Disturbance of T-cell homeostasis could lead to intestinal inflammation. Naive CD4 T cells undergoing spontaneous proliferation, a robust proliferative response that occurs under severe lymphopenic conditions, differentiate into effector cells producing Th1- and/or Th17-type cytokines and induce a chronic inflammation in the intestine that resembles human inflammatory bowel disease. In this study, we investigated the key properties of CD4 T cells necessary to induce experimental colitis. α4β7 upregulation was primarily induced by mesenteric lymph node (mLN) resident CD11b+ dendritic cell subsets via transforming growth factor beta (TGFβ)/retinoic acid-dependent mechanism. Interestingly, α4β7 expression was essential but not sufficient to induce inflammation. In addition to gut-homing specificity, expression of gut Ag specificity was also crucial. T-cell acquisition of the specificity was dramatically enhanced by the presence of γδ T cells, a population previously shown to exacerbate T-cell-mediated colitis. Importantly, interleukin (IL)-23-mediated T-cell stimulation was necessary to enhance colitogenicity but not gut antigen reactivity of proliferating CD4 T cells. These findings demonstrate that T-cell colitogenicity is achieved through multiple processes, offering a therapeutic rationale by intervening these pathways.

**Keywords:** α4β7 integrin; CD4 T cells; colitis; gut antigens; IBD

Immune reactions at the intestinal mucosal interface are tightly regulated not only to prevent unnecessary activation of immune cells against innocuous commensal antigens (Ags) but also to mount protective immune responses to pathogen-associated Ag. Dysregulation of the processes is thought to lead to chronic inflammation seen in inflammatory bowel disease (IBD). However, what initiates the inductive processes and the precise features of effector T cells that mediate the inflammation remain unclear.

Experimentally, T-cell-mediated experimental colitis is a widely used murine model to study pathogenesis of IBD. Naive CD4 T cells transferred into lymphopenic recipients undergo spontaneous proliferation and concomitantly differentiate into effector cells producing proinflammatory cytokines, mainly interferon (IFN)γ and interleukin (IL)-17. The proliferation is induced in response to Ag derived from commensal organisms as well as self. Gut-draining mesenteric lymph node (mLN) is a major site where the proliferation takes place and potential colitogenic T cells are generated. Expression of gut-homing α4β7 integrin, also known as the lymphocyte Peyer’s patch adhesion molecule,thought be to be an essential step for effector cells to enter the intestinal mucosa. It was reported that dendritic cells (DCs) from the gut-draining lymphoid tissues including mLN and Peyer’s patches, or from small intestine lamina propria, have a key role in generating gut-tropic effector cells. The vitamin A metabolite, retinoic acid (RA), has an additional role in upregulating α4β7 on T cells. Among DCs in the mLN, CD103+ subsets were shown to express retinal dehydrogenase 2, a key enzyme to synthesize RA, and to enhance gut-homing-molecule expression in activated T cells. However, mechanisms underlying the generation of gut Ag-specific colitogenic T cells in vivo remain unclear.

Both Th1 and Th17-type effector cells have been implicated in the pathogenesis of intestinal inflammation. We previously reported that γδ T cells have an additional role in generating colitogenic T cells in part by enhancing Th17 differentiation. Thus, lymphopenic mice harboring γδ T cells were highly susceptible, whereas lymphopenic mice deficient in γδ T cells were resistant to the disease despite the fact that the overall T-cell proliferation and expansion were comparable. Particularly interesting is that IL-17 production of γδ T cells appears to be directly linked to the Th17-promoting functions. IL-17-producing γδ T-cell subsets are phenotypically distinct from IFNγ-producing γδ T-cell subsets. They express receptor for IL-23, a cytokine known to stimulate γδ T cells to produce IL-17. Whether IL-23 is important for Th17-promoting
γδ T-cell functions during T-cell-induced colitis has not formally been tested.

