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TCR-Mediated Involvement of CD4$^+$ Transgenic T Cells in Spontaneous Inflammatory Bowel Disease in Lymphopenic Mice[1]

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Spontaneous colitis resembling ulcerative colitis developed in 3 of 10 independent TCR transgenic (Tg) mouse lines maintained under specific pathogen-free conditions. All three susceptible lines were CD4 lymphopenic, whereas resistant lines had normal numbers of CD4$^+$ T cells. Thus, cytochrome *c*-specific 5C.C7 TCR Tg mice developed colitis only when crossed onto a SCID- or Rag-1-deficient background. A second line of lymphopenic cytochrome *c*-specific Tg mice bearing the AND TCR also developed colitis. In both cases, CD4$^+$ T cells expressing the Tg-encoded TCR were preferentially activated in inflamed colons compared with lymph nodes or spleens. In contrast, Tg$^+$CD4$^+$ T cells remained quiescent in both inflamed and unaffected colons in another line of susceptible Tg mice carrying a TCR specific for myelin basic protein, suggesting a fortuitous cross-reactivity of the IE^k-restricted cytochrome *c*-reactive AND and 5C.C7 TCRs with an Ag present in the gut. The percentage of CD4$^+$ T cells expressing only endogenous TCR α-chains was increased consistently in inflamed colons in AND as well as 5C.C7 Rag-1$^{-/-}$ TCR Tg mice, suggesting that polyclonal CD4$^+$ T cells were also involved in the pathogenesis of spontaneous colitis. Moreover, our data indicate that some α-chain rearrangement was still occurring in TCR Tg mice on a Rag-1$^{-/-}$ background, since activated CD4$^+$ T cells expressing endogenously rearranged α-chains paired with the Tg-encoded β-chain were detected consistently in the colons of such mice. ***The Journal of Immunology*, 1999, 162: 7208–7216.**

Ulcerative colitis and Crohn's disease are chronic inflammatory conditions of the human bowel associated with significant morbidity and mortality. Despite the fact that both conditions were first described many years ago, their causes remain uncertain. Recent years have seen the emergence of a number of models of spontaneous colitis in genetically manipulated mice and rats (1–4). Although associated with a variety of distinct cellular and/or molecular abnormalities, these models have led to the identification of two shared factors in the pathogenesis of rodent colitis, namely a disturbance in the balance between immunoregulatory CD4$^+$ T lymphocyte subsets and/or cytokines, and the involvement of luminal microflora or their products in the initiation and/or progression of disease.

The effector cells responsible for rodent colitis have been examined in mice lacking cytokines and/or lymphocyte development genes. IL-2$^{-/-}$, IL-2Rα-chain$^{-/-}$, and IL-10$^{-/-}$ mice, for example, all develop a form of colitis (2, 3, 5), the characteristic features of which persist when they are crossed with B cell-deficient mice (6, 7). By contrast, colitis does not occur in the IL-2$^{-/-}$ Rag-2$^{-/-}$ (T cell-deficient) combination (6), whereas IL-2$^{-/-}$ β_2-microglobulin$^{-/-}$ (CD8$^+$ T cell-deficient) mice develop an accelerated form of the disease in which peripheral and colonic CD4$^+$ T cells are activated, confirming a primary role for CD4$^+$ cells in pathogenesis in this model (8). Mice deficient in T cells as a result of disruptions of TCR α- or β-chain genes have also been shown to be susceptible to colitis (4). Once again, B cells do not appear to be required for disease induction (9, 10). Moreover, in human CD3ϵ transgenic (Tg)[3] mice (Tgϵ26), in which a very early arrest in T cell development prevents development of a normal thymic microenvironment (11), transplantation of adult mice with normal T cell-depleted bone marrow cells results in a lethal wasting syndrome associated with severe colitis. This particular model therefore underscores the critical role of the thymus in the generation of T cells required for homeostasis of the mucosal immune system. Subfractionation of CD4$^+$ T cells from normal donors has revealed the presence of two distinct populations of cells, one capable of acting as inducers and effectors of colitis and the other with regulatory properties that protect against development of disease. Thus, transfer of sorted CD4$^+$CD45RB^{high} T cells into immunodeficient rats (12) or mice (13) leads to colitis, while disease can be prevented by cotransfer of CD4$^+$CD45RB^{low} T cells.

A possible role for gut flora in the pathogenesis of murine colitis has been suggested by two complementary lines of evidence. First, colitis can be prevented by maintaining the animals under germ-free conditions (2, 14, 15), whereas disease susceptibility is restored by reconstitution of the gut flora with mixtures of common commensal bacteria such as *Bacteroides sp*. (16). Correlations between infection with *Helicobacter hepaticus* and disease severity have also been noted in the adoptive transfer model described

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[3] Abbreviations used in this paper: Tg, transgenic; H&E, hematoxylin and eosin; MBP, myelin basic protein; SPF, specific pathogen free.

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above (17). Whether the colitogenic T cell response is induced by specific Ag, superantigen, or a mitogenic factor derived from gut organisms remains unclear, since the specificity of responding T cells has not been defined. In addition, it is uncertain whether the causative Ags are expressed only by gut flora, or whether epitopes are shared between enterobacterial Ags and the enterocytes themselves.

In this work, we describe the first models in which $CD4^+$ T cells expressing a prerearranged Tg TCR are involved in the pathogenesis of spontaneous colitis in CD4 lymphopenic mice. The two lines of mice showing susceptibility to colitis express related TCRs reactive with the C-terminal peptide of cytochrome *c*. Evidence for the pathogenic importance of cross-reactivity with the cytochrome *c* peptide was obtained by demonstrating that $CD4^+$ T cells from another susceptible mouse strain expressing a different specificity (to myelin basic protein (MBP)) were not involved in the disease process. In addition, susceptibility to colitis was found to correlate with CD4 lymphopenia, and to be associated with the presence in the colon of a polyclonal population of activated $CD4^+$ T cells that did not express the Tg-encoded specificity.

Materials and Methods

Mice

All mice were bred and maintained at the Centenary Institute Animal Facility under SPF conditions. Regular serological testing for a panel of 16 mouse pathogens (murine hepatitis virus, parvovirus, rotavirus, murine cytomegalovirus, Theiler's murine encephalitis virus, pneumonia virus of mice, sendai virus, lymphocytic choriomeningitis virus, ectromelia, *Mycoplasma pulmonis*, reovirus 3, adenovirus, polyoma virus, Hantaan virus, Tyzzer's disease (*Bacillus* piliformis), and *CAR Bacillus*) was conducted by the Murine Virus Monitoring Service (Adelaide, South Australia). Sentinel mice were screened every 3 mo for *Bordetella bronchoseptica*, *Corynebacterium kutscheri*, *Klebsiella sp.*, *Pasteurella multocida*, *Pasteurella pneumotropica*, *Psuedomonas aeruginosa*, *Staphylococcus aureus*, *Streptobacillus moniliformis*, *Citrobacter freundii*, *Streptococcus pneumoniae*, *Erysipelothrix rhusiopathiae*, and *Salmonella sp*. All mice on an immunodeficient background were provided with sterile food, water, cages, and bedding. Fecal samples from mice with colitis were negative for *Citrobacter* by culture on numerous occasions. No nematodes or protozoa were found in repeated stool specimens, nor were they visible in the lumen of the gut on H&E-stained sections.

