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Expression of Dual TCR on DO11.10 T Cells Allows for Ovalbumin-Induced Oral Tolerance to Prevent T Cell-Mediated Colitis Directed against Unrelated Enteric Bacterial Antigens¹

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The triggering Ag for inflammatory bowel disease and animal models of colitis is not known, but may include gut flora. Feeding OVA to DO11.10 mice with OVA-specific transgenic (Tg) TCR generates Ag-specific immunoregulatory CD4⁺ T cells (Treg) cells. We examined the ability of oral Ag-induced Treg cells to suppress T cell-mediated colitis in mice. SCID-bg mice given DO11.10 CD4⁺CD45RB^{high} T cells developed colitis, and cotransferring DO11.10 CD45RB^{low}CD4⁺ T cells prevented CD4⁺CD45RB^{high} T cell-induced colitis in the absence of OVA. The induction and prevention of disease by DO11.10 CD4⁺ T cell subsets were associated with an increase in endogenous TCR α chain expression on Tg T cells. Feeding OVA to SCID-bg mice reconstituted with DO11.10 CD4⁺CD45RB^{high} attenuated the colitis in association with increased TGF- β and IL-10 secretion, and decreased proliferative responses to both OVA and cecal bacteria Ag. OVA feeding also attenuated colitis in SCID-bg mice reconstituted with a mix of BALB/c and DO11.10 CD45RB^{high} T cells, suggesting that OVA-induced Treg cells suppressed BALB/c effector cells. The expression of endogenous non-Tg TCR allowed for DO11.10-derived T cells to respond to enteric flora Ag. Furthermore, feeding OVA-induced Treg cells prevented colitis by inducing tolerance in both OVA-reactive and non-OVA-reactive T cells and by inducing Ag-nonspecific Treg cells. Such a mechanism might allow for Ag-nonspecific modulation of intestinal inflammation in inflammatory bowel disease. *The Journal of Immunology*, 2004, 172: 1515–1523.

The regulation of the mucosal immune response to enteric flora and dietary Ags is essential for the maintenance of mucosal homeostasis. CD4⁺ T cells are central to both the induction and regulation of mucosal inflammation (1, 2). Abnormal CD4⁺ T cell responses to enteric bacterial flora are a common feature of experimental models of intestinal inflammation and human inflammatory bowel disease (IBD)³ (3–5). The inflammation is due to excessive Th1 or Th2 effector responses (2, 6) in association with loss of regulatory elements of the immune response (7). Understanding how these processes are involved in the pathogenesis of IBD is difficult, because the target Ag(s) is not known.

The murine colitis model of CD4⁺CD45RB^{high} T cell transfer into SCID mice has provided insight into the effector and regulatory T cell (Treg) mechanisms involved in intestinal inflammation (8, 9). This model requires enteric flora to induce inflammation, yet the specific triggering Ag is not known (1, 10, 11). The requirement for TCR recognition of Ag for Treg cell function is less clear. In a study of *Helicobacter* infection in the CD45RB^{high} colitis model, Treg cell function required previous Ag exposure (12). Other studies suggested that Treg cells can suppress immune pathology triggered by pathogens without previous exposure (10), including *Helicobacter* infection (13). In addition, in vitro-generated OVA TCR-specific T regulatory 1 (Tr1) cells required OVA to suppress colitis in vivo and did not share Ag specificity with the effector T cells, suggesting Ag-driven bystander suppression (9, 14). Therefore, the requirement for Ag specificity of naturally occurring or in vivo-induced Treg cells in the context of mucosal inflammation remains unclear.

In this study, we used DO11.10 mice as a source of T cells with a defined TCR specificity to follow Ag-specific T cells in the SCID-transfer colitis model. The DO11.10 mouse carries a transgene encoding the TCR specific for chicken OVA_{323–339} peptide on >97% of peripheral T cells (15). OVA-induced oral tolerance in DO11.10 mice involves induction of Treg cells that secrete IL-10 and TGF- β and are associated with the expansion and activation of CD4⁺CD25⁺ T cells (16, 17). It remains unclear whether these peripherally induced Treg cells can alter T cell-mediated mucosal inflammation or whether the tolerance is restricted to OVA Ag.

Our results indicate that DO11.10 CD4⁺CD45RB^{high} T cells induced colitis in SCID-bg mice in the absence of OVA, and OVA Tg CD4⁺CD45RB^{low} T cells prevented colitis induced by

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³ Abbreviations used in this paper: IBD, inflammatory bowel disease; Treg cell, T regulatory cell; Tr1, T regulatory 1; Tg, transgenic; MNC, mononuclear cell; MLN, mesenteric lymph node; CBA, cecal bacterial Ag; RAG, recombination-activating gene.

DO11.10-derived CD4⁺CD45RB^{high} without the need for OVA. These findings were associated with the expression of dual TCR, allowing for non-OVA Ag activation of DO11.10 T cells. Furthermore, feeding OVA to SCID-bg mice reconstituted with DO11.10 CD4⁺CD45RB^{high} T cells prevented colitis in association with tolerance against both OVA- and bacterial-derived Ag. More importantly, feeding OVA to mice reconstituted simultaneously with DO11.10- and BALB/c-derived CD4⁺CD45RB^{high} T cells significantly diminished the severity of the colitis. The expression of dual TCR on DO11.10-derived T cells allowed for oral Ag to induce the development of Treg cell function that influence non-OVA specific responses. This is the first demonstration that oral tolerance against a defined Ag can prevent mucosal inflammation associated with T cell stimulation by unrelated Ag(s).

Materials and Methods

Mice

DO11.10 mice were purchased from Charles River Laboratories (Wilmington, MA) and bred under standard pathogen-free conditions in the central animal facility of McMaster University. Congenic C.B.17/SCID-bg mice and BALB/c mice were obtained from Harlan (Indianapolis, IN). Female mice between 7 and 12 wk of age were used in these studies. All animal experiments were performed in accordance with institutional guidelines as approved by the Animal Care Review Board of McMaster University. Donor and recipient mice in our colony were routinely screened for *Helicobacter* sp. infection by PCR capable of detecting ribosomal sequences common to all *Helicobacter* species and were free of infection (data not shown).

Isolation and purification of CD45RB^{high} and CD45RB^{low} CD4⁺ spleen cells

Spleen cells from DO11.10 or BALB/c mice were used as a source of CD4⁺ T cells for reconstitution of SCID-bg recipient mice. CD4⁺ T cell subsets from the spleens were isolated and sorted as described previously (8). Briefly, single-cell suspensions were depleted of B220⁺, MAC-1⁺, and CD8⁺ cells by negative selection using M-450 sheep anti-rat IgG-coated Dynabeads (DynaL Biotech, Oslo, Norway). Purified anti-CD8 α , anti-CD11b, anti-MAC1 were obtained from BD PharMingen (Mississauga, Ontario, Canada). CD4⁺CD45RB^{high} and CD4⁺CD45RB^{low} fractions were sorted by FACS Vantage SE (BD Biosciences, San Jose, CA) under sterile conditions. The purity of each subpopulation was >98%. For preparation of purified transgenic (Tg) CD45RB^{high} and CD45RB^{low} CD4⁺ T cell subsets, CD4⁺ T cells derived from naive DO11.10 mice were labeled with FITC-conjugated anti-CD45RB, PE-conjugated anti-KJ1-26 mAbs, and CyChrome-conjugated anti-CD4 mAb. The purity of sorted Tg CD4⁺ T cell subsets was analyzed before their injection into SCID-bg recipients and was >98%.