Here, we demonstrate that gut-homing proinflammatory effector cells are preferentially generated within the gut-draining mLN via CD11b⁺ DCs. Expression of the gut-homing molecule, α4β7 integrin, was necessary to induce inflammation, although α4β7 expression itself was not sufficient to do so. Both T-cell reactivity against fecal extract Ag and proinflammatory cytokine production were essential features of colitogenic T cells, and the reactivity was enhanced by the presence of γδ T cells stimulated by IL-23. Our results highlight multiple pathways through which Th17-type colitogenic cell generation is regulated.

**RESULTS**

**CD4 T cells undergoing spontaneous proliferation upregulate α4β7 integrin expression in the mLN**

α4β7 integrin expression is essential for activated T cells to enter the gut tissues. Targeting α4β7-mediated trafficking has thus been an efficacious approach to attenuate intestinal inflammation in IBD as well as graft-versus-host diseases. Using a model of spontaneous T-cell proliferation, a robust T-cell response that occurs under lymphopenic conditions leading to a chronic intestinal inflammation, we examined the role of α4β7 expression in activated T cells during T-cell-mediated intestinal inflammation. Naïve CD4 T cells were transferred into T-cell-deficient T-cell receptor (TCR)β−/− recipients and examined for α4β7 expression. Consistent with the previous results, α4β7⁺ CD4 T cells were primarily generated in the gut-draining mLN, whereas the proportion of α4β7⁺ cells in the peripheral skin-draining peripheral LN (pLN) was very low (Figure 1a). Preferential accumulation of α4β7⁺ T cells in the mLN was still pronounced 3 weeks post transfer (data not shown). As activated T cells recirculate, we treated the recipients with FTY720, a Sl1P receptor agonist that blocks lymphocyte egress from the lymphoid tissues, to localize the precise location where activated T cells acquire α4β7 expression in vivo. TCRβ−/− mice were treated daily with FTY720 starting at ~16 h after T-cell transfer. A 16-h lag was given to allow the T cells to engage with Ag-presenting cells (APCs) to receive proper stimulation signals. As shown in Figure 1b, FTY720 treatment further increased the accumulation of α4β7⁺ T cells in the mLN, whereas their numbers in the pLN significantly decreased, a finding consistent with a study recently reported by Ishii and colleagues. Therefore, α4β7 acquisition of activated T cells mainly occurs in the mLN, suggesting that APCs residing within the mLN preferentially imprint gut-homing specificity by upregulating α4β7. Of note, the extent of T-cell proliferation between the two sites (that is, pLN versus mLN) was comparable, indicating that the differential expression of α4β7 in T cells is not because of the level of T-cell activation (not shown).

**CD11b⁺ DCs within the mLN upregulate α4β7 via transforming growth factor beta (TGFβ) and RA-dependent mechanism**

To directly examine whether APCs residing within the mLN are responsible for α4β7 upregulation, whole pLN and mLN cells isolated from TCRβ−/− mice were used as APCs to stimulate ovalbumin (OVA)-specific OT-II CD4 T cells with OVA peptide in vitro. Consistent with the in vivo results (Figure 1), cells from mLN were highly efficient in generating α4β7⁺ OT-II T cells (Figure 2a). Specifically, we noticed that adding recombinant TGFβ alone significantly increased α4β7 upregulation (~30%, Figure 2a), which was further increased to ~50% by adding TGFβ and IL-6 (Figure 2a). The generation of α4β7⁺ T cells without these cytokines was very low (Figure 2a). Interestingly, cells from the pLN were still unable to generate α4β7⁺ OT-II T cells in the presence of both TGFβ and IL-6 (Figure 2a). T-cell proliferation and CD44 upregulation were comparable between the conditions, indicating that the differential α4β7 expression is not because of activation status. Importantly, T-cell production of IL-17 was efficiently induced regardless of the origin of APCs (data not shown), indicating that the pLN APCs are functionally equivalent to the mLN APCs in activating Ag-specific T cells. mLN cells from TCRβ−/− and Rag−/− mice were equivalent in upregulating α4β7 expression in cocultured OT-II cells, suggesting that B cells are dispensable (Figure 2b). Vitamin A metabolite RA has been shown to be critical in inducing α4β7 expression in activated T cells. Consistent with this, adding RA receptor antagonist LE540 completely abolished the α4β7 expression (Figure 2c), suggesting that RA produced by mLN DCs has a key role in mLN APC-mediated expression of α4β7. The level of overall T-cell activation was comparable in these conditions (data not shown). We set out to further examine whether there are specific APC subsets among the mLN cells highly specialized in inducing α4β7 expression. Different DC subsets from the mLN were thus isolated and cocultured with OT-II cells. We found that CD11b⁺ DCs were the major cell type inducing α4β7 expression (Figure 2d). On the other hand, CD11b⁺ macrophages and other DC subsets including CD8⁺ DCs or CD11b⁻ CD8⁻ DCs were unable to upregulate α4β7 (Figure 2d). It was previously reported that gut-homing α4β7⁺ CD8 T cells are preferentially generated by CD103⁺ DCs but that induction of α4β7⁺ on CD4 T cells is equally induced by both CD103⁺ and CD103⁻ DCs. When CD103 expression of different mLN DCs was compared, the proportion of CD103⁺ DCs was comparable between the subsets (Figure 2e). Therefore, CD11b⁺ DC subsets appear to be a unique population that induces gut-homing specificity during spontaneous proliferation.