This study made use of a panel of eight lines of cytochrome *c*-specific Tg mice bearing the TCR derived from the 5C.C7 T cell clone (18), which recognizes a number of distinct Ags, including the C-terminal epitope of pigeon cytochrome *c* in association with IE^k; the C-terminal epitope of moth cytochrome *c* in association with IE^k, $IE\alpha^k\beta^b$, or $IE\alpha^d\beta^b$; IA^s as an alloantigen; and the subset of superantigens specific for Vβ3, including mls-2^a, mls-3^a, mls-4^a, mls-5^a, and staphylococcal enterotoxin A. The Tg lines, including -D (19) and -I (20), were generated by coinjection of a TCR Vα11.1 JαC7 construct that lacked the 3′ α enhancer (21, 22), and a Vβ3 Dβ1.1 Jβ1.2 construct that included either the Ig heavy chain enhancer (21) or the endogenous 3′ β-chain enhancer (22) in the case of -D and -I. On the original B10.BR background, the Tg$\alpha\beta$-encoded specificity (abbreviated as $Tg\alpha^+$) is expressed by 60–90% of peripheral $CD4^+$ T cells in the -D line (19, 23), and >95% of peripheral $CD4^+$ T cells in the -I line (20). The remaining cells express the Tg-encoded β-chain paired with an endogenously rearranged α-chain (termed $Tg\alpha^-$). Despite being constrained by expressing only the single Tg-encoded β-chain, $Tg\alpha^-$ cells constitute a polyclonal repertoire capable of recognizing a wide variety of foreign Ags. They do not, however, show reactivity to either cytochrome *c* or self Ags (such as the $Tg\alpha^+$ Id) in vivo or in vitro (Refs. 20, 22, 23, and 30 and B. Fazekas de St. Groth, unpublished data).

The -D line was crossed with 1) C57BL/6 SCID mice (kindly provided by C. Sidman, The Jackson Laboratory, Bar Harbor, ME, at the sixth backcross generation and subsequently backcrossed for a further four generations); 2) B10.BR SCID mice (derived by crossing sixth backcross C57BL/6 SCID with B10.BR for a further four generations); and 3) C57BL/6 Rag-$1^{-/-}$ mice (24) (kindly provided by L. Corcoran, Walter and Eliza Hall Institute, Melbourne, Australia, at the sixth backcross generation and subsequently backcrossed for a further two generations). After multiple rounds of backcrossing and intercrossing, lines of H-2^b, H-2^{bk}, and H-2^k TCR Tg -D mice on a SCID or Rag-$1^{-/-}$ background were established and maintained by crossing with Tg-negative SCID or Rag-$1^{-/-}$ mice of the appropriate MHC genotype. The percentage of $Tg\alpha^+$ cells within peripheral blood and lymph node $CD4^+$ cells from H-2^{bk} and H-2^k -D Rag-$1^{-/-}$ mice is >99%. On the nonselecting H-2^b background, peripheral $CD4^+$ T cells are undetectable.

Cytochrome *c*-specific Tg mice expressing the AND TCR (25) (kindly provided by E. Handman, Walter and Eliza Hall Institute, Melbourne, Australia, at the tenth backcross to B10.BR) were also maintained on the B10.BR background. The AND TCR comprises an identical β-chain to that of the 5C.C7 TCR, combined with an α-chain that uses Vα11.1 in combination with the Jα84 segment, in which 11 of 19 amino acids are different from the JαC7 segment found in the 5C.C7 α-chain (18, 26). This receptor configuration is sufficient to reconstitute a similar spectrum of Ag reactivity to that of the 5C.C7 receptor, including a low level of alloreactivity to IA^s (27). However, since the AND TCR α-chain construct includes the 3′ α enhancer missing from the 5C.C7 α-chain construct, allelic exclusion of endogenous α-chain rearrangement is significantly more efficient than in the 5C.C7 TCR Tg mice.

MBP-specific TCR Tg mice (28) (kindly provided by C. Janeway, Yale University, New Haven, CT, at the fourth backcross onto B10.PL) were backcrossed to B10.PL (The Jackson Laboratory) for a further four generations and then crossed with B10.BR to provide H-2^{ku} F_1 mice for experimental use. The MBP-specific α-chain construct also includes the 3′ α enhancer and mediates efficient allelic exclusion of endogenous α-chain rearrangement.

Clinical and pathological diagnosis of colitis

The presence of early colitis was indicated by soft stool with mucus, which progressed to chronic diarrhoea with intestinal bleeding, as well as rectal prolapse in a substantial proportion of cases. Necropsy was performed on any animal that manifested diarrhea, rectal prolapse, significant weight loss, or failure to thrive. H&E-stained sections of caecum, ascending colon, and descending colon were examined "blind" by a veterinary pathologist (M.F.). Selected sections of caecum and colon assessed as being either normal, moderately, or severely affected were stained by the Warthin Starry silver method to identify organisms with morphology consistent with that of *Helicobacter sp*.

Cell preparation and adoptive transfer

For isolation of colonic lymphocytes, the colons were separated into inflamed and unaffected groups on the basis of visual inspection (confirmed subsequently by H&E staining of a paraformaldehyde-fixed sample from the rectum). Lymphocytes were then isolated by the technique of van der Heijen and Stok (29) using collagenase/dispase digestion, followed by centrifugation through a 40/80% discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden). Unless otherwise stated, all manipulations of lymphocytes were performed in tissue culture medium (30). Because fewer than 10^5 lymphoid cells could be obtained from each unaffected colon, they were pooled in groups of six, whereas cells from each inflamed colon (>10^6/mouse) were analyzed separately. Approximately 40% of cells isolated from the colon and falling within a size gate appropriate for lymphocytes expressed CD4, irrespective of the presence of inflammation.

For Percoll purification of high density (resting) peripheral lymphocytes, single cell suspensions were prepared from pooled spleen and peripheral lymph nodes. The cells were washed twice, resuspended at 10^7/ml, loaded onto a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient, and centrifuged at 2500 rpm for 25 min at 20°C. Resting cells were removed from the 60/70% interface. For adoptive transfer, 1–5 × 10^7 Percoll-purified peripheral lymphocytes were injected via the lateral tail vein.

