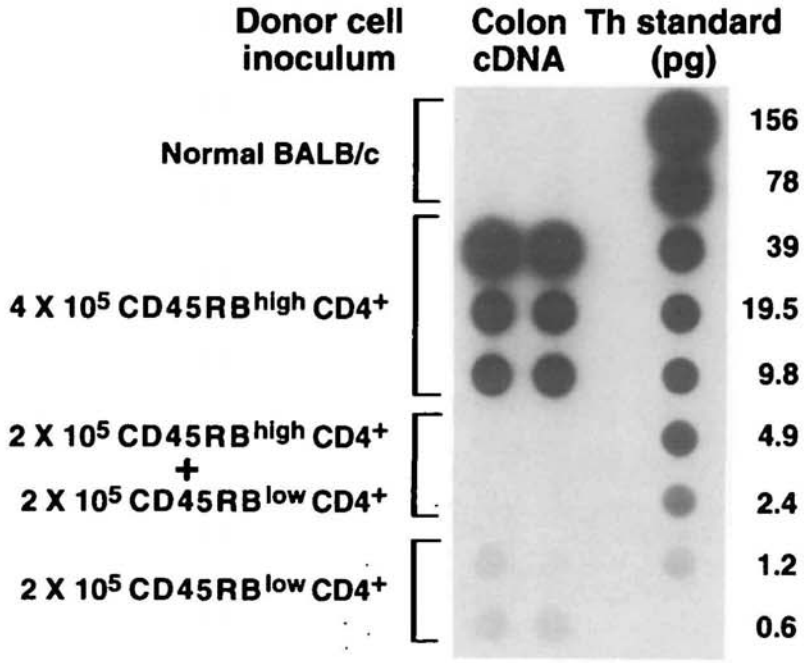


**Fig. 4.** Increased expression of IFN-γ mRNA in the colons of C. B-17 *scid* mice reconstituted with $CD45RB^{high}CD4^+$ T cells. Dot blot shows the expression of IFN-γ cDNA for the indicated samples with a standard curve constructed from $T_h$ standard cDNA. Each sample dot contains 100 units of HPRT, as described in Methods. Units of IFN-γ were defined with reference to the standard curve. Each row of dots represents individual animals analysed in duplicate.

from those derived and maintained under germ free conditions (61). The intestinal lesion in the conventionally housed mice had some similarities to the intestinal lesions described here, notably, restriction to the colon, marked leukocytic infiltration and epithelial cell hyperplasia. However, cytokine gene expression in the colon was not studied in the IL-2 deficient mice and it is not known whether the immune mediated pathology that occurs in the colon in these two models is mechanistically similar.

The $CD45RB^{high}$ and $CD45RB^{low}CD4^+$ T cell subsets are widely thought to represent naive and memory T cells respectively (15,37). However, the finding that the $CD45RB^{high}$ cells induce a lethal inflammatory disease which is inhibited by the $CD45RB^{low}$ population suggests that these subsets contain cells with distinct functional properties and is not consistent with a simple precursor–product relationship. It has been suggested that the $CD45RB^{high}$ and $CD45RB^{low}$ subsets may also distinguish functionally specialized peripheral $CD4^+$ T cells, similar to the $T_h1$- and $T_h2$-like clones respectively, which have been described in the mouse and human (16). $T_h1$ cells produce IL-2 and IFN-γ and are active in cell-mediated immune responses, whereas $T_h2$ cells produce IL-4, IL-5, and IL-10 and are the principal helpers for Ig production (38). Consistent with this idea, we have recently shown that the $CD45RB^{high}$ population isolated from *L. major* infected BALB/c mice produced IFN-γ in response to *L. major* antigens *in vitro* and conferred a healing $T_h1$-like response against the parasite when transferred to C. B-17 *scid* mice. In contrast, the $CD45RB^{low}$ population produced IL-4 upon recall responses *in vitro* and transferred a non-healing $T_h2$-type response against the parasite to C. B-17 *scid* mice (F. Powrie *et al.*, submitted).