![Figure 1](https://via.placeholder.com/150)

**Figure 1** α4β7 expression on CD4 T cells was enhanced in mesenteric lymph node. (a) α4β7 expression on CD4 T cells on day 5 following CD4 T cells’ adoptive transfer to TCRβ−/− mice. Data shown are representative of individually tested recipients. All experiments were repeated more than three times and similar results were observed. (b) Total number of α4β7⁺ CD4 T cells from the indicated organ after FTY720 treatment. Data shown are the mean ± s.d. of six mice in two independent experiments. **P<0.01; ***P<0.001.
Indeed, α4β7 expression in T cells was directly associated with colitogenic potential. α4β7+ or α4β7− CD4 T cells were isolated from the mLN of TCRβ+/− mice that had received CD4 T cells 3 weeks earlier and subsequently transferred into naive TCRβ+/− recipients. α4β7+ T-cell recipients exhibited severe weight loss and colonic inflammation, whereas α4β7− T-cell recipients did not show any signs of weight loss and intestinal inflammation (Figures 3a and b). Consistent with this, the accumulation of α4β7+ T-cell subsets in the mLN and colon lamina propria was greater than that of α4β7− cells (Figure 3c), confirming the importance of α4β7 expression in T-cell entry to the intestine. The accumulation of IL-17+ donor T cells was significantly higher in both mLN and colon lamina propria of α4β7+ T-cell recipients (Figure 3c). Notably, Il17a expression of α4β7+ and α4β7− T cells was comparable when measured prior to transfer (Figure 3d). These results suggest that the different susceptibility to the inflammation of α4β7+ and α4β7− T cells is not associated with Th17-effector phenotypes of the donor T cells.

As T-cell-induced colitis in T cells is attenuated in germ-free conditions, gut-homing colitogenic T cells are likely reactive to commensal Ag. We thus examined gut Ag reactivity of TCRβ+/− recipient T cells isolated from the mLN and pLN of each group and transferred into naive TCRβ+/− mice (Figure 4a), indicating that γδ T cells are not necessary for the generation of gut-homing effector cells. Consistent with our previous findings, α4β7+ T cells isolated from TCRβ+/− mice highly expressed Th17 type cytokines compared with the same phenotype cells isolated from TCRβδ−/− mice (Figure 4b). Other cytokines tested including IL-21, tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor and IFNγ were similarly expressed (Figure 4b). Therefore, T cells primed in the presence of γδ T cells better become IL-17-producing effector cells. α4β7+ T cells were then isolated from the mLNs of each group and transferred into naive TCRβ+/− mice. α4β7+ T cells isolated from TCRβ+/− mice induced severe weight loss as shown above, whereas α4β7− T cells isolated from TCRβδ−/− mice were unable to induce noticeable weight loss (Figure 4c). The colon tissue of TCRβδ−/−-originated α4β7+ T-cell recipients was heavily infiltrated with inflammatory cells, whereas that of TCRβδ−/−-originated α4β7−
independent experiments. *P<0.05; **P<0.01. (c) Absolute number of the total cells and IL-17-producing donor T cells from the indicated organ are shown. Data are mean±s.d. Experiments were repeated twice and similar results were observed. *P<0.05; **P<0.01. (d) Real-time quantitative PCR analysis of il17a expression on α4β7+ and α4β7− CD4 T cells prior to the transfer. All samples were performed in duplicates and normalized to GAPDH. Data shown are representative from six to seven individually tested mice. (e) α4β7+ and α4β7− donor T cells were re-isolated from the mLN and pLN, CFSE-labeled and cocultured with APCs pulsed with fecal extract Ag as described in Methods. CFSE dilution was determined using FACS analysis. Experiments were repeated twice, and similar results were obtained.

