

An Oral CD3-Specific Antibody Suppresses T-Cell-Induced Colitis and Alters Cytokine Responses to T-Cell Activation in Mice

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BACKGROUND & AIMS: New therapeutic approaches are needed for inflammatory bowel diseases. A monoclonal antibody against CD3 (anti-CD3) suppresses T-cell-mediated autoimmune diseases such as experimental allergic encephalomyelitis. We explored the effects of anti-CD3 in mice with colitis. **METHODS:** Severe combined immunodeficient mice were given injections of CD4⁺CD45RB^{high} T cells to induce colitis. Four weeks later, the mice were given 2 or 5 μ g/day of anti-CD3 or hamster immunoglobulin (Ig)G (control), via gavage, for 5 or 10 days. The effect of oral anti-CD3 on cytokine responses was studied by activating T cells using intraperitoneal injections of anti-CD3 monoclonal antibody 2 days after oral administration of the antibody. We collected intestine samples for histology analysis and cells were analyzed by flow cytometry. Cytokines in sera were analyzed by cytometric bead array. **RESULTS:** Oral administration of anti-CD3 protected the mice from wasting disease and intestinal inflammation. Analyses of spleen and mesenteric lymph node cells showed no differences in total cell counts, or percentages of CD4⁺ and forkhead box P3⁺ regulatory T cells, between mice given anti-CD3 or the control immunoglobulin. Colitis therefore was not suppressed by induction of forkhead box P3⁺ regulatory T cells, or depletion or limited expansion of T cells. Oral administration of anti-CD3 ameliorated the enteropathy induced by intraperitoneal injection of the antibody. In mice with enteropathy, oral anti-CD3 reduced levels of inflammatory cytokines such as interferon- γ , tumor necrosis factor- α , and interleukin (IL)-6; it also increased levels of the anti-inflammatory cytokines IL-10 and transforming growth factor- β . The effects of oral anti-CD3 required IL-10. **CONCLUSIONS:** Oral administration of anti-CD3 to mice induces changes in the mucosal immune response that prevent colitis, independent of specific antigen, and reduce T-cell activation in an IL-10-dependent manner. Oral anti-CD3 therefore might be developed for the treatment of patients with inflammatory bowel disease.

Keywords: IFN γ ; TNF- α ; IL-6; TGF- β .

(IBD), including the newer biologic therapies, aim to induce and maintain clinical remission while minimizing adverse effects (reviewed by Talley et al² and Triantafyllidis et al³). However, these treatments for IBD are not curative,^{2,3} leaving the need to develop therapeutics that change the immune regulation of mucosal inflammation and alter the natural course of the disease.

Feeding antigen (Ag) can lead to oral tolerance via T-cell anergy, deletion, or initiation of T-cell suppression by regulatory T cells (Treg) (reviewed by Faria and Weiner^{4,5} and Strober et al⁶). Ag-specific and nonspecific means of inducing oral tolerance, as a treatment for “autoimmune” diseases, presents a potentially powerful way to alter chronic inflammatory conditions. Recently, orally administered anti-CD3 ϵ monoclonal antibody (referred to as anti-CD3 in this article) also was shown to induce “oral tolerance,” in an Ag-nonspecific fashion.⁷ Indeed, feeding anti-CD3 to mice undergoing induction of experimental allergic encephalomyelitis, suppressed the development of the neurologic disease.⁸ This treatment was associated with an increase in T cells expressing latency-associated peptide (LAP) most prominently in mesenteric lymph nodes (MLNs) and Peyer's Patches (PPs).

Feeding anti-CD3 increased the in vitro suppressive activity of the CD4⁺LAP⁺ cells, which produced more interleukin (IL)-4, IL-10, and transforming growth factor (TGF)- β , and less IL-2.⁸ In addition, feeding anti-CD3 was effective in preventing diabetes, arthritis, lupus, arteriosclerosis, and transplantation rejection in mouse models.^{7,9} We previously showed that feeding ovalbumin (OVA) prevented T-cell-induced colitis by inducing tolerance in both OVA-reactive and non-OVA-reactive T cells, through induction of Ag-nonspecific Treg cells.¹⁰ Here, we examined the efficacy of feeding anti-CD3 monoclonal antibody (mAb) in preventing disease in the T-cell transfer

Abbreviations used in this paper: Ag, antigen; Foxp3, forkhead box P3; hIgG, hamster immunoglobulin gamma; IBD, inflammatory bowel disease; IEL, intraepithelial leukocyte; IL, interleukin; IP, intraperitoneal; LAP, latency-associated peptide; LPL, lamina propria leukocyte; mAb, monoclonal antibody; MLN, mesenteric lymph node; OVA, ovalbumin; PP, Peyer's Patch; SCID, severe combined immunodeficient; TCR, T-cell receptor; TGF, transforming growth factor; Treg, regulatory T cells.