**Table 2.** Cytokine mRNA levels in the colon of T cell-restored C. B-17 *scid* mice

| Phenotype of $CD4^+$ T cell inoculum | Units of cytokine mRNA/$10^5$ units of HPRT mRNA | | |
|---|---|---|---|
| | IFN-γ | TNF-α | IL-10 |
| [a]$4 \times 10^5$ $CD45RB^{high}$ | 47 ± 11 | 3300 ± 590 | 9 ± 1 |
| $2 \times 10^5$ $CD45RB^{low}$ | <1.3 | 2000 ± 330 | 9 ± 4 |
| $2 \times 10^5$ $CD45RB^{high}$ + $2 \times 10^5$ $CD45RB^{low}$ | <1.3 | 240 ± 14 | 5 ± 1 |
| BALB/c | <1 3 | 480 ± 53 | 17 ± 2 |

Cytokine mRNA levels were determined by semi-quantitative PCR. Data represent the mean value of duplicate samples from two animals per group plus SE, apart from [a], which contains three animals per group plus SE. IL-4 mRNA levels in all samples were below the sensitivity of the assay (1.3 units/$10^5$ units of HPRT).

The colitis induced by the $CD45RB^{high}$ population has the hallmarks of a $T_h1$ response. Firstly, the infiltrates contained high numbers of $CD4^+$ T cells and macrophages but few eosinophils. Secondly, IFN-γ mRNA levels were elevated at least 40-fold compared with colons from normal BALB/c mice, $CD45RB^{low}$-restored mice, or $CD45RB^{high}$- and $CD45RB^{low}$-restored mice. This level of IFN-γ mRNA was of the same magnitude as in lymphoid organs from mice making strong $T_h1$ responses to *L. major* (L. Barcomb Caddle, unpublished observations). TNF-α mRNA was also elevated in the animals with colitis; however, the presence of this cytokine was not predictive of colitis as the

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**Table 3.** Reconstitution of CD4+ T cells in C. B-17 *scid* mice restored with CD4+ T cell subsets

| Phenotype of CD4+ T cell inoculum | Cell yield ($\times 10^6$) | CD4+ T cells | |
|---|---|---|---|
| | | % | Total ($\times 10^6$) |
| [a]2 – 4 × $10^5$ CD45RB$^{high}$ | 37.9 ± 13 | 3.9 ± 1.3 | 1.1 ± 0.3 |
| [b]2 × $10^5$ CD45RB$^{low}$ | 2.6 ± 0.9 | 20.6 ± 1.8 | 0.54 ± 0.17 |
| 2 – 5 × $10^5$ CD4+ | 4.6 ± 0.9 | 18.2 ± 4.3 | 0.7 ± 0.15 |
| Unreconstituted SCID | 3.2 ± 0.67 | 1.0 ± 0.1 | 0.03 ± 0 006 |
| BALB/C | 63.8 ± 0.73 | 32.3 ± 0.7 | 20.5 ± 0.22 |

The percentage of CD4+ T cells was determined by FACS analysis. Data represent the mean of five animals per group plus SE. [a]$n$ = 7, [b]$n$ = 3.

CD45RB$^{low}$-restored mice also had elevated TNF-$\alpha$ mRNA levels. The presence of these cytokines does not necessarily implicate them in the pathogenesis of the disease and studies to address this are currently under way. In contrast to $T_h1$ cytokine mRNA, the $T_h2$ cytokines IL-4 and IL-10 were not elevated in the colons of the reconstituted C. B-17 *scid* mice. Indeed, IL-4 mRNA levels in all samples were below the limit of sensitivity of the assay, although IL-4 mRNA in $T_h2$ responses to *L. major* in BALB/c mice were easily within the sensitivity range of this assay (L. Barcomb Caddle, unpublished observations).