T-cell recipients displayed only mild inflammation (Figure 4d). Consistent with the disease progression and intestinal inflammation, donor cell accumulation in both lymphoid and intestinal tissues were significantly greater in recipients of TCRβ−/−-derived α4β7+ T cells (Figure 4e). Likewise, the level of IL-17-producing T cells in the mLN and colon was significantly higher in TCRβ−/−-originated α4β7+ T-cell recipients (Figure 4f).

γδ T cells directly promote the generation of gut Ag-reactive effector cells

Our results demonstrate that α4β7+ cells generated without γδ T cells do not express Th17-type cytokines and induce colitis, suggesting a possibility that the lack of Th17-type cytokine expression could be the main reason that these cells are non-colitogenic. Alternatively, γδ T cells may control T-cell acquisition of gut Ag reactivity. To test this possibility we harvested α4β7+ T cells from TCRβ−/− and TCRβδ−/− recipients 3 weeks post transfer and cocultured with splenic DCs pulsed with fecal extracts as demonstrated in Figure 3e. CD4 T cells harvested from TCRβ−/− mice dramatically proliferated when cocultured with APCs pulsed with fecal extract Ag, whereas T cells harvested from TCRβδ−/− mice failed to proliferate in the same conditions (Figure 5a). T-cell proliferation was completely blocked by anti-MHCII (Y3P) monoclonal antibody (Figure 5a). The proliferating T cells produced IL-17A (Figure 5b) and IFNγ (data not shown). Therefore, γδ T cells may enhance the generation of gut Ag-reactive colitogenic cells.

To directly test this possibility in vivo, we adoptively transferred purified lymphoid γδ T cells (isolated from the secondary lymphoid tissues) into TCRβδ−/− mice. Fluorescence-activated cell sorting (FACS)-purified naive CD4 T cells were then transferred into the recipients 7 days post γδ T cell transfer as illustrated in Figure 5c. The donor CD4 T cells were subsequently isolated from the mLN of recipients 7 days following CD4 T cells transfer and cocultured with fecal extract Ag-pulsed APCs. A control group of TCRβδ−/− mice received naive CD4 T cells without γδ T-cell transfer (Nil group in Figure 5c), and CD4 T cells were used for coculture experiments. CD4 T cells activated in the presence of γδ T cells transferred 7 days earlier exhibited a significant gut Ag reactivity based on CFSE dilution (Figure 5d) as well as IL-17 production (Figure 5e). By contrast, CD4 T cells activated without γδ T cells did not proliferate or produce IL-17 (Figures 5d and e). Therefore, γδ T cells appear to directly enhance the generation of gut Ag-reactive IL-17-producing colitogenic T cells in vivo.