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Crohn's disease and ulcerative colitis are chronic conditions with a prevalence of up to 396/100,000 persons.¹ Current treatments of inflammatory bowel disease

model of colitis and in modulating in vivo T-cell activation responses.

Our data show that feeding anti-CD3 was effective in preventing T-cell-induced colitis in the CD4⁺CD45RB^{high} transfer model. We also showed that oral anti-CD3 prevented systemic T-cell activation and this was dependent on IL-10. Oral anti-CD3 mAb treatment is a novel approach for the prevention and treatment of T-cell-induced colitis. This approach of inducing tolerance is not dependent on identifying the specific Ag triggering inflammation and may be of value in treating patients with IBD.

Materials and Methods

Mice

Severe combined immunodeficient (SCID) and BALB/c mice were obtained from Taconic (Germantown, NY). C57BL/6J and B6.129P2-*IL10*^{tm1Cgn}/J (*IL-10*^{-/-}) mice from high standard pathogen-free barrier-maintained production colonies, showing a low incidence of spontaneous colitis (additional details are available in the Supplementary Materials and Methods section), were purchased from Jackson Laboratories (stock #002251 and #000664; Bar Harbor, ME). Mice were maintained under standard pathogen-free conditions in the central animal facility (Division of Comparative Medicine, University of Toronto). All animal experiments were performed in accordance with institutional guidelines and were approved by the Animal Care Review Board of the University of Toronto.

T-Cell Transfer Model of Colitis

In the T-cell transfer model, colitogenic CD4⁺CD45RB^{high} T cells induce, whereas CD4⁺CD45RB^{low} T cells prevent, colitis.^{11,12} A total of 3×10^5 CD4⁺CD45RB^{high} T cells in 500 μ L sterile phosphate-buffered saline were injected intraperitoneally (IP) for each SCID recipient. Healthy controls received 1.5×10^5 CD4⁺CD45RB^{low} T cells along with injection of colitogenic T cells. Mice were monitored twice weekly as described.¹⁰ Starting during week 4 of the experiment, SCID recipients of colitogenic T cells were gavaged with 2 or 5 μ g/day of anti-CD3 or hamster IgG (hIgG) isotype control, for 5 or 10 days.

Model of In Vivo T-Cell Activation

BALB/c, C57BL/6, or *IL10*^{-/-} mice were fed 5 μ g/day/dose of anti-CD3 or hIgG for 5 days by gavage. After 2 days of resting, mice were injected IP with 50 μ g of anti-CD3 and monitored for 24 hours. Mice were assessed for clinical signs of enteropathy, including activity level, appearance of the coat, perianal state, and behavior at 2-, 4-, 6-, 8-, and 24-hour time points. Mice were killed at 2 or 24 hours after injection.

Histologic Examination

The intestines were fixed in 10% neutral buffered formalin and stained with H&E (Pathology Core, Centre for Phenogenomics, Toronto, Ontario, Canada). Colons were graded for severity of inflammation^{10,11} by an experienced pathologist (C.S.) blinded to the experimental set-up. In some experiments, weight-to-length ratios of colons were used as an objective measure of colon inflammation.¹¹ Microphotographs were acquired using a BX51, UIS2 series microscope with a 20 $\times$ /0.5 UPlanFL N objective, DP71 digital camera and imaging software (Olympus, Markham, Canada).

Cell Sorting and Flow Cytometry

BALB/c splenic CD4⁺ T-cell subsets for transfer were isolated and sorted from spleens as previously described.¹⁰ Briefly, antibody-labeled single-cell suspensions were depleted of B220⁺, CD11b⁺, and CD8⁺ cells using Dynabeads (Invitrogen Dynal AS, Oslo, Norway). CD4⁺CD45RB^{high} and CD4⁺CD45RB^{low} fractions were sorted on a FACSaria (BD Becton Dickinson, San Jose, CA) with a purity of greater than 98%. Isolation of lamina propria leukocytes (LPLs) and intraepithelial leukocytes (IELs) was performed as described¹³ with modification outlined in the Supplementary Materials and Methods section. For FCM analysis, single-cell suspensions were stained with relevant mAb (detailed in the Supplementary Materials and Methods section). Data were acquired on a FACSLSR II or a FACSCanto II (BD). Data were analyzed using FlowJo Software (Treestar, Ashland, OR).