Flow cytometry

Single cell suspensions were washed with PBS containing 5% FCS and 5 mM sodium azide (FACS wash). Aliquots of 10^6 cells were stained in 96-well round-bottom PVC microtiter plates (ICN, Costa Mesa, CA). For three-color analysis, the 5C.C7 and AND TCRs were detected in a two-stage stain using a combination of biotinylated RR8.1 (rat anti-Vα11) (31) and unconjugated KJ25 (hamster anti-Vβ3) (32), followed by streptavidin-conjugated Quantum Red (Sigma, St. Louis, MO), FITC-conjugated anti-hamster Ig (Caltag, South San Francisco, CA), and PE-conjugated anti-CD4 (Caltag). For detection of CD44, CD69, or CD45RB in three-color stains, FITC-conjugated IM7 (33) (PharMingen, San Diego, CA), H1.2F3 (34) (PharMingen), or 16A (PharMingen) were substituted for Vβ3 staining. All Abs were diluted in FACS wash. A total of 5 × 10^4 events, gated for lymphocytes on the basis of forward and side scatter profiles, were routinely collected using a FACScan (Becton Dickinson, Mountain View, CA).

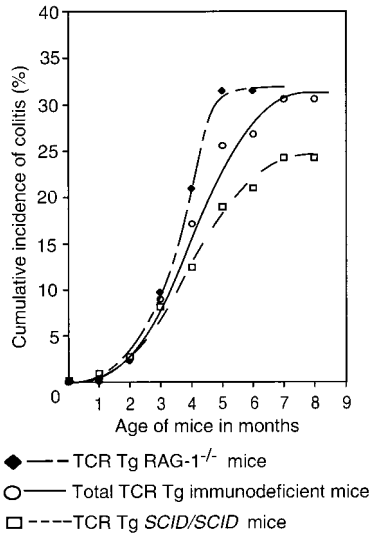

FIGURE 1. Cumulative incidence of spontaneous colitis in immunodeficient TCR Tg mice expressing IE^k. The data were derived from a retrospective analysis of 111 SCID and 140 Rag-$1^{-/-}$ -D TCR Tg mice on an H-2^k or H-2^{bk} background.

For four- and five-color analysis of cells from 5C.C7 and AND TCR Tg mice, cells were stained with combination of anti-CD4 PE, allophycocyanin-conjugated RR8.1, KJ25 detected by Texas Red-conjugated anti-hamster Ig (Caltag) and FITC-IM7, -H1.2F3 or -16A, together with propidium iodide exclusion. MBP-specific TCR Tg T cells were detected using a combination of anti-CD4 PE, biotinylated anti-Vβ8.2 (F23.2) (35), plus avidin Texas Red (Molecular Probes, Eugene, OR), and anti-Id 19G11-AP (36), together with either FITC-IM7, -H1.2F3, or -16A. Alternatively, IM7, H1.2F3, and 16A were biotinylated (PharMingen), and KJ25 or F23.2 was detected in the FITC channel. Samples were analyzed using a Becton Dickinson FACStar^{Plus} and 2–5 $\times$ 10^5 events, gated for lymphocytes on the basis of forward and side scatter profiles, and collected for analysis using the Cellquest (Becton Dickinson) or FlowJo programs (Treestar, San Carlos, CA).

PBL counts

Lymphocyte counts were performed by analyzing 100 μl heparinized blood in a Sysmex K-1000 Automated Hematology Analyzer (Toa Medical Electronics, Kobe, Japan).

Statistics

Cumulative incidence data for -D TCR Tg (pooled H-2^{bk} and H-2^k) mice with Rag-$1^{-/-}$ vs SCID genotype, and for -D TCR Tg (pooled Rag-$1^{-/-}$ and SCID) mice of H-2^{bk} vs H-2^k genotype were compared using the log rank test for the paired Kaplan-Meier curves (37). Comparison of PBL counts was performed using a one-way ANOVA, followed by comparison of the group means using the Studentized Range, *Q* test (38).

Results

Incidence of spontaneous colitis in intact TCR transgenic mice on an immunodeficient background

The morbidity and mortality of a total of 1607 mice from the -D TCR Tg SCID and Rag-$1^{-/-}$ colonies were analyzed retrospectively by studying routine records from the animal facility over a period of 2 yr. Of the 251 mice expressing the Tg TCR and either H-2^k or H-2^{bk}, approximately 30% developed clinical signs of disease within the first 6 mo of life, with the peak incidence occurring between the ages of 2 and 6 mo (Fig. 1). Very few new cases were diagnosed after the age of 6 mo. Although the incidence in mice on a Rag-$1^{-/-}$ background was marginally higher than in those on a SCID background (Fig. 1), the difference was not statistically significant ($0.1 < p < 0.5$), nor was there a difference between the incidence in mice expressing H-2^k vs H-2^{bk} (not shown). Mice for which the cause of death was unknown were recorded as being negative for spontaneous colitis, and amounted to less than 10% of the total. Hence, the calculated incidence of colitis may have underestimated the actual frequency of disease by up to 10%. No cases of spontaneous colitis occurred either in immunodeficient mice lacking a significant number of peripheral T cells, i.e., those not carrying both the Tg TCR and the appropriate MHC gene (IE^k) required for positive selection in the thymus, or in -D TCR Tg immunocompetent mice kept under the same SPF conditions (data not shown).

Pathology

Postmortem examination of animals manifesting clinical signs of colitis revealed an enlarged, rigid, thickened colon with loose luminal contents. The stomach, small intestine, and liver were all normal. In severe cases, mesenteric lymphadenopathy and gross splenomegaly accompanied the intestinal pathology. Representative H&E sections are shown in Fig. 2. Pronounced mucosal hyperplasia was a prominent feature in virtually all affected mice, usually accompanied by a mixed infiltrate comprising neutrophils and mononuclear cells in the lamina propria and, in severe cases, the submucosa and serosa. In approximately one-third of affected mice, lymphoid cells predominated. At the sites of maximal inflammation, there were mucosal ulceration and crypt abscess formation. Compared with human inflammatory bowel disease, the histological appearance had more features in common with ulcerative colitis than with Crohn's disease.

Comparison of the severity of lesions at different levels of the gut revealed a consistent pattern, with the most severe lesions being located in the descending colon and rectum, while the lesions in the caecum were of intermediate severity and the ascending colon typically showed the least involvement. Examination of silver-stained sections showed variable numbers of organisms with morphology consistent with that of *Helicobacter sp.* within crypt lumina; these were generally mixed with bacteria of different morphological types. No obvious correlation between the numbers of organisms with *Helicobacter* morphology and severity of lesions was apparent.