IL-23-mediated stimulation of γδ T cells is important for Th17 differentiation but not for gut Ag reactivity

γδ T cells express IL-23 receptor29,30 and are the first cells to respond to IL-23 during experimental autoimmune encephalomyelitis (EAE).13 IL-23 induces IL-17 production by γδ T cells, amplifying proinflammatory Th17-type immune responses.15,31 Whether IL-23-induced γδ T-cell activation is needed to support colitogenic T-cell development was thus examined. TCRβδ−/− mice received naive CD4 T cells together with wild-type or IL-23R−/−
The presence of Th17 differentiation such as IL-17 is associated with weight loss and heightened expression of gut Ag reactivity of CD4 T cells. We performed adoptive transfer experiments described in Figure 5c using IL-23R−/− and IL-17A−/− γδ T cells. As demonstrated in Figure 6e, CD4 T cells activated with wild-type γδ T cells underwent significant proliferation as determined with CFSE dilution. Likewise, CD4 T cells activated with IL-23R−/− or IL-17A−/− γδ T cells, although they failed to induce colitis, displayed similar gut Ag reactivity (Figure 6e). Consistent with the clinical score, the colon of IL-17A−/− γδ T cells was heavily infiltrated with inflammatory cells, whereas those recipients of CD4 T cells alone or together with IL-23R−/− γδ T cells exhibited only a mild inflammation (Figure 6f).

**DISCUSSION**

During spontaneous proliferation, colitogenic effector T cells are generated and severe intestinal inflammation develops. However, the precise features for effector T cells to be colitogenic have not been formally explored. The present study aims to examine characteristics of colitogenic T cells and mechanisms by which they are generated.
z4β7-dependent gut trafficking is essential for colitogenicity. Consistent with a recent report,20 T-cell upregulation of z4β7 gut-homing integrin was only noted in the gut-draining mLN, whereas T cells similarly proliferating in the pLN failed to do so. The upregulation was TGFβ/RA-dependent. The finding that the upregulation is most pronounced when T cells are stimulated by CD11b+ DCs strongly suggests that these DC subsets may be the source of TGFβ and/or RA. It was recently reported that IRF4-dependent CD103+CD11b+ DCs represent the major migratory DCs in the lamina propria that drive Th17 differentiation,32 although no correlation between CD103 expression and DC functions to upregulate z4β7 expression was observed from the current study. Whether CD103+CD11b+ DCs and CD103–CD11b+ DCs function differently in upregulating z4β7 expression and generating colitogenic T cells remains to be examined. It is interesting to note that z4β7 expression on T cells within the draining mLN is not associated with effector phenotype, Th17. When IL-17 expression of CD4 T cells that do or do not acquire z4β7 expression was compared, both populations were equally capable of expressing IL-17, indicating that Th17 differentiation during spontaneous proliferation is independent of z4β7 expression.

Differential ability for z4β7+ T cells to become colitogenic may be linked to their Ag reactivity. We directly tested this question by ex vivo coculturing in vivo-activated effector cells with APCs pulsed with fecal extract Ag. Supporting the hypothesis, z4β7+ T cells generated within the mLN strongly reacted to gut Ag stimulation, whereas z4β7- T cells generated in the same mLN failed to respond to the stimulation despite that their Th17 differentiation is not affected by the lack of z4β7 expression. On the basis of these findings, we would argue that there are CD11b+ DC subsets preferentially presenting colitogenic gut Ag to naïve T cells and providing necessary signals to generate gut-tropic effector cells.33 Indeed, we observed that after intraluminal Ag administration, mLN DC subsets that present the Ag were of CD11b+ subsets (Freeman and Min, unpublished observation). Of note, Matsuda et al.34 previously found a highly restricted repertoire diversity of T cells that expanded within immunodeficient mice, concluding that the expansion of T cells requires the activation of Ag-specific T cells. The concept of Ag-specific colitogenic T-cell responses agrees with the gut Ag reactivity reported in this study. When we compared TCRβ repertoire diversity of T cells generated with or without γδ T cells using FACS analysis, we found that the TCR Vδ distribution between the conditions was indistinguishable (Do and Min, unpublished observation). Sequence analysis of responding cells may be necessary to directly compare their clonal diversity.