Cytokine Analysis

Sera were collected at 2 or 24 hours after injection of anti-CD3 and analyzed for IL-2, IL-4, IL-6, IL-10, IL-17a, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ using a Cytometric Bead Array (#560485; BD Biosciences). Data were acquired on a FACSCalibur (BD) and analyzed using FCAP Array software (BD). TGF- β levels were measured using an enzyme-linked immunosorbent assay (#88-8350, eBioscience).

Statistical Analysis

Data were expressed as the mean $\pm$ standard error of the mean for parametric data or the median with interquartile range for nonparametric data. Statistical analysis was performed using the unpaired Student *t* test for independent samples or 1-way analysis of variance (ANOVA) with post-test comparison. The Mann-Whitney *U* test was used for nonparametric data. Two-way ANOVA was used to analyze time-course data. The differences between the 2 groups were considered significant when the *P* value was less than .05.

Results

Oral Anti-CD3 mAb Prevents Wasting Disease and Intestinal Inflammation in T-Cell Transfer Model of Colitis

To determine if oral anti-CD3 treatment holds potential in treating IBD, we evaluated this therapeutic approach in the T-cell transfer model of colitis. SCID mice were reconstituted with BALB/c-derived CD4⁺CD45RB^{high} T cells. In addition to oral treatment groups, control mice were reconstituted with CD4⁺CD45RB^{high} T cells and CD4⁺CD45RB^{low} T cells as healthy controls, or with CD4⁺CD45RB^{high} T cells only. Initially, oral anti-CD3 or control hIgG treatments were administered for 5 days,^{8,14–16} starting during week 4 after T-cell transfer. This time point was chosen to allow for T-cell expansion and homing, which happen after cell transfer before oral treatment. Mice fed anti-CD3 mAb at 5 μ g/dose/day over 5 days lost significantly less weight than hIgG-fed controls or recipients of CD45RB^{high} T cells alone (Figure 1A). In both the colitis control mice and the hIgG isotype-fed controls, colonic histopathology showed hyperplastic epithelium, goblet cell depletion, and infiltration of mononuclear cells in the epithelium; and, in some instances, into the lamina propria (Figure 1C and E).