Phenotypic characterization of T cells in colitic intestines

The finding that susceptibility to spontaneous colitis correlated with expression of IE^k in -D Tg Rag-$1^{-/-}$ mice suggested a pathogenic role for peripheral IE^k-restricted $CD4^+$ T cells bearing the Tg-encoded receptor. However, IE^k may have mediated positive selection of $CD4^+$ T cells in the thymus, without necessarily acting as the restriction element for Ag recognition by Tg-expressing T cells in the peripheral lymphoid tissue and colon. It was therefore necessary to confirm that activated Tg-expressing T cells, which have generally been assumed to comprise the entire T cell repertoire in such mice, were selectively and exclusively present in inflamed colons. When this was done by using flow cytometry to examine cells isolated from colons of affected H-2^{bk} -D TCR Tg Rag-$1^{-/-}$ mice, the most striking finding was that an average of 62% of colonic $CD4^+$ cells failed to express the Tg-encoded specificity, expressing instead an endogenously rearranged TCR α-chain paired with the Tg-encoded β-chain (Figs. 3 and 4*A*). This phenotype has previously been described on 20–40% of peripheral $CD4^+$ T cells in H-2^{bk} -D TCR Tg Rag-$1^{+/+}$ mice (19, 23). The TCR-$\alpha\beta$ was coexpressed with CD3 in both cases, as expected (not shown). Such cells will subsequently be referred to as Tgα^-, to distinguish them from those expressing the Tg-encoded specificity (Tgα^+). The presence of such cells in mice on a Rag-$1^{-/-}$ background indicated that rearrangement of endogenous TCR α-chains was not entirely prevented by targeted inactivation of the

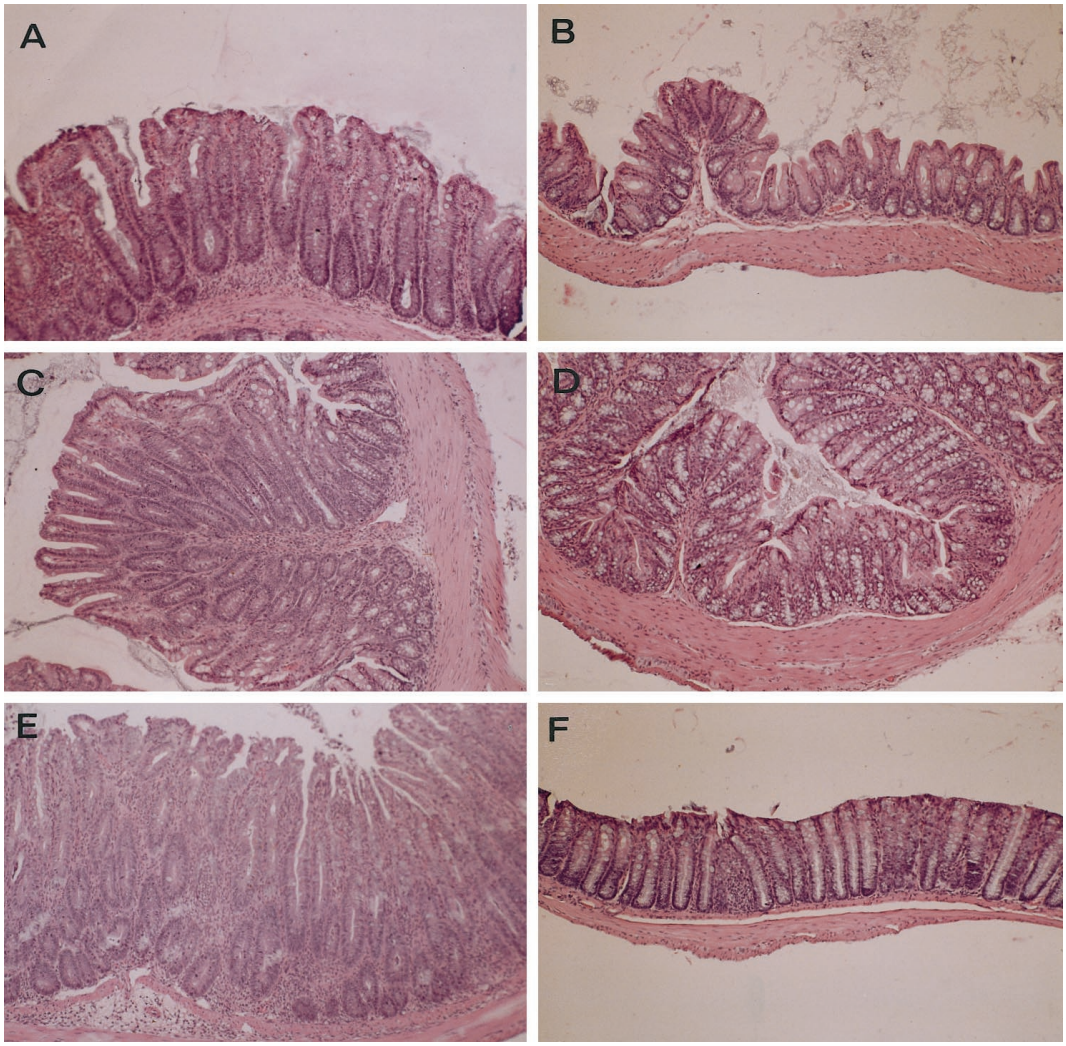

FIGURE 2. Histology of spontaneous colitis in immunodeficient TCR Tg mice. H&E-stained sections from caecum (*A* and *B*), ascending colon (*C* and *D*), and descending colon (*E* and *F*) of colitic (*A*, *C*, and *E*) and unaffected (*B*, *D*, and *F*) -D Rag-$1^{-/-}$ mice are shown. Magnification, ×100.

Rag-1 gene. Since TCR Tg-negative Rag-$1^{-/-}$ mice had no detectable peripheral $CD3^+$ cells, expression of the prerearranged Tg-encoded β-chain appeared to have allowed cells with a functional endogenous α rearrangement to express surface TCR without the need for a simultaneous productive rearrangement of the endogenous β locus.

$Tg\alpha^-$ cells were also detected in uninflamed colons, where they comprised 6% of $CD4^+$ cells, compared with fewer than 1% in the peripheral lymphoid tissues (Fig. 4*A*). Their presence in the colon of unaffected animals suggested selective expansion and/or migration of a small oligo- or polyclonal $Tg\alpha^-$ population in response to recognition of Ag derived from the normal gut. Comparison of the activation status of colonic $CD4^+Tg\alpha^-$ cells from colitic and unaffected mice revealed that both populations expressed CD44, CD69, and CD45RB at levels consistent with an activated or memory phenotype (Fig. 4*B*, *left panel*). Moreover, a high percentage of the few $Tg\alpha^-$ cells located in peripheral lymphoid tissues also expressed activation/memory markers, consistent with expansion of cells with specificity for environmental Ags. Taken together, these findings provided convincing evidence in favor of a pathogenic role for a polyclonal population of T cells that lacked specificity for cytochrome *c*. However, analysis of the cytochrome *c*-specific ($Tg\alpha^+$) population present in the same tissue samples also provided data suggestive of their involvement in colitis. Thus, the number of colonic $CD4^+Tg\alpha^+$ cells was increased by approximately 4-fold in colitis, despite their relative decrease as a percentage of total cells, because the total number of colonic $CD4^+$ T cells increased by 10-fold (Fig. 4*A* and *Materials and Methods*). Moreover, the presence of colitis led to an increase in both the size of colonic $CD4^+Tg\alpha^+$ cells (mean forward scatter channel number 151 vs 134 in uninflamed colon) and the percentage of $CD69^{high}$, $CD44^{high}$, and $CD45RB^{low}$ cells within this population (Fig. 4*B*, *right panel*). In contrast, very few $CD4^+Tg\alpha^+$ cells from the spleen, peripheral, and mesenteric lymph nodes of colitic as well as unaffected mice expressed activation/memory markers. Thus, cytochrome *c*-reactive $Tg\alpha^+$ cells appeared to respond to an Ag that was present exclusively in the gut, in contrast to the polyclonal $Tg\alpha^-$ population that responded to a range of Ags, including those in the gut.