Reconstitution of SCID-bg mice with T cell subsets and OVA feeding

DO11.10-derived CD4⁺CD45RB^{high} and CD4⁺CD45RB^{low} T cells were washed and resuspended at 2×10^6 cells/ml in sterile PBS. Seven- to 12 wk-old SCID-bg mice each received either CD4⁺CD45RB^{high} T cells (4×10^5 cells i.p.) alone or combined with CD45RB^{low}CD4⁺ T cells (2×10^5). In some experiments, CD4⁺ T cells subsets derived from BALB/c and DO11.10 mice were mixed and injected into SCID-bg mice. Mice were monitored twice weekly for change in body weight, evidence for soft stool, or diarrhea, bloody diarrhea, and rectal prolapse. Mice were sacrificed when they exhibited two or more of the following signs: 1) a loss of >15% of their original body weight, 2) the development of a large (>3-mm) rectal prolapse, or 3) extensive diarrhea or bloody stools. Some of the recipient mice were fed OVA (1 mg/ml; Sigma-Aldrich, St. Louis, MO) or PBS in the drinking water. This regimen of OVA feeding was previously shown to induce oral tolerance with suppression of OVA responses in DO11.10 mice (18). The average water consumption is ~4–5 ml/mouse/day. The drinking water was changed every 3 days.

Histological examination

Recipient SCID-bg mice were sacrificed 8–12 wk after reconstitution. The colon was opened longitudinally and separated into ascending, transverse, and descending colon and cecum. Tissues were fixed in 10% buffered formalin, sectioned, and stained with H&E. Each segment was analyzed for

the severity of intestinal inflammation and graded on a scale from 0 (no change) to 4 (most severe), as described previously (19). The scores at each segment were combined to provide an overall score of inflammation with a maximum score of 16.

Phenotypic analysis by flow cytometry

Preparation for flow cytometry analysis involved suspending 5×10^5 mononuclear cells (MNC) isolated from spleen or mesenteric lymph node (MLN) in PBS/0.2% w/v BSA supplemented with 0.1% w/v sodium azide. Cells were then incubated with relevant mAb for 30 min at 4°C and washed. Three-color flow cytometry acquisition was performed on FACScan (BD Biosciences). The following reagents and mAbs were obtained from BD PharMingen: FITC-conjugated hamster anti-CD3 ϵ (145-2C-11), PE- and CyChrome-conjugated anti-CD4 mAb (L3T4), PE-conjugated CD25 (IL-2R α -chain, p55), PE-conjugated anti-V α 2 TCR (B20.1), and FITC- and PE-conjugated rat IgG2b. FITC- and PE-conjugated mAb specific for DO11.10 Tg TCR clonotype (clone KJ1-26) were purchased from Caltag Laboratories (Burlingame, CA). A total of 5×10^5 events gated on lymphocytes was collected by FACScan using the CellQuest software, and the data was analyzed by WinList version 5.0 (Verity Software House, Topsham, ME).

Preparation of cecal bacterial Ag (CBA)

CBA was prepared as previously described (5). The cecum removed from BALB/c or DO11.10 mice was placed in PBS and mixed on a vortex. After removing residual tissue, the suspension was sonicated 10 times for 30 s on ice. The suspension was then centrifuged at $10,000 \times g$ for 10 min. The supernatant was collected, sterile filtered through a 0.22- μ m syringe filter, and stored at -20°C . The protein concentration in the supernatants was typically 1.5–3.0 mg/ml.

Proliferation assay

APCs were prepared from BALB/c spleen cells and pulsed with OVA (1 mg/ml) or CBA (200 μ g/ml) in six-well plates. After incubation for 18 h, the cells were washed and irradiated (3000 rad). Proliferation assays were performed as previously described (20). Briefly, 100 μ l of 5×10^6 lymphoid cells per milliliter was added to 96-well flat-bottom tissue culture plates along with Ag-pulsed APC (5×10^6 /well) in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M 2-ME (Sigma-Aldrich). Plate-bound anti-CD3 (5 μ g/ml) or Con A (1 μ g/ml) was used as positive control. Each treatment was set in triplicate and maintained for 5 days at 37°C in 5% CO₂. Cell cultures were pulsed with 1 μ Ci of [³H]thymidine for the last 16 h, and proliferation responses were determined by measuring [³H]thymidine incorporation.

Cytokine measurement

Cytokine production was measured in cell culture supernatants. Supernatants were stored at -20°C until analysis by ELISA specific for IL-2, TNF- α , and IL-10 performed according to manufacturer's specification (R&D Systems, Minneapolis, MN). TGF- β concentrations were determined using the commercially available TGF- β Quantikine kit (R&D Systems). Data were analyzed against the linear portion of the generated standard curve using calibrated standards provided by the manufacturers.

Statistical analysis

Data were expressed as mean \pm SEM. Statistical analysis was performed using the two-tailed Student's *t* test for independent samples. Mann-Whitney was used for nonparametric data. One-way ANOVA was used for time course data. The differences between the mean of two groups were considered significant when the value of *p* < 0.05.

Results

Expression of CD45RB and CD25 on dual-TCR-expressing T cells in DO11.10 mice

A variable proportion of DO11.10 T cells in peripheral lymphoid tissue express a second TCR due to incomplete allelic exclusion at the TCR α locus (i.e., Tg β -chain plus non-Tg α -chain) (21). A significant proportion of DO11.10 lamina propria lymphocytes express the activated/memory-like T cell phenotype, suggesting that enteric bacterial activated these Tg T cells through a nonclonotypic TCR (21, 22). Single- and dual-TCR-expressing T cells can be

distinguished by flow cytometry analysis of the relative expression of CD3 vs the clonotypic marker of the Tg TCR, KJ1-26, based on the fixed stoichiometry of expression of total TCR to CD3 (21, 23). A typical distribution pattern (Fig. 1A) of the expression of KJ1-26 vs CD3 fluorescence on DO11.10-derived splenocytes showed that $\sim 12.2 \pm 2.8\%$ of $CD3^+KJ1-26^+$ cells expressed an intermediate intensity of KJ1-26, i.e., a $<1:1$ ratio with CD3. V α 2 represents a non-Tg V α chain that is rarely expressed by cells with the high KJ1-26:CD3 ratio, but is found on cells with a lower KJ1-26:CD3 ratio (Fig. 1A), indicating that the T cells with lower KJ1-26:CD3 ratio express both the clonotypic TCR KJ1-26 and a second TCR. V α 2 is just one representative example of non-Tg TCR V α chain rearrangement (21).