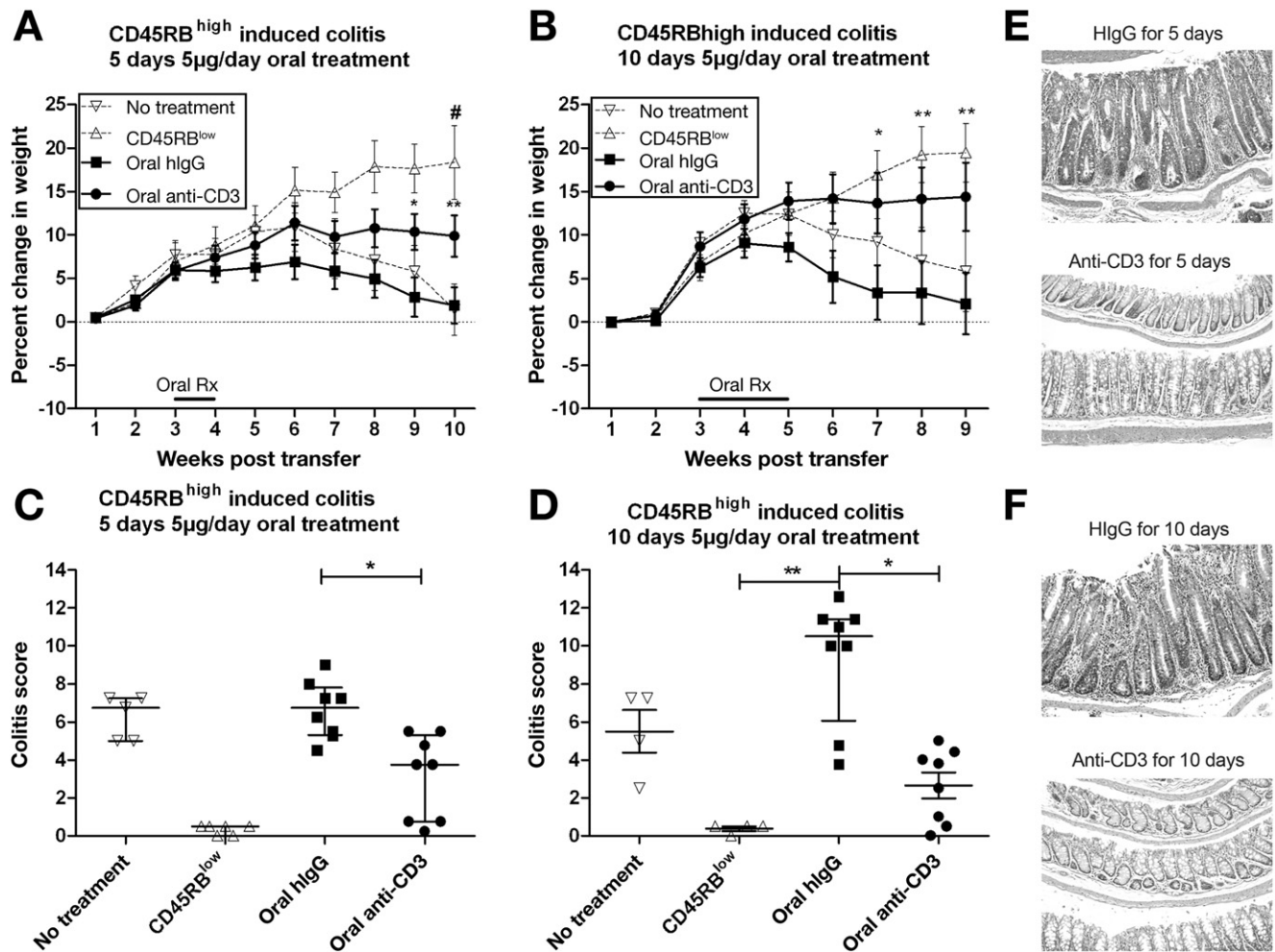


Figure 1. Oral Anti-CD3 prevents wasting and pathology in the T-cell transfer model of colitis. SCID recipients of CD4⁺CD45RB^{high} T cells were fed 5 μg of anti-CD3 or hlgG starting at week 4 postreconstitution. Colitis controls received colitogenic CD4⁺CD45RB^{high} T cells alone, healthy controls received 1.5×10^5 CD4⁺CD45RB^{low} along with colitogenic T cells. (A) Depicted are percentages (mean $\pm$ standard error of the mean) of changes in weights in all groups of mice treated for 5 days (data pooled from 3 experiments, for a total of $n = 8$ mice for oral treatments, $n = 5$ mice for controls; $\#P < .05$, $**P < .01$; 2-way ANOVA). (B) Depicted are percentages (mean $\pm$ standard error of the mean) of changes in weights in all groups of mice treated for 10 days (data pooled from 2 experiments; for a total of $n = 8$ for each oral treatment, $n = 4$ for controls; $*P < .05$, $**P < .01$; 2-way ANOVA). (C and D) Depicted are colitis scores from all SCID recipients as described in panels A or B. Data points represent individual mice, bars represent the median and interquartile ranges for each group ($*P < .05$, $**P < .01$, 1-way ANOVA, Kruskal-Wallis, Dunn comparison of all pairs). (E and F) Representative photographs at 20 $\times$ magnification of H&E-stained sections from colons of oral-treated SCID recipients.

Compared with hlgG isotype-fed controls, SCID mice transferred with CD4⁺CD45RB^{high} T cells and fed anti-CD3 had significantly less histopathologic evidence of colitis, as quantified by scoring of colonic inflammation (Figure 1C and E). However, oral anti-CD3-treated SCID recipients of CD4⁺CD45RB^{high} gained significantly less weight and tended to show more signs of inflammation in the colon than mice co-transferred with protective CD4⁺CD45RB^{low} T cells (Figure 1A and C). Therefore, we sought to increase the efficacy of oral anti-CD3 treatment in the T-cell transfer model of colitis.

Mice fed anti-CD3 at a 5 μg/day dose for 10 days lost significantly less weight (Figure 1B) and showed a significantly lower degree of inflammation in the colon than hlgG-treated controls (Figure 1D and F). Moreover, weight curves and inflammation scores of the anti-CD3-treated mice were no longer significantly different from the recip-

ients of CD4⁺CD45RB^{low} T cells, indicating greater efficacy of the 10-day treatment regimen (Figure 1). A lower daily dose of 2 μg over 5 days failed to confer protection (Supplementary Figure 1). Together, these results indicate that oral anti-CD3 was effective in preventing T-cell-induced colitis.