Association of CD4 lymphopenia with susceptibility of TCR Tg Rag-$1^{+/+}$ mice to spontaneous colitis

It is clear that colitis is a feature of some but not all mice with a disturbed T cell repertoire (4). Moreover, in our model the disease has never been identified in seven independent lines of TCR Tg mice on an immunosufficient background, derived from founders injected with the same 5C.C7 constructs as the -D line and maintained in the same SPF colony for a similar period of time. The

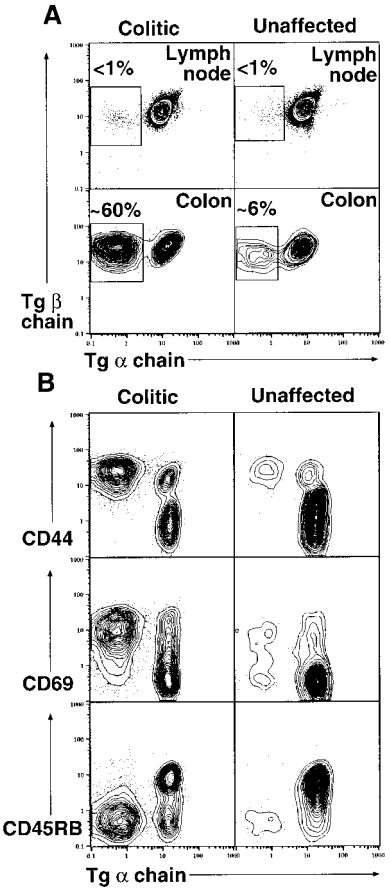

FIGURE 3. Representative flow-cytometric profiles of $CD4^+$ T cells isolated from immunodeficient -D TCR Tg mice on a Rag-$1^{-/-}$ H-2^{bk} background. *A*, TCR expression by $CD4^+$ cells isolated from immunodeficient -D TCR Tg mice on a Rag-$1^{-/-}$ H-2^{bk} background. Simultaneous staining for CD3 expression showed that the cells expressing Tg β-chain were uniformly $CD3^+$, irrespective of Tg α-chain expression (not shown). *B*, Expression of an activated/memory phenotype by a proportion of $CD4^+Tg\alpha^+$ T cells isolated from the colon of colitic -D Rag-$1^{-/-}$ H-2^{bk} mice.

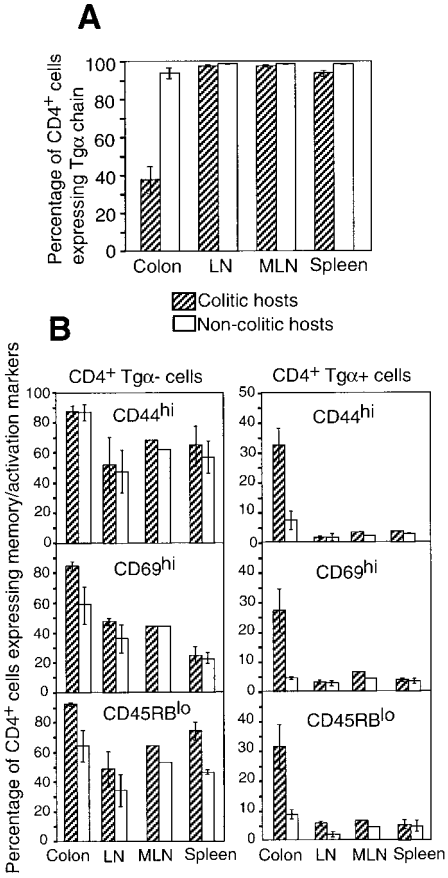

FIGURE 4. Flow-cytometric analysis of $CD4^+$ T cells isolated from immunodeficient -D TCR Tg mice on a Rag-$1^{-/-}$ H-2^{bk} background. Three groups of mice aged 2–4 mo, 6–7 mo, and 8–9 mo were examined in separate experiments. The data represent the mean +/− SEM of the pooled data from the three experiments, involving a total of 6 colitic and 18 unaffected mice, apart from the mesenteric lymph node (MLN) data that were derived from 1 colitic and 6 unaffected mice. *A*, Percentage of $CD4^+Tg\alpha^+$ T cells in colon, spleen, mesenteric lymph nodes, and pooled peripheral lymph nodes. The remainder of the $CD4^+$ T cells were $Tg\alpha^-$ (expressing a combination of Tgβ and endogenously rearranged α-chains, as illustrated in Fig. 3). *B*, Expression of activation/memory markers in the cells derived from the analysis described in *A*.

most striking difference between these lines and the susceptible -D Rag-$1^{-/-}$ mice was that the latter had a low CD4 T cell count. To determine whether lymphopenia was indeed an important predisposing factor for colitis, in the same way as it is for other autoimmune diseases (39), CD4 counts were performed on two imported TCR Tg lines that were found to be susceptible to spontaneous colitis within a few months of being rederived by C sectioning into the SPF colony (Fig. 5).

The first of these lines, expressing the cytochrome *c*-specific AND TCR (25), uses an identical β-chain to that of the 5C.C7 TCR expressed by the -D Tg line described above, combined with a closely related α-chain (see *Materials and Methods*). Efficient allelic exclusion by the AND TCR α-chain construct prevents production of sufficient $Tg\alpha^-$ cells to fully populate the peripheral $CD4^+$ T cell compartment (Fig. 5), since thymic positive selection of a single TCR, like that of multiple TCRs recognizing a single peptide-MHC specificity, is kinetically limited (reviewed in Ref. 40). In AND TCR Tg mice suffering from spontaneous colitis, the distribution (not shown) and activation status (Fig. 6*A*) of $CD4^+Tg\alpha^+$ vs $Tg\alpha^-$ cells were very similar to those in affected -D TCR Tg Rag-$1^{-/-}$ mice, suggesting a similar mechanism of disease induction. Moreover, the histological findings were indistinguishable from those in -D TCR Tg SCID and Rag-$1^{-/-}$ mice (not shown).

Spontaneous colitis with an identical histological picture (not shown) was also observed in a second line of mice expressing a TCR of different specificity, namely to MBP in association with IA^u (28). In this case, the incidence of disease was restricted to the Tg^+ offspring of crosses between H-2^u Tg mice and a congenic H-2^k line (B10.BR), which were more severely CD4 lymphopenic than the parental H-2^u line (Fig. 5). Interestingly, in contrast to preferential activation of $Tg\alpha^+$ cells noted in inflamed colon from both the cytochrome *c*-specific models described above, the percentage of activated cells within the MPB-reactive $Tg\alpha^+$ population of H-2^{ku} mice with colitis was uniformly low both in the colon and in peripheral lymphoid tissue (Fig. 6*B*). Thus, the degree of lymphopenia as well as the specificity of the TCR influence the incidence of disease.