T cells expressing dual TCR, i.e., T cells expressing intermediate levels of KJ1-26 and a lower KJ1-26:CD3 ratio, have a higher percentage of $CD45RB^{low}$ (50.7 ± 2.2 vs $15.3 \pm 4.9\%$; $n = 3$) and $CD25^+$ (20.5 ± 3.1 vs $2.0 \pm 0.2\%$; $n = 3$) than those T cells expressing high level of KJ1-26:CD3 ratio (Fig. 1A). Similar re-

sults were obtained from DO11.10 MLN (Fig. 1B). Accordingly, only $4.7 \pm 1.1\%$ of spleen $KJ1-26^+CD45RB^{high}$ T cells express intermediate level of KJ1-26:CD3 ratio, whereas the number for $KJ1-26^+CD45RB^{low}$ T cells expressing dual TCR was $43.2 \pm 6.9\%$. Similarly, spleen $KJ1-26^+CD25^+$ T cells also contain significantly higher number of dual-TCR-expressing T cells than Tg $CD25^-$ T cells (52.1 ± 4.8 vs $6.8 \pm 1.0\%$; $p < 0.05$; $n = 3$). The finding that T cells with $CD45RB^{low}$ and $CD25^+$ phenotype, a marker of memory/activated T cells and naturally occurring Treg cells, also contain a significant percentage of dual-TCR-bearing T cells is not limited to Tg mice, because $>50\%$ of dual-TCR-expressing T cells (V α 2 plus another V α chain) from BALB/c mice were $CD4^+CD45RB^{low}$ T cells, whereas only 18.1% of single V α 2-expressing T cells expressed $CD4^+CD45RB^{low}$. This suggests that, although only a small portion of Tg T cells express a second TCR with endogenous V α chain, a majority of those dual-TCR-expressing cells are represented in the T cell subsets expressing phenotypic markers of activation/memory and Treg cells.

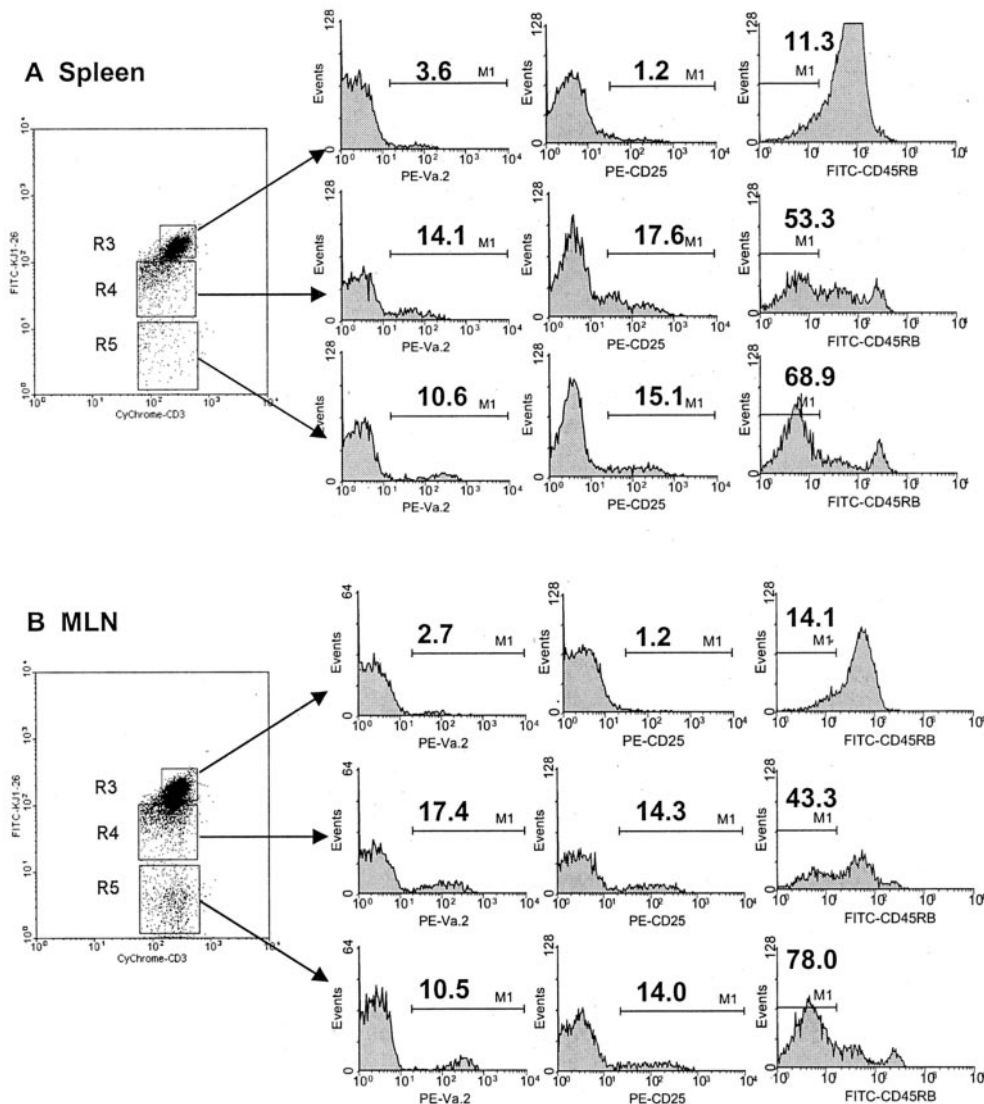


FIGURE 1. Dual-TCR T cells are enriched in naturally occurring Treg cells in naive DO11.10 Tg peripheral T cells. Splenocytes (A) or MLN cells (B) were stained for three-color fluorescence analysis with different combinations of FITC/PE-conjugated anti-KJ1-26, CyChrome-anti-CD3, PE-anti-V α 2, PE-anti-CD25, or PE-anti-CD45RB. According to the distribution of KJ1-26 $^+$ vs CD3 $^+$ expression, T cells from naive DO11.10 mice can be divided into three groups. Gate R3 contains cells with a high KJ1-26:CD3 ratio; and gate R4 contains cells with a lower ratio. Gate R5 contains T cells that do not express clonotypic TCR. Frequencies of a second non-Tg TCR V α chain (V α 2), CD25 $^+$, and CD45RB low T cells in gates R3, R4, and R5 are depicted in single-color histograms.

SCID-bg mice reconstituted with OVA-specific TCR Tg (DO11.10) CD45RB^{high}CD4⁺ T cells developed colitis in the absence of OVA Ag

Given that DO11.10 T cells can express nonclonotypic TCR, one would predict that CD4⁺CD45RB^{high} T cells could induce colitis on transfer into SCID mice in the absence of OVA. However, the only previous report used preactivated DO11.10 CD4 T cells to induce colitis on transfer into SCID mice (11). Therefore, to determine whether nonactivated CD4⁺CD45RB^{high} T cells from DO11.10 mice were able to induce colitis in the absence of OVA, we transferred CD4⁺CD45RB^{high} T cells from OVA naive DO11.10 mice into SCID-bg mice. These mice developed weight loss starting at ~4–5 wk after cell transfer (Fig. 2). Diarrhea and rectal prolapse developed by 8–10 wk. The time course and severity of the colitis was similar to that seen after BALB/c-derived CD4⁺CD45RB^{high} T cells were transferred into SCID-bg mice (data not shown). Histological examination of SCID-bg mice reconstituted with DO11.10 CD4⁺CD45RB^{high} T cells showed a transmural inflammation that was most severe in the transverse colon (Fig. 3A) and was similar to conventional BALB/c into SCID transfer colitis (19).