Oral Anti-CD3 Does Not Induce Changes in T-Cell Numbers or Phenotype in Transfer Model of Colitis

To determine how feeding anti-CD3 prevented colitis, we examined the changes in T-cell phenotype within the spleen and MLNs. In comparison with the other experimental groups, recipients of both CD4⁺CD45RB^{high} and CD4⁺CD45RB^{low} T cells (healthy controls) tended to show reduced numbers of cells recovered from the spleen

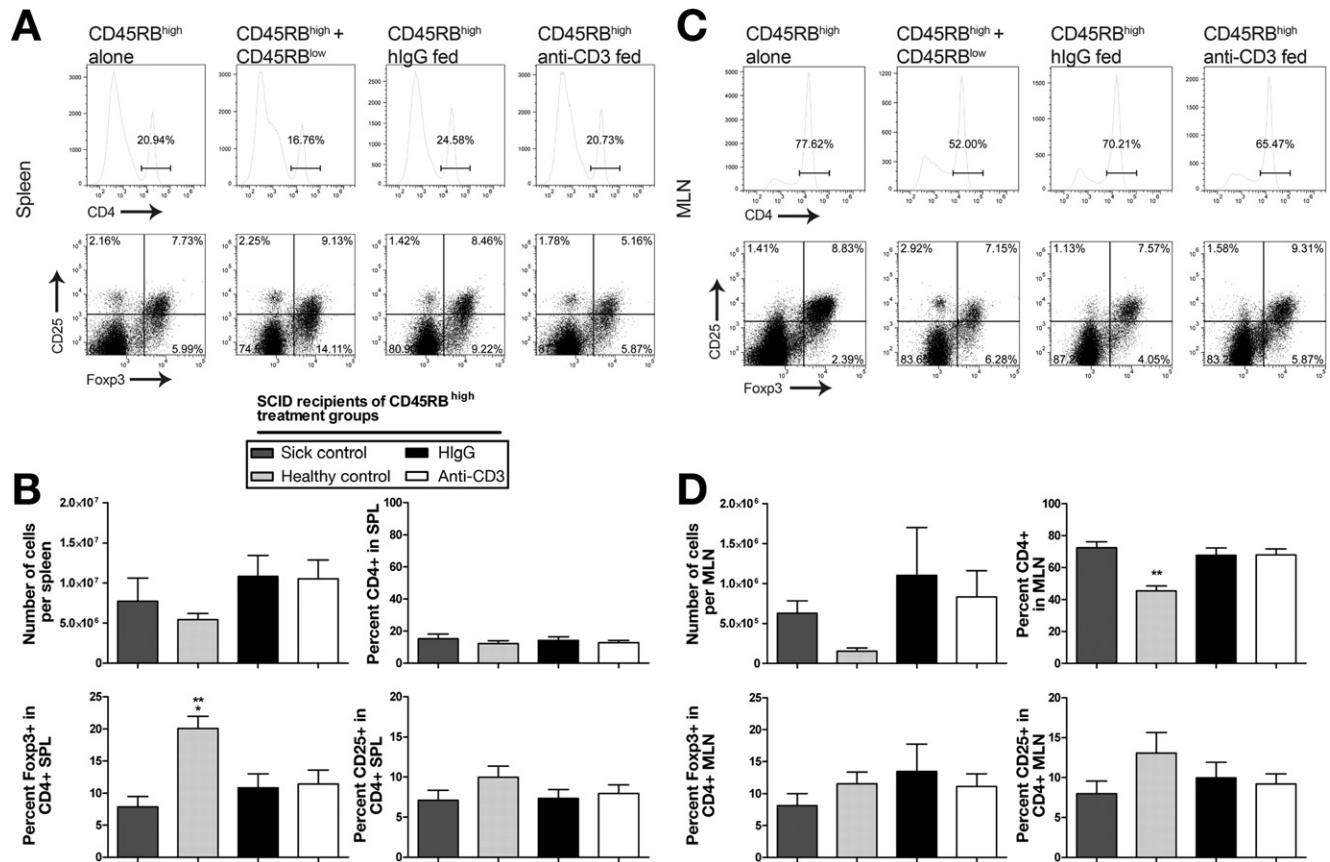


Figure 2. Oral anti-CD3 did not induce changes in cell numbers or regulatory phenotype in the spleen or mesenteric lymph node. Cells harvested from spleens and MLNs of SCID recipients treated for 10 days as described in Figure 1, were analyzed for expression of CD4, CD25, and Foxp3. (A) Depicted are representative histograms and dot plots of SPL in all treatment groups for expression of CD4 (histogram), CD25, and Foxp3 (dot plot). (B) Columns represent mean $\pm$ standard error of the mean of the number of cells recovered from the spleen, the percentage of CD4⁺ T cells in the spleen, or the percentage of Foxp3⁺ and CD25⁺ cells within CD4⁺ gated SPL (* P < .05, CD45RB^{low} vs no treatment; ** P < .01, CD45RB^{low} vs oral anti-CD3 and oral hlgG, 1-way ANOVA). (C) Shown are representative histograms and dot plots of MLN for all treatment groups for expression of CD4 (histogram), CD25, and Foxp3 (dot plot). (D) Columns represent the mean $\pm$ standard error of the mean of the number of cells recovered from the MLN, the percentage of CD4⁺ T cells in the MLN, and mean $\pm$ standard error of the mean of the percentage of Foxp3⁺ and CD25⁺ cells within CD4⁺ gated T cells in the MLN. (** P < .01, CD45RB^{low} vs no treatment, oral anti-CD3, and oral hlgG, 1-way ANOVA.)