Adoptive transfer of colitis to immunodeficient hosts

The data described in the previous section suggested that development of colitis in lymphopenic animals required the involvement of oligo- or polyclonal $CD4^+Tg\alpha^-$ cells, whereas

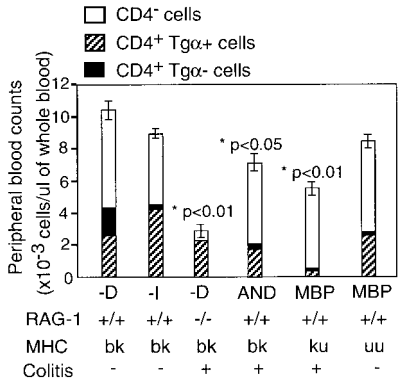

FIGURE 5. $CD4^+$ lymphopenia and TCR specificity are correlated with susceptibility to colitis. Total lymphocyte counts are shown as mean ± SEM for five mice per group. Filled areas represent the mean proportions of $CD4^+Tg\alpha^+$ (gray fill) and $CD4^+Tg\alpha^-$ (black fill) cells within total PBL (excluding monocytes and granulocytes). Statistical significance of the difference between mean lymphocyte counts was determined using a one-way ANOVA ($p < 0.0001$), followed by comparison of means via the Q method. Means marked ∗ are significantly different from the -D Rag-$1^{+/+}$ group.

$CD4^+Tg\alpha^+$ cells could be involved or not, depending on the specificity of the TCR. The ability of TCR specificity to determine disease involvement pointed to the possibility of a previously unrecognized cross-reactivity of the two related cytochrome *c*-specific TCRs with an Ag present in the gut or its contents. To examine the relative importance of $CD4^+Tg\alpha^-$ and $Tg\alpha^+$ populations in the pathogenesis of colitis, lymphocytes from -D Rag-$1^{+/+}$ mice, containing a mixture of $CD4^+Tg\alpha^-$ and $Tg\alpha^+$ cells in the ratio 1:3, were transferred into syngeneic non-Tg Rag-$1^{-/-}$ recipients. Overt colitis was seen within 2–4 wk of transfer, the incidence of disease being 58% in a series of transfer experiments involving 26 recipients. The animals that failed to develop disease by 4 wk remained well for the maximum observation period of 8 wk and showed no histological evidence of disease.

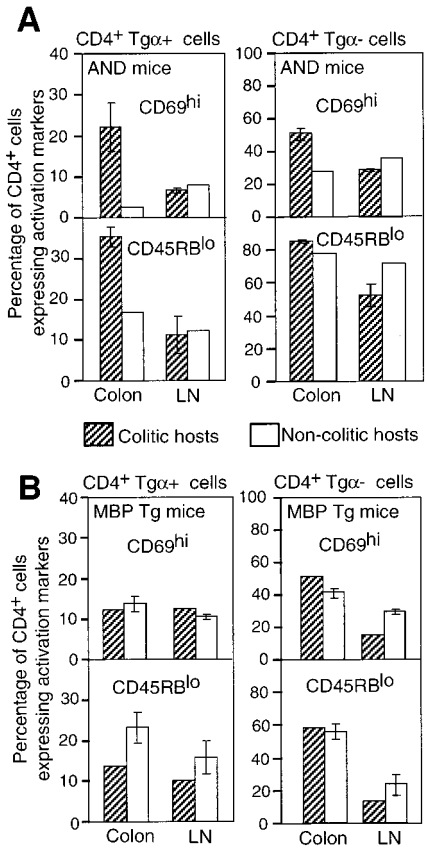

FIGURE 6. Cytochrome *c*-reactive $CD4^+$TCR Tg cells from AND TCR Tg mice are involved in spontaneous colitis, in contrast to MBP-specific $CD4^+$TCR Tg cells. Expression of activation/memory markers in the $CD4^+Tg\alpha^+$ and $Tg\alpha^-$ cells of colitic vs noncolitic AND TCR Tg mice (*A*) and MBP-specific TCR Tg mice (*B*).

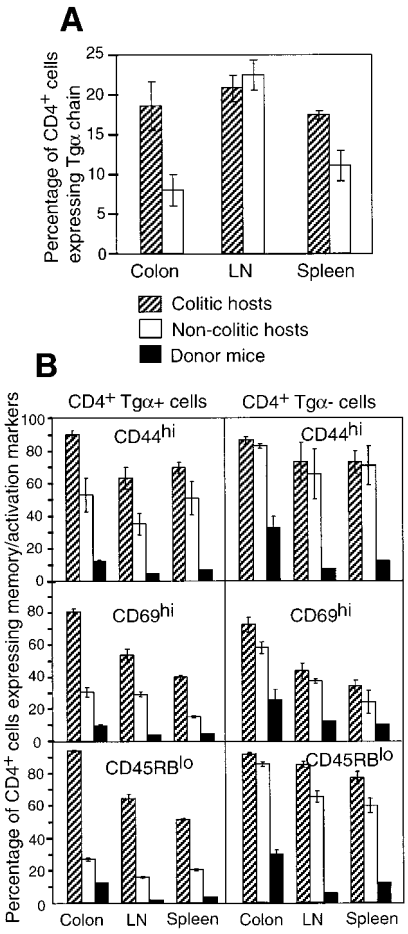

FIGURE 7. Phenotype of $CD4^+$ T cells isolated from the colons, lymph nodes, and spleens of H-2^{bk} Rag-$1^{-/-}$ mice adoptively transferred with Percoll-purified high density lymphocytes from -D TCR Tg Rag-$1^{+/+}$ donors. The data are derived from two experiments in which a total of 7 of 14 mice showed evidence of colitis at necropsy. *A*, Percentage of $CD4^+Tg\alpha^+$ cells in colon, pooled peripheral lymph nodes, and spleen. *B*, Expression of activation/memory markers by the cells derived from the analysis described in *A*, compared with a cohort of 10 unmanipulated -D TCR Tg mice (donor mice).

Donor-derived lymphocytes isolated from the colon, spleen, and lymph nodes of adoptive Rag-$1^{-/-}$ recipients were compared with those from a cohort of 10 unmanipulated -D Rag-$1^{+/+}$ mice. The percentage of $Tg\alpha^+$ cells among $CD4^+$ cells recovered from the peripheral lymph nodes after adoptive transfer was <25% (Fig. 7*A*), compared with >70% in the inoculum (not shown). The explanation for this decline was an imbalance in spontaneous proliferation upon adoptive transfer to immunodeficient mice, such that more $CD4^+$ $Tg\alpha^-$ than $Tg\alpha^+$ cells divided after transfer (W.-P. Koh, P. Bertolino, and B. Fazekas de St. Groth, unpublished data). Consistent with the data from spontaneous disease in -D Rag-$1^{-/-}$ mice (Fig. 4), $CD4^+Tg\alpha^+$ cells underwent a greater degree of activation in the inflamed colon than in the peripheral lymphoid

tissues (Fig. 7*B*, *left panels*). The percentage of Tgα^+ cells among colonic CD4$^+$ cells was also significantly higher in colitic vs noncolitic recipients (Fig. 7*A*), in contrast to the pattern observed in intact mice that developed spontaneous disease (Fig. 4). The colons of the immunodeficient hosts that did not succumb to disease contained very few lymphocytes, the percentage of colonic CD4$^+$Tgα^+ cells was relatively low, and expression of activation markers was intermediate between the high level in adoptive recipients that did develop colitis and the low level in intact -D TCR Tg donors (Fig. 7*B*, *left panels*). The proportion of activated CD4$^+$Tgα^+ cells in the peripheral lymphoid tissues was also intermediate between that in colitic recipients and the very low level in intact donors.