DO11.10 CD45RB^{low}CD4⁺ T cells prevented colitis in SCID-bg mice reconstituted with DO11.10 CD45RB^{high}CD4⁺ T cells

To determine whether DO11.10 CD45RB^{low}CD4⁺ T cells could control the colitis induced by DO11.10 CD45RB^{high} T cells in the absence of OVA stimulation, 2×10^5 DO11.10 CD45RB^{low} T cells were injected into SCID-bg mice together with 4×10^5 DO11.10 CD45RB^{high} T cells. In contrast to mice receiving DO11.10 CD45RB^{high} T cells alone, animals reconstituted with both T cell subsets failed to lose weight (Fig. 2) and failed to develop colonic inflammation (Fig. 3B). These data indicate that DO11.10 CD4⁺CD45RB^{low} T cells functioned as Treg cells in the absence of Ag specifically recognized by the Tg TCR.

Phenotype of DO11.10-derived CD4⁺ T cell subsets after transfer into SCID-bg mice

Previously, studies showed that BALB/c-derived CD4⁺ T cell subsets repopulated the peripheral lymphoid tissues of SCID mice (8, 11). Our data showed that DO11.10 T cells were identifiable in SCID-bg recipients (Table I). Therefore, DO11.10 Tg CD4⁺ T cell subsets were able to reconstitute SCID-Bg mice even in the absence of the specific Ag OVA. The number of CD4⁺ T cells in spleen and MLN of SCID-bg mice reconstituted with Tg

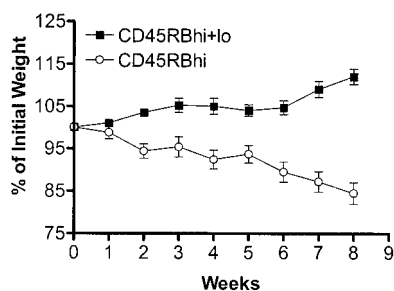


FIGURE 2. Induction of colitis in SCID-bg mice reconstituted with naive CD45RB^{high}CD4⁺ T cells derived from DO11.10 mice. Eight- to 12-wk-old female C.B-17 SCID-bg mice were reconstituted with either 4×10^5 CD45RB^{high}CD4⁺ T cells or 4×10^5 CD45RB^{high} plus 2×10^5 CD45RB^{low} CD4⁺ T cells derived from DO11.10 mice. The change in weight over time is expressed as percentage of initial body weight. Data are representative of three independent experiments, each containing three to five mice per group.

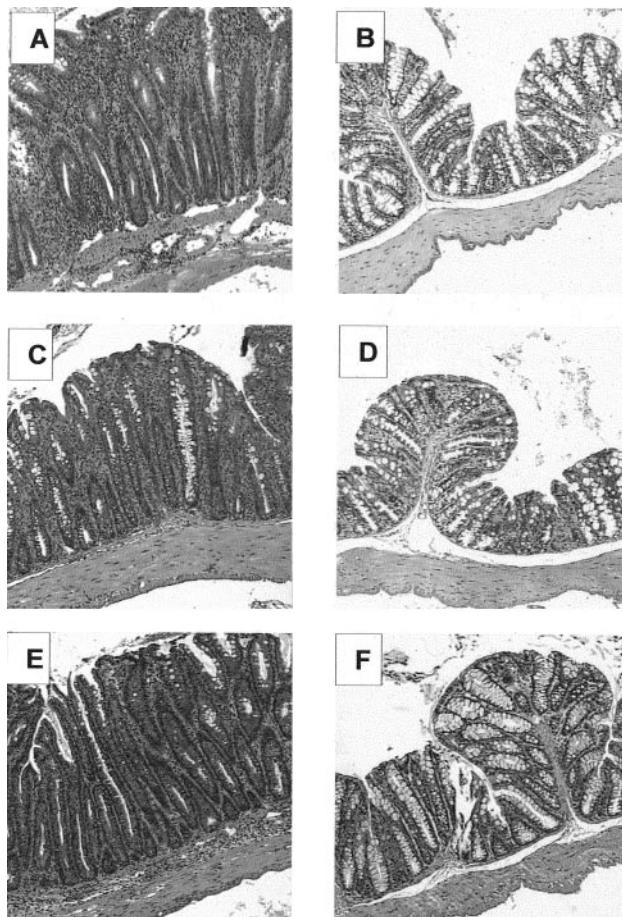


FIGURE 3. Representative photomicrographs of transverse colon from SCID-bg mice that was reconstituted with purified CD4⁺ T cell subsets. A, Severe colitis in a SCID-bg recipient of DO11.10 CD4⁺CD45RB^{high} T cells. B, Lack of colitis in a SCID-recipient of DO11.10 CD4⁺CD45RB^{high} plus DO11.10 CD4⁺CD45RB^{low}. C, PBS-fed SCID-bg recipient of DO11.10 CD4⁺CD45RB^{high} T cells. D, OVA-fed SCID-bg recipient of DO11.10 CD4⁺CD45RB^{high} T cells. E, PBS-fed SCID-bg recipients of mixed CD4⁺CD45RB^{high} T cells from both DO11.10 and BALB/c mice. F, OVA-fed SCID-bg recipients of mixed DO11.10 and BALB/c CD4⁺CD45RB^{high} T cells. H&E stain; original magnification, $\times 100$.

CD45RB^{high} subsets was higher than that found in SCID-bg mice reconstituted with both CD45RB^{high} and CD45RB^{low} T cell subsets (Table I).

As described for conventional BALB/c into SCID transfer model (19), the T cells that emerged in SCID-bg recipients of DO11.10 CD4⁺CD45RB^{high} T cells lost their naive phenotype with a majority of spleen CD4⁺ T cells expressing CD45RB^{low}. This change was independent of the presence or absence of colitis (Table I). In addition to the loss of CD45RB^{high} expression, there was a significant decrease in the percentage of CD4⁺ T cells expressing the clonotypic Tg TCR marker KJ1-26 (Fig. 4 and Table I). The decrease in expression of the clonotypic marker was also seen in T cells recovered from mice reconstituted with both CD45RB^{high} and CD45RB^{low} T cells.

Previously, Iqbal et al. (6) showed that transfer of CD4⁺ T cells from a DO11.10 mouse backcrossed onto recombination-activating gene (RAG)-2 deficient background failed to induce colitis in SCID mice. This suggested that, in the absence of non-Tg TCR, these T cells could not react with bacteria-derived Ag. To determine whether DO11.10 T cells inducing colitis were associated with the expression of a nonclonotypic TCR, T cells from SCID-bg

Table I. Phenotypic analysis of CD4⁺ T cells in SCID-bg mice reconstituted with DO11.10 CD4⁺ T cell subsets^a

| | DO11.10 Donors | CD45RB ^{high} Recipients | CD45RB ^{high + low} Recipients |
|---|----------------|-----------------------------------|---|
| Total CD4 ⁺ T cells (×10 ⁶) | | | |
| Spleen | 26.2 ± 2.3 | 1.87 ± 0.3 | 0.76 ± 0.08 ^b |
| MLN | 6.5 ± 1.8 | 0.74 ± 0.23 | 0.13 ± 0.02 ^b |
| CD4 ⁺ CD45RB ^{low} T cells ^c | | | |
| Spleen | 22.6 ± 3.1 | 67.7 ± 6.6 | 78.7 ± 2.7 |
| MLN | 18.7 ± 0.3 | 68.2 ± 5.7 | 70.2 ± 8.2 |
| Vα.2 ⁺ KJ1-26 ⁺ T cells ^d | | | |
| Spleen | 4.6 ± 0.8 | 24.3 ± 3.1 | 11.9 ± 1.5 ^b |
| MLN | 2.12 ± 0.28 | 11.3 ± 1.9 | 6.2 ± 1.4 ^b |

^a At the time of sacrifice, spleen and MLN cells were isolated, and three-color flow cytometry was performed with relevant fluorescence-conjugated Abs (see *Materials and Methods*).