There is good evidence that $T_h1$ immunity can be pathogenic both in response to known infectious agents and in certain autoimmune diseases in which the aetiology is unknown. For example, IFN-$\gamma$ has been implicated in the pathology associated with tuberculoid leprosy (2), cerebral malaria (1), and diabetes in non-obese diabetic rats (39,40). It has been suggested that development of these deleterious $T_h1$ responses may be inhibited by the expansion of $T_h2$-like cells which secrete IL-4 and IL-10 (41). These cytokines have been shown to inhibit the effector functions of $T_h1$ cells, both at the level of $T_h1$ cytokine synthesis (42 – 44) and macrophage activation (45,46) *in vitro*, and recently we have shown that IL-4 and IL-10 synergize to inhibit $T_h1$ responses *in vivo* (47). Since the CD45RB$^{low}$ but not the CD45RB$^{high}$ population contains cells with the characteristics of $T_h2$ cells (6), it is possible that production of IL-4 and IL-10 by the CD45RB$^{low}$ population may account for the suppressive properties of this subset. This hypothesis is currently being tested. However, the experiments described here do not rule out the possibility that the regulatory population contained within the CD45RB$^{low}$ subset has its own discrete cytokine repertoire distinct from $T_h2$-type cells. Relevant to this multiple organ inflammation occurs in mice with targeted disruption of the transforming growth factor-$\beta$1 gene (48,49).

In spite of the similarities in the induction and regulation of wasting disease that occur with CD4+ T cell subset transfer into immunodeficient rats and mice, the pathologic lesions that presumably led to the wasting and mortality were very different in the two studies. Target organs significantly affected in the CD45RC$^{high}$CD4+ T cell reconstituted nude rats included lung, liver, thyroid, pancreas, and stomach, whereas the only consistent severe pathologic lesion evident in the CD45RB$^{high}$CD4+ T cell-restored C. B-17 *scid* mice was colitis, although occasional lung, liver, and thyroid lesions were observed. Several explanations can be advanced. Firstly, differences in the MHC between C. B-17 *scid* mice and nude rats may result in the activation of T cells with different target organ specificity. MHC genes have been linked to susceptibility to a number of autoimmune diseases (50). Secondly, nude rats contain B cells whereas C. B-17 *scid* mice do not. Recently it has been suggested that B cells may diversify the T cell repertoire by effective presentation to T cells with low affinity TCR (51). Thirdly, it is possible, although unlikely, that the different target organs in rat and mouse may reflect the tropism of distinct infectious agents.

Whatever the nature of the stimulus to which the CD45RB$^{high}$ CD4+ T cell population is responding, our results demonstrate that CD4+ T cells exist in normal animals which are capable of causing lethal inflammatory responses and that under normal circumstances these cells are held in check by a phenotypically distinguishable subpopulation of CD4+ T cells. Elucidation of the mechanism by which the CD45RB$^{low}$ cells inhibit the CD45RB$^{high}$ population from inducing colitis may have general relevance for immunoregulation, as CD4+ T cell populations capable of actively suppressing inflammatory responses to a wide range of stimuli have been described, including immunity to *L. major* (52), and tolerance to alloantigen (53,54), diabetes (55,56), SGVHD (57), and multiple organ autoimmune disease (58,59).

After this work was submitted, Morrissey and co-workers reported that the CD45RB$^{high}$CD4+ T cell population from C. B-17 mice induced a wasting disease and colitis when injected into C. B-17 *scid* mice. However, in their study the role of the CD45RB$^{low}$CD4+ T cell population was not examined (60). These results, taken together with our own, indicate that the induction of colitis with the CD45RB$^{high}$CD4+ T cell subset is a general phenomenon and not restricted to prevailing environmental conditions at any one institute.

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## Abbreviations

| | |
|---|---|
| HPRT | hypoxanthine phosphoribosyltransferase |
| IFN | interferon |
| PCR | polymerase chain reaction |
| PE | phycoerythrin |
| SGVHD | syngeneic graft-versus-host disease |
| $T_h$ | T helper cells |
| TNF | tumour necrosis factor |

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