Of note, gut-homing specificity alone was not sufficient to be colitogenic for those effector T cells generated during spontaneous proliferation. Following naive T-cell transfer, z4β7+ T cells were equally generated within the mLN of TCRβ-/- recipients, a condition resistant to T-cell-mediated colitis.3 When isolated and retransferred into naive susceptible TCRβ-/- recipients, z4β7+ T cells generated within TCRβ-/- mice remained non-colitogenic, which was in good contrast to z4β7+ T cells generated within TCRβ+/-- mice. Therefore, gut-homing T cells generated in susceptible and resistant lymphopenic conditions appear to be distinct, particularly in regard to Ag specificity, as T-cell reactivity to commensal Ag is thought to be essential for colitogenicity.4,35 In support with this, z4β7+ T cells generated within susceptible TCRβ-/- mice displayed a strong reactivity to ex vivo stimulation with fecal extract Ag, whereas z4β7+ T cells generated within resistant TCRβ-/- mice did not exhibit such reactivity. Therefore, resistance versus susceptibility may be determined at the level of Ag specificity of spontaneously proliferating T cells.

A mechanism behind the induction of effector cells with different Ag specificity is unclear. It is possible that Ags presented possibly by CD11b+ DCs may be different depending on the recipients. Alternatively, stimulatory functions of DCs to prime gut Ag-specific T cells could be different in these conditions. As the presence of γδ T cells is the major difference between these conditions, we propose that γδ T cells may be responsible for enhancing gut Ag uptake and/or migration of gut Ag-bearing APCs to the draining mLN. Indeed, γδ T cells pretransferred into TCRβ-/- mice were sufficient to restore gut Ag specificity of proliferating T cells and colitogenicity. How γδ T cells exert such immunoregulatory functions remains to be determined. It was previously reported that γδ T cells can promote DC maturation via CD40L–CD40 interaction.36 It will be interesting to examine whether such cell-to-cell interaction operates in this setting.

It is important to emphasize that IL-23 stimulation of γδ T cells has an important role in this process. This is based on the observation that CD4 T cells activated in the presence of γδ T cells deficient in IL-23R fail to restore colitis. Interestingly, however, the roles for γδ T cells in inducing gut Ag-reactive T cells appear to be dissociated from the role for enhancing the generation of colitogenic Th17-type cells. When IL-23R--/- γδ T cells are transferred into TCR β-/- mice, gut Ag reactivity is efficiently acquired, although both Th17 differentiation and colitogenicity are not observed. IL-23 is highly expressed on IL-17-producing γδ T-cell subsets,3 and IL-23 stimulation induces γδ T-cell IL-17 production.13,31 However,
IL-17A produced by activated γδ T cells is not involved in this process, as both Th17 differentiation and colitis induction efficiently occurred in IL-17A-/- γδ T-cell recipients. We previously reported that only CCR6+ but not CCR6- γδ T cells are capable of promoting Th17 differentiation and colitis.3 CCR6+ γδ T cells produce both IL-17A and IL-17F. Moreover, IL-23 stimulation of γδ T cells induces the production of both IL-17A and IL-17F (data not shown). Therefore, IL-17F produced by activated γδ T cells may mediate such regulatory functions. It is also possible that IL-17A (or IL-17F) produced by γδ T cells also directly or indirectly supports APC functions to enhance gut Ag-reactive T-cell generation.

Overall, we identified that T-cell expression of the gut-tropic adhesion molecule α4β7, gut Ag specificity and Th1/Th17 phenotype are required to mediate severe colitis and that γδ T cells have a crucial role in this process in part by IL-23. Future investigation should focus on a cellular pathway to develop a better therapeutic strategy to intervene chronic intestinal inflammation such as IBD.

METHODS

Mice
C57BL/6, C57BL/6-Rag1-/-, CD45.1 C57BL/6, C57BL/6 TCRβ-/- and C57BL/6 TCRβδ-/- mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). CD45.1 C57BL/6-RAG1-/- OT-II mice were kindly provided by Dr William Paul (NIH). IL-23R-/- and IL-17A-/- mice were previously reported.25,26 All the mice were maintained under specific pathogen-free facility located in the Lerner Research Institute. All animal experiments were performed in accordance with the approved protocols for the Cleveland Clinic Foundation Institutional Animal Care and Usage Committee.