^b Statistical difference as determined by *p* < 0.05 compared with CD45RB^{high} recipients.

^c Percentage of gated CD4⁺ T cells expressing phenotypes defined. Data pooled from two independent experiments and represented as mean ± SEM.

^d Percentage of cells expressing CD3 and KJ1-26.

recipients were stained with mAb specific for a non-Tg TCR Vα2 chain. In the donor DO11.10 mice, only 4.6 ± 0.8% (*n* = 4) of the splenic CD4⁺ T cells (KJ1-26⁺) expressed a second non-Tg Vα2, whereas there was a 2- to 4-fold increase in cells expressing non-Tg Vα TCR chain in the spleen from both CD45RB^{high} alone or combined CD45RB^{high} and RB^{low} recipients (Fig. 4 and Table I). Furthermore, analysis of the expression of the Tg Vβ8 TCR chain as detected by F23.1 mAb (specific for Vβ8.1, -8.2, -8.3) on CD4⁺ T cells, showed that colitic mice exhibited a 2-fold increase in non-Tg KJ1-26⁻CD4⁺ T cells, suggesting that a higher percentage of CD4⁺ T cells expressed endogenous non-Tg α-chains as compared with SCID-bg mice reconstituted with both DO11.10 CD45RB^{low} and RB^{high} T cells (Fig. 4B). These data indicate that the emergence of a second non-Tg TCR could permit effector T cells to respond to non-OVA Ag such as those present in the gut flora.

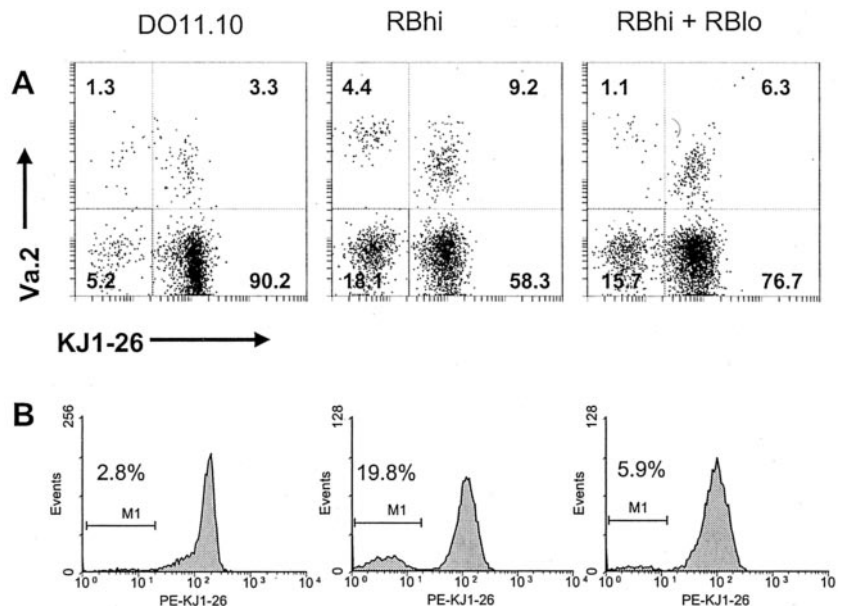
Oral administration of OVA attenuated the colitis in SCID-bg mice reconstituted with OVA-specific TCR DO11.10 CD45RB^{high}CD4⁺ T cells

Feeding OVA to DO11.10 mice induced oral tolerance to OVA Ag via the induction of regulatory-type T cells (16). To determine

whether feeding OVA would prevent T cell-mediated colitis, we fed OVA to SCID-bg mice reconstituted with DO11.10 CD4⁺CD45RB^{high} T cells. SCID-bg recipients were allowed to drink water containing OVA (1 mg/ml) starting 1 day after reconstitution. The DO11.10 CD45RB^{high}-transferred SCID-bg mice fed OVA were significantly protected from the development of weight loss (Fig. 5A), and this was associated with a significant reduction in the severity of colitis (Figs. 5B, and 3, C and D). At the time of sacrifice, the mean histological colitis score was 8.56 ± 0.8 for PBS-fed SCID-bg mice reconstituted with DO11.10-derived CD4⁺CD45RB^{high} T cells, whereas the average score for OVA-fed recipients was 4.93 ± 0.76 (*p* < 0.002). OVA feeding did not induce any difference in weight loss or intestinal inflammation in the mice reconstituted with both DO11.10 CD4⁺CD45RB^{high} and CD4⁺CD45RB^{low} T cells (Fig. 5). Feeding OVA did not alter the colitis in SCID-bg mice reconstituted with BALB/c-derived CD4⁺CD45RB^{high} T cells (data not shown).

To determine whether the effect of OVA feeding on the intestinal inflammation in the DO11.10 CD4⁺CD45RB^{high} T cell-reconstituted SCID-bg mice was due to Ag-specific CD4⁺ T cell deletion, the cells from spleens and MLN of these recipients were isolated, and total numbers of CD4⁺ T cells and CD4⁺KJ1-26⁺ T

FIGURE 4. TCR expression on MLN cells from SCID-bg mice reconstituted with Tg CD4⁺ T cells subsets. Eight to 10 wk after reconstitution, MLN cells were isolated from SCID-bg recipients and stained with PE- or FITC- or CyChrome-conjugated CD4, KJ1-26, F23.1, and/or Vα.2 mAbs (see *Materials and Methods*). *A*, Representative dot plots of three-color flow cytometry analysis gated on CD4⁺ T cells, comparing expression of the Tg TCR as detected by KJ1-26 clonotype-specific Ab and a second TCR Vα chain as detected by TCR Vα.2. *B*, Histograms depict the expression of Tg TCR on T cells gated for expression of CD4⁺F23.1⁺ (specific for Vβ8.1, -8.2, -8.3) T cells.