(Figure 2B, Supplementary Figure 2B). Within CD4⁺ gated splenic lymphocytes, recipients of CD45RB^{low} T cells had a significantly higher percentage of forkhead box P3 (Foxp3)⁺ cells compared with the other groups (Figure 2A and B, Supplementary Figure 2A and B). Recipients of CD4⁺CD45RB^{high} T cells without oral treatment, as well as recipients fed anti-CD3 or hlgG isotype control, showed similar total cell counts in the spleen (Figure 2B, Supplementary Figure 2B). Moreover, the percentages of splenic T cells expressing the regulatory T-cell markers Foxp3 and CD25 were unchanged (Figure 2A and B, Supplementary Figure 2A and B). In MLNs, only mice co-transferred with CD4⁺CD45RB^{high} T cells and CD4⁺CD45RB^{low} T cells had significantly less CD4⁺ cells within gated lymphocytes (Figure 2C and D). Oral anti-CD3 and control hlgG-treated mice, as well as untreated recipients of CD4⁺CD45RB^{high} T cells, had similar levels of CD4⁺ T cells, and showed no difference in expression of Foxp3 or CD25 within gated CD4⁺ lymphocytes in the MLN (Figure 2C and D).

Feeding Anti-CD3 mAb Suppresses Signs of T-Cell-Induced Enteropathy

Systemic administration of anti-CD3 causes polyclonal T-cell activation, leading to a cytokine release syndrome and acute damage to the small bowel.¹⁷ Here, we assessed the effects of feeding anti-CD3 on T-cell-induced enteropathy that follows systemic in vivo T-cell activation after injection of anti-CD3. Compared with hlgG-fed controls, we observed significantly decreased clinical signs in mice fed anti-CD3 before systemic T-cell activation with IP anti-CD3 (Figure 3A and B, Supplementary Figure 3A). There was a significant difference in the villus-to-crypt ratios between anti-CD3 vs hlgG-fed mice 24 hours after IP injection of anti-CD3, indicating amelioration of gut damage by oral anti-CD3 (Figure 3C and D, Supplementary Figure 3B). Similar to our observations in the T-cell transfer model of colitis, we did not observe significant differences in total cell numbers, percentage of CD4⁺ cells, or T cells expressing the regulatory phenotype marker Foxp3 in spleen, MLNs, or PPs of mice fed anti-