In contrast to preferential activation of the CD4$^+$Tgα^+ population in inflamed vs uninflamed colon, the polyclonal CD4$^+$Tgα^- population was highly activated in all Rag-1$^{-/-}$ recipient mice, irrespective of the presence of colonic inflammation (Fig. 7*B*, *right panels*). The disparity between expression of CD44 and CD69 by cells in the peripheral lymphoid tissues, in contrast to coexpression by >70% of cells isolated from the colon, suggested that many of the circulating cells may not have been recently exposed to Ag.

Analysis of CD4$^+$ T cells from the colon and peripheral lymphoid tissues of Rag-1$^{-/-}$ recipients of cells from a second line of 5C.C7 TCR Tg mice (termed -I (20)), which express the Tg α-chain on a greater percentage of CD4$^+$ cells both before and after adoptive transfer, revealed a very similar pattern of recruitment and activation within the CD4$^+$Tgα^+ and Tgα^- populations (not shown). Thus, adoptive transfer confirmed the correlation between colitis and preferential homing and/or proliferation of activated cytochrome-reactive CD4$^+$Tgα^+ in the colon.

Discussion

The TCR Tg models of spontaneous colitis described in this work are unique in that CD4$^+$ T cells expressing a prerearranged, class II-restricted Tg TCR play a role in disease pathogenesis. The evidence that such a role is indeed the result of Ag-specific activation via the TCR is based on comparison of findings in the four different disease models. Thus, the disease-dependent increase in the percentage of colonic cells expressing activation/memory markers was restricted to the population bearing the cytochrome *c*-reactive Tg-encoded TCR in the first three models, namely intact Tg -D mice on a Rag-1$^{-/-}$ background (Fig. 4), intact lymphopenic AND Tg mice on a Rag-1$^{+/+}$ background (Fig. 6*A*), and syngeneic Rag-1$^{-/-}$ recipients of -D cells (Fig. 7). By contrast, in the fourth model, T cells bearing a Tg-encoded TCR specific for the unrelated Ag, MBP, showed no evidence of preferential activation in inflamed colons (Fig. 6*B*). The latter phenotype resembles that described in a previous report of colitis following transfer of cells from anti-H-Y TCR Tg donors into SCID recipients, which also demonstrated activation of an oligoclonal CD4$^+$Tgα^- population, but not of Tgα^+ cells (41). The specificity of the Tg-encoded TCR was therefore crucial in determining whether T cells showed preferential activation in the inflamed colon, indicative of Ag-specific activation.

None of the reported ligands of the 5C.C7 and AND TCRs are known to be selectively present in the colon of animals developing colitis. These include the C-terminal epitope of pigeon cytochrome *c* in association with IE^k, the C-terminal epitope of moth cytochrome *c* in association with IE^k, IE$\alpha^k\beta^b$ or IE$\alpha^d\beta^b$, IA^s as an alloantigen, and the subset of superantigens specific for Vβ3, including mls-2^a, mls-3^a, mls-4^a, mls-5^a, and staphylococcal enterotoxin A. This suggests that these two cytochrome-reactive TCRs display a fortuitous cross-reactivity with an Ag present in either the gut or its contents. Database searches have to date failed to reveal any bacterial sequences that would be predicted to act as ligands for the 5C.C7 TCR, based on published studies of its specificity for a panel of synthetic peptides mutated by means of global amino acid replacement of the original moth cytochrome *c* epitope (42). Moreover, no direct data are available to implicate colonic bacteria in the pathogenesis of colitis in our colony, which is kept under SPF but not germfree conditions. However, the anatomic distribution of inflammation correlates with that of the bacterial load in the colon, being most severe in the descending colon and caecum (Fig. 2). Involvement of gut flora would be consistent with other reports of spontaneous colitis in rodents, in which it was found that animals kept under germfree conditions failed to develop disease (2, 15, 16, 43).

More recently, *H. hepaticus* has been implicated in murine colitis (17), and bacteria with morphology consistent with that of this organism were seen in variable numbers in sections of caecum and colon in the present study, although there was no obvious correlation with disease severity. Rather than acting as a primary pathogen, however, it seems likely that additional factors are necessary for full manifestation of clinical disease in association with *H. hepaticus* infection. For example, inoculation of *H. hepaticus* into germfree mice generally produced only mild lesions of a somewhat different morphology to those seen in spontaneous disease (44), and in a study of spontaneous disease in immunodeficient mice, organisms were detected in normal mucosa as well as at sites of inflammation (45). Furthermore, no *Helicobacter* type organisms could be demonstrated in a form of spontaneous colitis in nude rats with very similar pathological features to the murine disease (43). It does not therefore appear that Koch's postulates have yet been fulfilled for *Helicobacter* species and colitis in mice.

Alternatively, molecular mimicry may explain the involvement of colonic flora in rodent models of colitis. In support of this hypothesis, reactivity by lamina propria T cells to determinants on the surface of enterocytes has been demonstrated in human IBD (46); moreover, it has been suggested that sharing of these epitopes by enterobacteria may initiate both cell-mediated and humoral immunity, which is subsequently directed against cells in the intestinal mucosa itself (47). Although colitis has been prevented by raising disease-prone mice under germfree conditions, there are no studies to date in which the disease process was reversed after initiation, by removal of a putative pathogenic organism. Hence, it is possible that particular gut organisms are required for initiation, but not maintenance of colitis, a finding that would imply but not prove a pathogenic role for cross-reactivity to enterocytes.

In addition to the monoclonal population of CD4$^+$ cytochrome-specific Tgα^+ T cells, polyclonal CD4$^+$Tgα^- T cells appear to be involved in the pathogenesis of spontaneous colitis in all of the TCR Tg models described in this work, presumably through specific recognition of different peptides restricted by the same or different MHC molecules. Indeed, preliminary data derived from experiments using the adoptive transfer model described in this work have demonstrated that colonic inflammation induced by CD4$^+$Tgα^- cells is a prerequisite for recruitment of activated CD4$^+$Tgα^+ cells to the colon, suggesting that the specific Ag recognized by the Tgα^+ cells may be released in sufficient quantities only as a result of prior inflammation (W.-P. Koh, P. Bertolino, and B. Fazekas de St. Groth, in preparation). Once again these preliminary findings are consistent with CD4$^+$Tgα^+ reactivity either to a gut microbe, or more likely to a true self Ag expressed by enterocytes themselves. Alternatively, the Tg TCR-specific stimulus may be a superantigen reactive with Vβ3, although if this were the case, availability of the superantigen would also have to be limited except during inflammation.