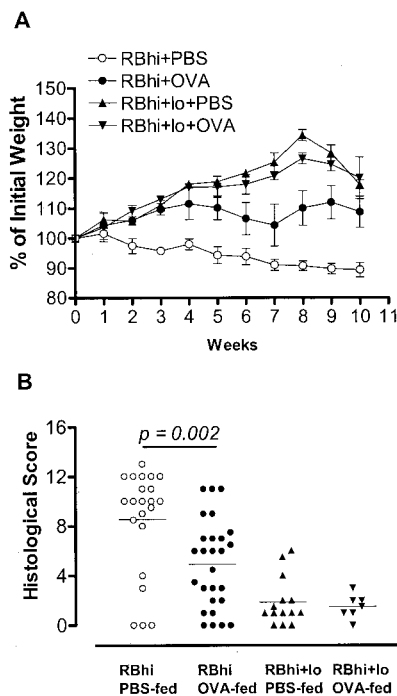


FIGURE 5. OVA feeding attenuated the development of colitis in SCID-bg mice reconstituted with DO11.10 CD45RB^{high}CD4⁺ T cells. **A**, Weight changes in SCID-bg mice reconstituted with 4×10^5 CD45RB^{high}CD4⁺ spleen T cells from DO11.10 mice alone or in combination with 2×10^5 CD45RB^{low}CD4⁺ T cells. Half of each group was fed OVA in drinking water (1 mg/ml). The differences in body weight between OVA-fed and PBS-fed CD45RB^{high}CD4⁺ T cells recipients were statistically significant ($p < 0.05$ by ANOVA). Results are representative of five independent experiments with four to five mice per group. **B**, Histological score of colitis in SCID-bg mice reconstituted with CD4⁺ T cell subsets from DO11.10 mice. Inflammation was scored at cecum and proximal, middle, and distal colon, as described in *Materials and Methods*. Each data point represents the score of an individual mouse. The bar represents the mean of the inflammatory score for the group.

cells were determined by FACS analysis (Table II). OVA-fed RB^{high} recipients did show decreased CD4⁺ and CD4⁺KJ1-26⁺ T cell numbers; however, in mice reconstituted with both RB^{high} plus RB^{low} T cells and fed OVA, there was no decrease in either CD4⁺ or KJ1-26⁺ T cell numbers (Table II). This suggested that the decrease of total CD4⁺ and CD4⁺KJ1-26⁺ T cells numbers in CD4⁺CD45RB^{high} recipients by OVA feeding is related to a diminished inflammation compared with PBS-fed CD4⁺CD45RB^{high} recipients.

Proliferation and cytokine responses of T cells from OVA-fed SCID-bg recipients of DO11.10 CD45RB^{high} T cells

To investigate whether feeding OVA to CD45RB^{high}-reconstituted SCID-bg mice led to Ag-specific tolerance, we measured in vitro proliferation and cytokine responses to both OVA and Ag derived from CBA. MNC from spleen were isolated from OVA- and PBS-fed SCID-bg recipients of DO11.10-derived CD4⁺CD45RB^{high} T cells. These cells were restimulated in vitro with CBA- or OVA-pulsed APC. There was significant proliferation in the spleen of the PBS-fed SCID-bg mice receiving DO11.10 CD4⁺CD45RB^{high} T cells in response to both OVA and CBA. In contrast, OVA feeding significantly reduced the proliferative response to both CBA and OVA (Fig. 6A). Cytokine production of MNC from OVA-fed and PBS-fed SCID-bg recipient mice was measured in spleen cells restimulated with CBA and OVA-pulsed APC in vitro; IL-2, TNF- α , TGF- β , and IL-10 levels were measured by ELISA in supernatants collected 4 days after in vitro stimulation (Fig. 6B). OVA-fed mice had a significant decrease in IL-2 and TNF- α responses to both CBA and OVA stimulation. In contrast, production of the regulatory cytokine TGF- β in response to both CBA and OVA stimulation was significantly increased. IL-10 production was undetectable in MNC from both PBS- and OVA-fed SCID-bg mice receiving CD4⁺CD45RB^{high} T cells, in response to CBA- and OVA-pulsed APC. Therefore, OVA feeding induced tolerance to both the specific Ag OVA and to the unrelated Ags in gut flora. The effect of OVA feeding on proliferative and cytokine responses in these reconstituted mice may be due to induction of anergy of T cells expressing dual TCRs or due to the induction of Th3-type Treg cells that nonspecifically down-regulate all T cell responses.

OVA feeding prevented colitis in SCID-bg mice reconstituted with a mixed transfer of CD45RB^{high}CD4⁺ T cells from BALB/c and DO11.10 mice

To further examine whether feeding OVA would prevent non-OVA Tg T cell-mediated colitis, we transferred equal numbers of CD4⁺CD45RB^{high} T cells from BALB/c and DO11.10 mice (1:1 ratio; total of 4×10^5 per mouse) to SCID-bg mice. Half of the recipients were fed OVA Ag (or PBS as control). The time course of disease onset and the intestinal inflammation induced by mixed CD4⁺CD45RB^{high} T cells was similar to that seen in mice receiving T cells derived from either BALB/c or DO11.10 mice alone. OVA feeding mice reconstituted with the combination of both BALB/c and DO11.10 CD45RB^{high} T cells significantly prevented the weight loss (Fig. 7A) and the severity of colonic inflammation (Fig. 3, E and F). The overall inflammation score for OVA-fed group was 4.9 ± 1.1 compared with 10.1 ± 0.9 in the non-OVA-fed group (Fig. 7B; $p < 0.01$).

Table II. Total number of CD4⁺ T cells and CD4⁺KJ1-26⁺ T cells recovered from recipients that were fed with OVA or PBS^a

| | Spleen ($\times 10^6$) | | | MLN ($\times 10^6$) | | |
|---|--------------------------|------------------------|--------------------------------------|--------------------------|-------------------------|--------------------------------------|
| | Cellularity | CD4 ⁺ | CD4 ⁺ KJ1-26 ⁺ | Cellularity | CD4 ⁺ | CD4 ⁺ KJ1-26 ⁺ |
| CD45RB ^{high} PBS-fed ($n = 15$) | 34.2 (4.3) | 1.9 (0.3) | 1.1 (0.2) | 1.8 (0.3) | 0.7 (0.2) | 0.4 (0.1) |
| CD45RB ^{high} OVA-fed ($n = 15$) | 26.6 (5.0) | 1.1 (0.2) ^b | 0.7 (0.18) ^b | 0.70 (0.15) ^b | 0.2 (0.04) ^b | 0.11 (0.02) ^b |
| CD45RB ^{high} + ^{low} PBS-fed ($n = 6$) | 11.0 (1.5) | 0.76 (0.08) | 0.25 (0.04) | 0.58 (0.05) | 0.12 (0.02) | 0.06 (0.006) |
| CD45RB ^{high} + ^{low} OVA-fed ($n = 5$) | 11.5 (1.5) | 0.81 (0.18) | 0.51 (0.11) | 0.96 (0.09) | 0.14 (0.04) | 0.12 (0.04) |

^a The data were pooled from three experiments and represent the mean (SEM) of the indicated groups of mice.

^b Statistical difference ($p < 0.05$) as determined by the Student *t* test compared with the CD45RB^{high} PBS-fed group.