Cell sorting and colitis induction
Whole LN naive CD4 T cells were obtained as previously reported.29 In brief, LN cells (axillary, cervical, inguinal and mesenteric LN) were pooled and total T cells were negatively purified through a magnetic separation. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-B220, anti-CD44, anti-F4/80, anti-CD11c and anti-CD11b antibodies (BD Biosciences, San Jose, CA, USA). CD4+CD25+CD44low naive T cells were further sorted using a FACS Aria cell sorter (BD Biosciences, San Jose, CA, USA). A total of 2.5 x 10^5 naive CD4 T cells were transferred to naive OT-II mice. After T-cell transfer, mice were weighed weekly and monitored for signs of disease. In some experiments, α4β7+ CD4 T cells were sorted from TCRβδ-/- or TCRβδδ-/- recipients on days 7 and 21 after transfer and transferred to naive TCRβδδ-/- recipients for colitis induction. γδ T cells were isolated from the secondary lymphoid tissues (LN and spleen) by cell sorting and adoptively transferred (2 x 10^6 cells) where indicated.

In vitro OT-II T-cell stimulation
Ovalbumin peptide-specific TCR transgenic CD4 T cells were obtained from CD45.1 OT-II TCR Tg Rag-/- mice. A total of 2 x 10^5 naive OT-II T cells were cultured with 2 x 10^6 APCs in the presence of 1 µg ml^-1 OVA peptide.23,25,26 A total of 5 ng ml^-1 human rTGF-β1 (Peprotech, Rocky Hill, NJ, USA) and 10 ng ml^-1 rIL-6 (Peprotech) were added in the culture. Cultures...
were incubated for 5 days and cells were analyzed for 4β expression. In some in vitro experiments, 1 μM RA receptor antagonist LE540 (Wako Chemical, Richmond, VA, USA) was added.

**Fecal Ag stimulation**

Fecal extract Ag was prepared from C57BL/6 TCRb/b mice as described in previous study. Sorted 4β+ CD4 T cells were cultured with CFSE. T cells were cultured with 0.3-mg fecal Ag-pulsed APC at a 1:1 ratio. After 5 days, CFSE dilution was assessed using FACS.

**FACS analysis**

Cells were stained with anti-CD4 (RM4-5), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD8 (53–6.7), anti-IL-17a (Ebio717), anti-IFNγ (XM1G2.2), anti-IL-17 (AF6-120.1), anti-CD45 (120), anti-CCR9 (ebioCW-12) and anti-4β (DTAK2X) (all Abs from e Bioscience). Cells were acquired using a FACS Calibur or LSR II (BD Biosciences) and analyzed using a FlowJo software (Treestar, Ashland, OR, USA). For Intracellular staining, cells were separately harvested and ex vivo-stimulated with PMA (10 ng ml⁻¹) and ionomycin (1 μM) for 4 h in the presence of 2 μM monensin (Calbiochem, San Diego, CA, USA) during the last 2 h of stimulation. Cells were immediately fixed with 4% paraformaldehyde, permeabilized and stained with fluorescence-conjugated antibodies.

**Real-time quantitative PCR**

FACS-sorted cells or colon tissues were disrupted using a TissueLyser II (Qiagen). cDNA was subsequently synthesized using a SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed using gene specific primers and probe sets (Applied Biosystem, Foster City, CA, USA) and ABI 7500 PCR machine (Applied Biosystem).

**Histology**

Colon tissues were fixed in 10% acidic acid/60% methanol. Slides were cut and stained with hematoxylin and eosin. Colon tissues were scored in a blinded manner as previously reported by two individuals and scores were averaged. In brief, colon tissues were assessed at both low and high magnification to get an overall score using the scoring system: 0: no sign; 1: low infiltration and inflammation; 2: medium infiltration/inflammation; 3: high infiltration/inflammation; 4: severe infiltration with moderate loss of goblet cells and crypt structure; 5: transmural infiltration, loss of goblet cells and crypt structure.

**Statistical analysis**

Statistical significance was determined using the Student’s t-test using the Prism 5 software (GraphPad, La Jolla, CA, USA). A P-value of <0.05 was considered statistically significant.

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