Tgα^+ T cells could in theory be recruited to the gut by means of coexpression of one or more endogenously rearranged α-chains, rather than via a cross-reaction with the Tg-encoded TCR. This is unlikely, however, for at least two reasons. First, cytochrome *c*-reactive CD4$^+$Tgα^+ T cells are activated in spontaneous colitis in intact AND TCR Tg and -D Rag-1$^{-/-}$ mice, in which α-chain allelic exclusion is highly efficient, as evidenced by their inability to generate sufficient CD4$^+$ cells to fully populate the peripheral compartment (Fig. 5). Second, T cells expressing only a single TCR-$\alpha\beta$ pair are of higher avidity for Ag than those in which the expression of the specific receptor is reduced by coexpression of a second α-chain (48). Thus, a population of CD4$^+$Tgα^- T cells, each expressing only a single α-chain, would compete effectively for activation by gut-derived Ag against Tgα^+ cells coexpressing the same repertoire of endogenously rearranged α-chains. Indeed, preferential recruitment of dual α-chain Tgα^+ cells to the gut could be a possibility only if they were to express a distinct repertoire of α-chains compared with Tgα^- cells, an assumption for which there is no evidence.

In addition to the role of the Tg TCR in the pathogenesis of colitis, the current study illustrates the crucial importance of a lymphopenic environment for induction of spontaneous disease. In the case of the TCR Tg models used in this study, lymphopenia is a consequence of the natural limit to the number of T cells of a single specificity that can be positively selected in the thymus (40). Thus, when a particular prerearranged TCR $\alpha\beta$ pair is expressed to the exclusion of almost all other TCRs, as in a Tg mouse with highly effective allelic exclusion (e.g., the AND mouse) or a TCR Tg on a Rag-deficient or SCID background, the production of thymic emigrants is insufficient to fill the peripheral T cell compartment to its normal level.

Although a link between lymphopenia and colitis was suggested from studies of TCR α- or β-chain knockout mice, the gross disruption in their lymphocyte compartment has made interpretation of the relative significance of each particular abnormality difficult. Furthermore, colitis-susceptible IL-2$^{-/-}$, IL-2R$\alpha^{-/-}$, and IL-10$^{-/-}$ mice suffer not from lymphopenia, but rather from progressive lymphocytosis (2, 3, 5). One hypothesis reconciling these apparently conflicting pathogenetic mechanisms is based on the suggestion by Papiernik (49) that the propensity of IL-2$^{-/-}$, IL-2R$\alpha^{-/-}$, and IL-10$^{-/-}$ mice to develop Th1-mediated colitis is due to the absence of a protective subset of activated, IL-10-producing, IL-2-dependent cells, which may well represent the crucial subpopulation within the protective CD45RB^{low} population previously defined by Powrie (13). If these cells are assumed to mediate protection against colitis, any unifying hypothesis must establish a link between absolute lymphopenia and a relative functional deficit in the protective population.

Previous investigators have suggested that immunoregulatory T cells may act via a direct T-T interaction (39); however, there is no evidence to support an imbalance between naive and regulatory T cells in lymphopenia. Consequently, an alternative explanation is necessary. One possibility, supported by recent in vitro data (50), is that immunoregulatory T cells exert their effects on other T cells indirectly via APCs. If true, then lymphopenia per se would disrupt the effectiveness of immunoregulatory T cells in vivo, since the number of APCs would be high relative to their potential regulators. By down-regulating APC stimulatory capacity, such regulatory T cells might act to raise the threshold for recognition of Ag so that naive T cells could not respond to relatively nonimmunogenic Ags derived, for example, from gut commensal organisms, inhaled Ags, and possibly even true self Ags. In the absence of a regulatory population, naive T cells would make a response to these environmental Ags. Such a scenario occurs following adoptive transfer of naive CD4$^+$ T cells into lymphopenic animals, and appears to be a prerequisite for development of colitis under these experimental conditions (W.-P. Koh, P. Bertolino, and B. Fazekas de St. Groth, in preparation).

A graphic illustration of the importance of lymphopenia in susceptibility to autoimmune disease is seen in experimental models in which gastritis and other organ-specific autoimmune diseases are induced in BALB/c and A/J mice by means of a diverse range of experimental maneuvers that render the mice nonspecifically CD4 lymphopenic. Such maneuvers include thymectomy at 3 days of age (51), transplantation of a neonatal thymus into a nude host (52), and expression of a Tg-encoded TCR α-chain in the absence of a Tg-encoded β-chain (53). In the best-studied model, namely 3-day thymectomy, regulatory cells are generated at a normal rate, and constitute a normal percentage of peripheral T cells, but the deficit in their absolute number is sufficient to precipitate autoimmune disease (51). In the colitis model described in this work, acquisition of resistance to disease by 6 mo of age in -D Rag-1$^{-/-}$ mice (Fig. 1), and by 4 wk after adoptive transfer in Rag-1$^{-/-}$ recipients of -D Rag-1$^{+/+}$ cells, may have been due to generation of a sufficiently large population of regulatory cells to prevent further activation of naive cells specific for colitogenic Ags.

The degree of CD4 lymphopenia required to confer susceptibility to colitis in the current study was influenced by the presence of a TCR Tg population with a pathogenic specificity. Thus, only moderate CD4 lymphopenia was required when a substantial proportion of T cells expressed a cytochrome-reactive receptor, whereas profound CD4 lymphopenia, of the degree seen in TCR α-chain knockout mice, was essential when the Tg TCR was of a neutral non-cross-reactive specificity, such as anti-MBP. Since generation of regulatory T cells via recognition of environmental Ag is likely to require a polyclonal T cell repertoire, it is not surprising that an increase in the size of the polyclonal CD4$^+$ population leads to a reduction in the incidence of disease in mice expressing a self-reactive TCR Tg population, as indicated by disease resistance in all eight 5C.C7 TCR Tg lines with poor allelic exclusion on the original Rag-1$^{+/+}$ background (Fig. 5 and not shown).

In contrast to previous hapten-induced models of colitis in rodents (54, 55), the monoclonality and ease of detection and characterization of the Tg CD4$^+$ effector T cells present in the current models make them uniquely suited to further studies of the precise cellular and Ag-dependent interactions that initiate and propagate a dysregulated, pathogenic mucosal immune response. Cells of the Tg specificity also represent a powerful tool for isolating the colitogenic Ag in this particular model. However, even without formal identification of the colitogenic Ag, the current cytochrome-specific TCR Tg model has the potential to provide novel insights into disease pathogenesis, as demonstrated by previous studies of spontaneous T-dependent autoimmune diseases mediated by TCR Tg CD4$^+$ cells (56, 57). Moreover, the ability to use cytochrome *c* to test the functional capacity of colitogenic T cells at various stages of disease, and to induce regulatory cells in situ, offers significant advantages over the currently popular polyclonal models. Thus, it will allow different protocols for immunization with specific Ag to be tested, with a view to rational intervention in human inflammatory bowel disease.

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