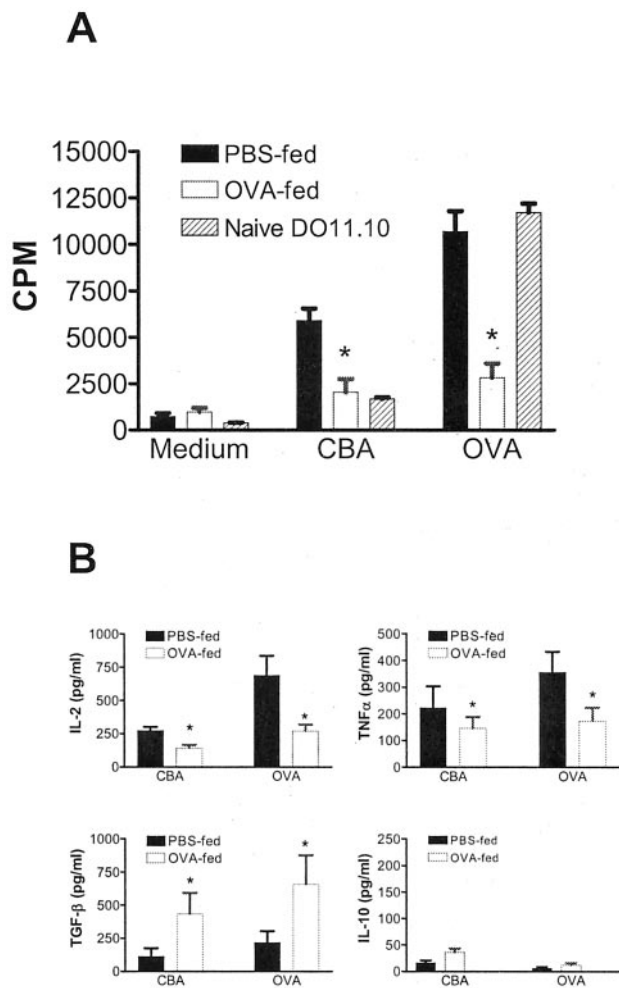


FIGURE 6. Effect of OVA feeding on proliferation and cytokine production by splenocytes from SCID-bg mice reconstituted with DO11.10 CD45RB^{high} T cells. *A*, Proliferative responses of splenocytes isolated from PBS-fed (■) or OVA-fed (□) recipient mice against CBA and OVA in vitro, with naive DO11.10 spleen cell as control (▨). APCs (irradiated spleen cells from normal BALB/c mice) were pulsed with CBA (200 μg/ml) or OVA (1 mg/ml) and added to cultures of MNC isolated from spleens of SCID-bg mice 9 wk after reconstitution with DO11.10 CD45RB^{high}CD4⁺ T cells. *B*, Cytokine production by splenocytes from PBS-fed (■) or OVA-fed (□) recipient mice. IL-2, TNF-α, TGF-β, and IL-10 levels were measured by ELISA in supernatants collected 4 days after in vitro stimulation with CBA and OVA for 4 days. Data represent mean ± SEM of two experiments. *, $p < 0.05$.

The cells from spleens and MLN of these animals were isolated, and percentages of CD4⁺ T cells and CD4⁺KJ1-26⁺ T cells were determined by FACS analysis (Table III). Although equal numbers of BALB/c and DO11.10 CD4⁺CD45RB^{high} T cells were injected into the recipient mice, there were only $6.98 \pm 0.67\%$ of CD4⁺ T cells recovered from spleen and $13.8 \pm 1.76\%$ CD4⁺ T cells recovered from MLN expressing the Tg marker KJ1-26, indicating that the non-Tg BALB/c mice-derived CD4⁺ T cells had expanded preferentially in the recipients. However, the marked decrease in total CD4⁺ T cells in both spleen and MLN after OVA feeding was not due to Ag-specific deletion of OVA-specific T cells, because total CD4⁺KJ1-26⁺ T cells in both spleen and MLN were similar between PBS-fed and OVA-fed SCID-bg mice reconstituted with same number of mixed CD4⁺CD45RB^{high} T cells (Table III). Therefore, the ability of OVA feeding to diminish colitis in mice reconstituted simultaneously with non-Tg BALB/c and

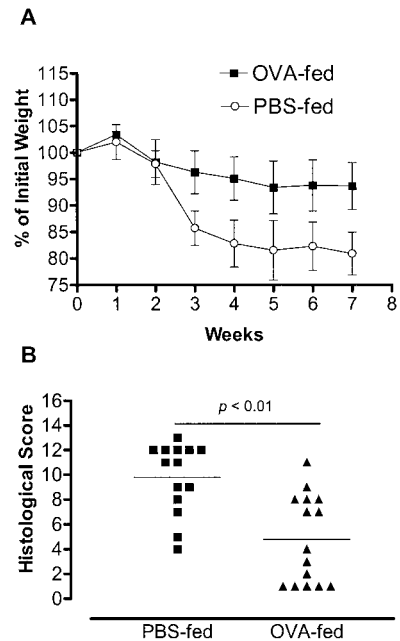


FIGURE 7. OVA feeding prevented the development of colitis in SCID-bg mice reconstituted with the combination of both DO11.10- and BALB/c-derived CD4⁺CD45RB^{high} T cells. *A*, Eight-week-old female SCID-bg mice were injected (i.p.) with CD4⁺CD45RB^{high} T cells from both DO11.10 and BALB/c mice (1:1 ratio; total, 4×10^5 cell/mouse). Half of the recipients were fed OVA (1 mg/ml) in drinking water. Weight changes are expressed as the mean percentage of initial body weight ± SEM. The differences in body weight between OVA-fed and PBS-fed groups were significant ($p < 0.05$). Results are representative of three independent experiments each with four to five mice per group. *B*, The intestinal pathology was analyzed at time of sacrifice as described in *Materials and Methods*. Data represent the histological score of individual mice. The bar represents the mean score for the group. *, $p < 0.05$, OVA-fed group vs PBS-fed group.

OVA-Tg CD4⁺CD45RB^{high} T cells suggests that OVA feeding induced the development of a Treg cell from the OVA-reactive Tg CD45RB^{high} T cells.

Discussion

Modulating T cell function in an effort to alter chronic inflammatory processes such as IBD requires understanding the requirements for Ag-specific and nonspecific activation of these cells. In this study we used Ag-specific Tg T cells in the SCID-transfer colitis model to examine the role of specific Ag and TCR interactions in the induction and prevention of intestinal inflammation. The data presented in this report demonstrate that naive Tg DO11.10 T cells cause colitis via activation through nonclonotypic TCR, and that activation of Treg cells through Ag-specific TCR interactions can inhibit proinflammatory effector T cell responses to unrelated Ag.

This study is the first to demonstrate that naive nonactivated DO11.10 CD45RB^{high}CD4⁺ T cells can cause colitis in SCID-bg mice in the absence of the specific Ag, OVA. This finding implies that the effector T cells were stimulated through a nonclonotypic TCR. Because CD45RB^{high} induction of colitis is dependent on the presence of gut flora (1, 11), it is reasonable to speculate that gut flora-derived Ag stimulated the relevant effector T cells through non-Tg TCR. This conclusion is supported by previous studies showing that naive CD4 T cells (presumably all are CD4⁺CD45RB^{high}) from DO11.10 mice backcrossed onto RAG-deficient background failed to induce colitis (6), and that enteric

Table III. Attenuation of disease in mixed CD45RB^{high} recipients by OVA feeding correlates with decreased total CD4⁺ T cells but not OVA-Tg CD4⁺ T cells

| | Cell Yield ($\times 10^6$) | CD4 ⁺ T Cells | | CD4 ⁺ KJ1-26 ⁺ T Cells | |
|----------------------|---------------------------------|--------------------------|-------------------------|--|-------------------------|
| | | % ^a | Total ($\times 10^6$) | % ^b | Total ($\times 10^4$) |
| Spleen | | | | | |
| PBS-fed ($n = 9$) | 36.5 \pm 7.3 | 5.66 \pm 0.72 | 1.81 \pm 0.35 | 6.98 \pm 0.67 | 11.13 \pm 1.37 |
| OVA-fed ($n = 10$) | 20.6 \pm 3.2 | 4.78 \pm 0.77 | 1.01 \pm 0.17* | 15.3 \pm 5.90* | 8.50 \pm 1.25 |
| MLN | | | | | |
| PBS-fed ($n = 9$) | 2.55 \pm 0.28 | 21.5 \pm 4.17 | 0.59 \pm 0.14 | 13.8 \pm 1.76 | 6.72 \pm 1.10 |
| OVA-fed ($n = 10$) | 1.53 \pm 0.30 | 19.3 \pm 5.470 | 0.27 \pm 0.07* | 29.5 \pm 7.76* | 6.78 \pm 1.68 |

^a The percentage of CD4⁺ T cells in total cells was determined by FACS analysis. Data represent the mean plus SE of two independent experiments.