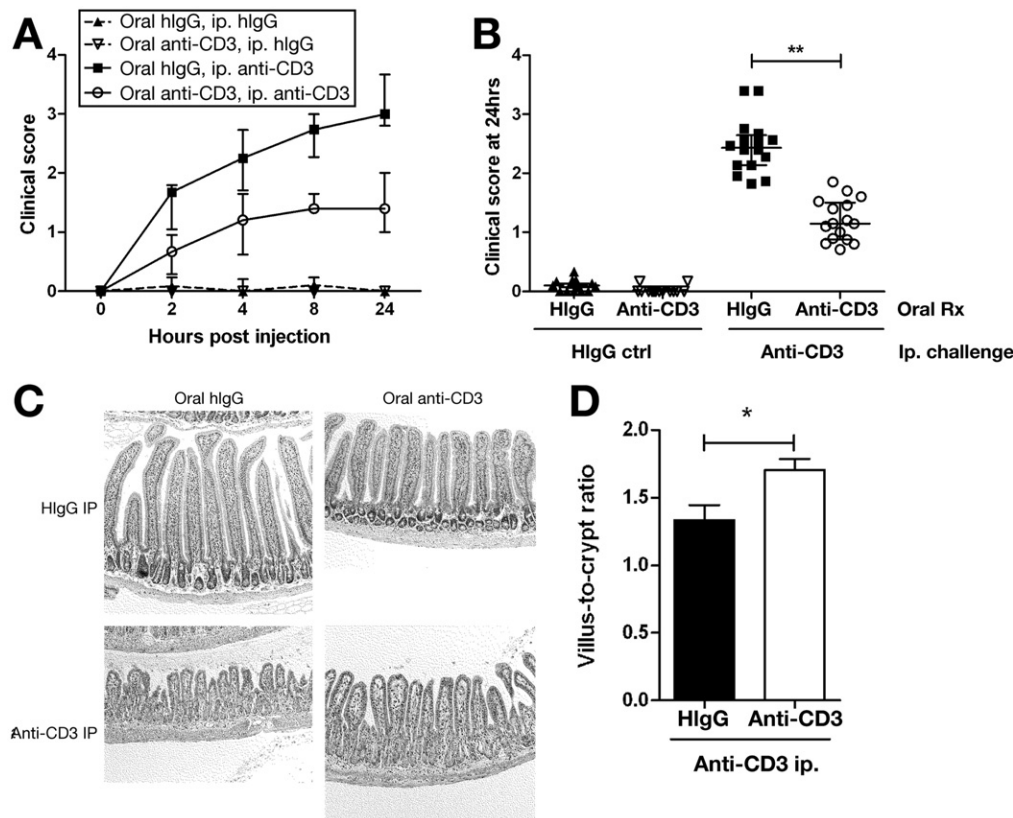


Figure 3. Oral anti CD-3 suppresses signs of T-cell-induced enteropathy. BALB/c mice were fed 5 μ g of anti-CD3 or control hlgG daily for 5 days and enteropathy induced by IP injection of a single 50- μ g dose of anti-CD3 or hlgG control. Mice were monitored and scored at 2, 4, 8, and 24 hours after injection. (A) Depicted is the median disease score with interquartile range at each time point. (B) Clinical scores for each treatment at 24 hours after injection, data points represent individual mice, bars represent the median $\pm$ interquartile range (4 experiments, $**P < .01$; 1-way ANOVA, Kruskal-Wallis). (C) Representative photographs, 20 $\times$ magnification of H&E-stained sections from the small bowel harvested at 24 hours. (D) Presented are villus-to-crypt ratios (mean $\pm$ standard error of the mean) for oral-treated anti-CD3-injected mice ($**P < .05$; 1-way-ANOVA).

CD3 vs hlgG before or after IP challenge with anti-CD3 (Figure 4).

Oral Anti-CD3 Increases Anti-Inflammatory Cytokines IL-10 and TGF- β While Reducing Proinflammatory Cytokine Secretion

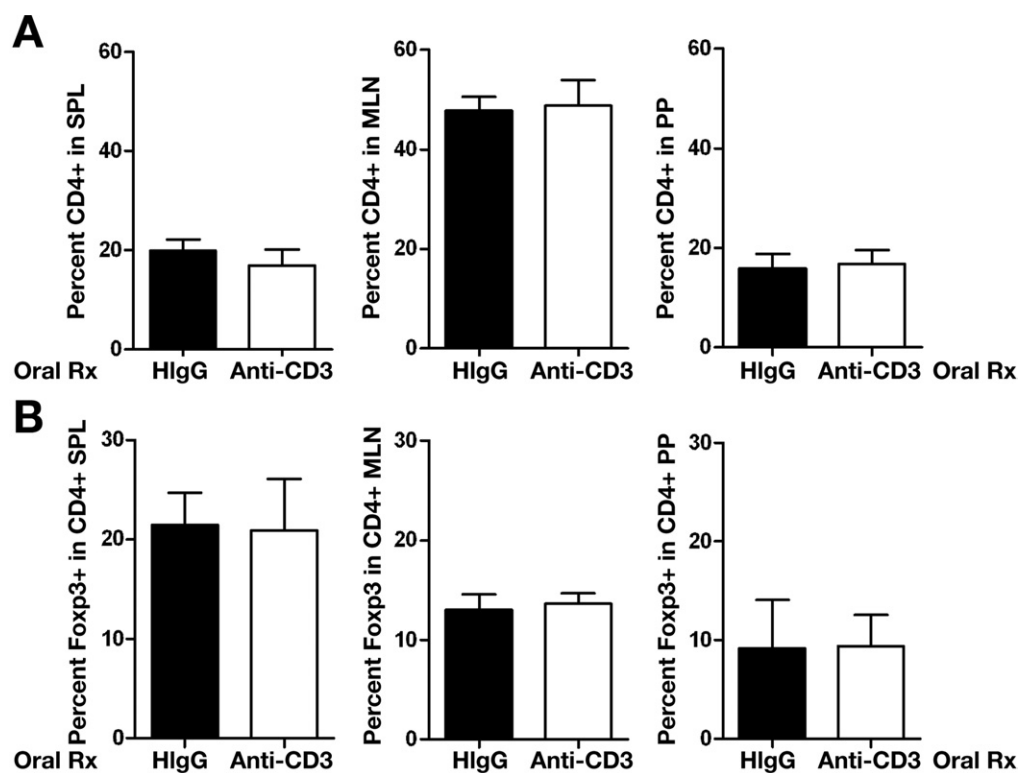
Levels of cytokines were measured in sera of anti-CD3 and hlgG-fed mice, at 2 or 24 hours after challenge with IP injection of hlgG isotype or anti-CD3 antibody. IFN γ and TNF- α were significantly lower in anti-CD3-fed mice, compared with hlgG isotype-fed controls, 2 hours after injection of anti-CD3 (Figure 5A and B). Twenty-four hours after IP anti-CD3 challenge, these levels had decreased, and differences between the oral treatments were no longer observed. IL-6 followed a similar time course. Isotype-fed controls expressed significantly higher levels of IL-6 compared with anti-CD3-fed control mice in response to IP anti-CD3 injection after 2 hours, and both groups returned to baseline levels by 24 hours after the challenge (Figure 5C). Although IL-17A was induced in response to IP challenge, there was no difference between the 2 oral treatment groups (Figure 5D). Two hours after IP anti-CD3 injection, IL-10 levels were increased in both oral treatment groups, but were not significantly different at that time point. However, 24 hours after IP anti-CD3 injection, IL-10 levels continued to increase in anti-CD3-fed mice and were at that point significantly higher compared with hlgG isotype-treated controls, in which levels remained the same as for the 2-hour time point (Figure 5E). Levels of TGF- β were significantly induced in mice