^b The percentage of KJ1-26⁺ T cells in CD4⁺ T cells was determined by FACS analysis. Data represent the mean plus SE of two independent experiments.

* $p < 0.05$, OVA-fed compared with PBS-fed groups.

bacteria can activate OVA-specific TCR Tg T cells in DO11.10 mice through a nonclonotypic TCR (21, 24). Indeed, we showed that the majority of OVA Tg CD4⁺CD45RB^{high} T cells transferred into SCID-bg mice become activated and replace their CD45RB^{high} phenotype with expression of the activation/memory phenotype, CD45RB^{low}, and express non-Tg V α chains. Therefore, these T cells express more than one TCR, i.e., one clonotypic TCR with both the Tg V α 13.1 and V β 8.2 chains (KJ1-26⁺) and a second TCR with the Tg V β 8.2 chain associated with a non-Tg V α chain.

The ability of T cells to express a dual TCR is not limited to these Tg mice in that T cells expressing dual TCR have been described in non-Tg rodents as well as in humans (23, 25). Dual-TCR-bearing T cells are generated in the thymus (26–28) and may contribute to the induction of autoimmunity (29, 30) and to the generation of TCR repertoire diversity (31). In addition, there are case descriptions of clonal expansion of dual-TCR-expressing T cells in patients with IBD, HIV infection, and T cell malignancies (32, 33). In spontaneous murine models of colitis in TCR Tg lymphopenic mice, T cells with dual α -chains paired with Tg-encoded β -chain were increased in the inflamed colons but not on noninflamed colon, suggesting polyclonal T cell activation (34). Thus, dual-TCR T cells may have an important role in the pathogenesis of IBD through immune recognition of cross-reactive and triggering Ags such as found in normal gut flora Ag. The question of why these cells respond to normal enteric flora in mice remains to be addressed.

The data presented in this report also showed that cotransfer of DO11.10 CD45RB^{low} T cells into SCID-bg mice prevented colitis-induced DO11.10-derived CD4⁺CD45RB^{high} T cells in the absence of the specific Ag, OVA. Therefore, Treg cells functioned independent of cognate Ag and influenced T cells that do not share Ag specificity. This finding raises the possibility that Treg T cells can also be activated through nonclonotypic TCR. The significant proportion of OVA Tg CD4⁺CD45RB^{low} T cells that express a second TCR chain, together with the finding that >50% of dual-TCR-bearing T cells express CD4⁺CD45RB^{low} phenotype in BALB/c mice, suggests that naturally occurring Treg cells maintain an extended TCR repertoire and are readily responsive to a wide range of self or nonself Ag. We cannot exclude the possibility of that Treg cell activation can occur through Ag-nonspecific polyclonal pathways or even via TCR-independent activation pathways such as LPS-Toll-like receptors interactions (35).

Dual-TCR expression on naturally occurring Treg cells might reflect the importance of endogenous TCR α -chains in the development of immune tolerance. This would help explain the development of colitis in TCR α -chain-deficient mice (36, 37), as well as in other TCR Tg mice backcrossed onto RAG-2-deficient mice

(34, 38, 39). Loss of the TCR α -chain was associated with the concomitant loss of suppressor activity of Ag-specific T suppressor cell lines (40).

The presence of T cells expressing two TCRs sets up a scenario where a specific Ag, e.g., OVA, influences T cell responses to non-OVA Ag. This is supported by the finding that feeding low-dose OVA to SCID-bg mice transferred with DO11.10 CD45RB^{high} T cells attenuated the development of colitis. This finding is analogous to the results described by Fossati et al. (41) who showed that triggering a second TCR on diabetogenic T cells prevented the induction of diabetes. OVA feeding was not associated with deletion of Tg T cells but was associated with suppression of T cell responses, i.e., a decrease in proliferation and proinflammatory cytokine, and increase in suppressive cytokines such as TGF- β , responses to both OVA and CBA stimulation in vitro. Therefore, OVA feeding not only induced tolerance in OVA-specific TCR-carrying T cells, but also tolerized responses to unrelated gut flora Ag. In addition, the finding that feeding OVA diminished colitis in mice reconstituted with both DO11.10- and BALB/c-derived CD45RB^{high} T cells indicates that OVA feeding induced a Treg cell that influenced both Tg and BALB/c effector T cells. In support of our argument, total CD4⁺KJ1-26⁺ T cells numbers recovered from MLN and spleen from both OVA-fed and PBS-fed recipients (BALB/c plus DO11.10 CD4⁺CD45RB^{high}) were similar, excluding the possibility of Ag-specific deletion. Therefore, feeding Ag induced cytokines associated with previously described Treg cells. These findings also suggest that Treg cells are involved in suppressing effector T cells that do not share Ag specificity.

The studies described in this report demonstrate that adoptive transfer of DO11.10 T cells into SCID-bg mice allowed us to follow Ag-specific T cell responses during the induction and regulation of colitis. Others using DO11.10-derived T cells showed that in vitro-generated DO11.10 Tr1 cells inhibited CD45RB^{high}-induced colitis but only when recipients were fed OVA (14), suggesting that the suppressive function of Tr1 clones inhibited the function of effector T cells responding to unrelated Ags. In contrast, OVA-specific Tr1 clones failed to prevent spontaneous autoimmune encephalomyelitis in myelin basic protein-specific Tg mice with a disrupted *RAG-1* gene, even in the presence of OVA Ag (39). The reasons for this discrepancy are not clear but may reflect the fact that these Tr1 clones were preactivated in vitro by OVA peptide, which may eliminate T cells expressing non-Tg TCR, thereby limiting their TCR repertoire diversity.

In conclusion, we demonstrated that the OVA TCR Tg CD4⁺ T cells, with their ability to express a second TCR, can act as both effector and Treg cells in the initiation and modulation of T cell-mediated colitis independent of cognate Ag. In addition, low-dose

OVA feeding induced T cell hyporesponsiveness to both OVA and CBA and attenuated the mucosal inflammation induced by OVA-specific TCR Tg T cells. More importantly, OVA-induced tolerance suppressed BALB/c CD45RB^{high} T cell-induced colitis. The fact that regulatory function can be induced directly from naive T cells by a model Ag unrelated to the Ag driving the colitis opens the possibility of new therapeutic approaches in this disease.

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