fed anti-CD3 but not in isotype-fed controls, in response to IP challenge with anti-CD3 (Figure 5F). In summary, these data showed that oral anti-CD3 induced anti-inflammatory cytokines IL-10 and TGF- β and reduced the secretion of proinflammatory cytokines secreted in response to polyclonal T-cell activation.

IL-10 Is Required for Oral Anti-CD3 to Exert Its Protective Effect in T-Cell-Induced Enteropathy

To assess whether IL-10 is required for oral anti-CD3 treatment to exert its protective effect, we examined the effect of oral anti-CD3 in IL-10-deficient mice. Anti-CD3-fed *IL10*^{-/-} mice showed a similar degree of enteropathy as hlgG control-fed mice, with similar clinical scores and histopathology in response to challenge with IP anti-CD3 (Figure 6A–D). Proinflammatory cytokines were detected at similar levels in *IL10*^{-/-} mice compared with wild-type mice at 24 hours after IP challenge (Supplementary Figure 3 for wild-type mice, Figure 6E for *IL10*^{-/-} mice). In contrast to our observations in the wild-type C57Bl/6 mice (Supplementary Figure 3), there was no difference in levels of proinflammatory cytokines between oral anti-CD3 and hlgG-fed *IL10*^{-/-} mice after challenge with IP anti-CD3 (Figure 6E). Surprisingly, TGF- β was reduced significantly in anti-CD3-fed compared with hlgG-fed *IL10*^{-/-} mice 24 hours after challenge with IP anti-CD3 (Figure 5 and Supplementary Figure 3 for wild-type mice, and Figure 7 for *IL10*^{-/-} mice). Therefore, in the absence of IL-10, oral anti-CD3 was unable to

Figure 4. Oral anti-CD3 does not alter percentages of CD4⁺ T cells or induce regulatory phenotype T cells. BALB/c mice were fed anti-CD3 or hIgG and injected with 50 μ g of anti-CD3 IP. Cells harvested from SPL, MLN, and PP were analyzed for CD4 and Foxp3 expression. (A) Depicted are mean $\pm$ standard error of the mean of the percentages of CD4⁺ cells within gated mononuclear cells in SPL, MLN, and PP. (B) Shown are mean $\pm$ standard error of the mean of the percentages of Foxp3⁺ cells within gated CD4⁺ T cells in SPL, MLN, and PP.



confer protection from clinical signs and small-bowel damage after polyclonal T-cell activation in vivo.

Oral Anti-CD3 Reduces Proinflammatory Cytokine Secretion in the Small Intestine

We fed naive BALB/c mice anti-CD3 or hIgG (5 μ g/day) for 3 consecutive days. Mice were killed 24 hours after the last feeding, IELs and LPLs then were isolated from small intestines and colons of these mice. In addition,

we obtained single-cell suspensions from PPs and MLNs. Percentages of Foxp3⁺ cells within CD4⁺ IELs or LPLs of the small or the large intestine did not differ between anti-CD3- and hIgG-fed mice (Supplementary Figure 5). Cells restimulated in vitro with phorbol-12-myristate-13-acetate and ionomycin, in the presence of monensin, were evaluated for expression of cytokines. There were significantly lower percentages of IFN γ - and IL-17A-expressing cells in small-bowel IELs of anti-CD3-

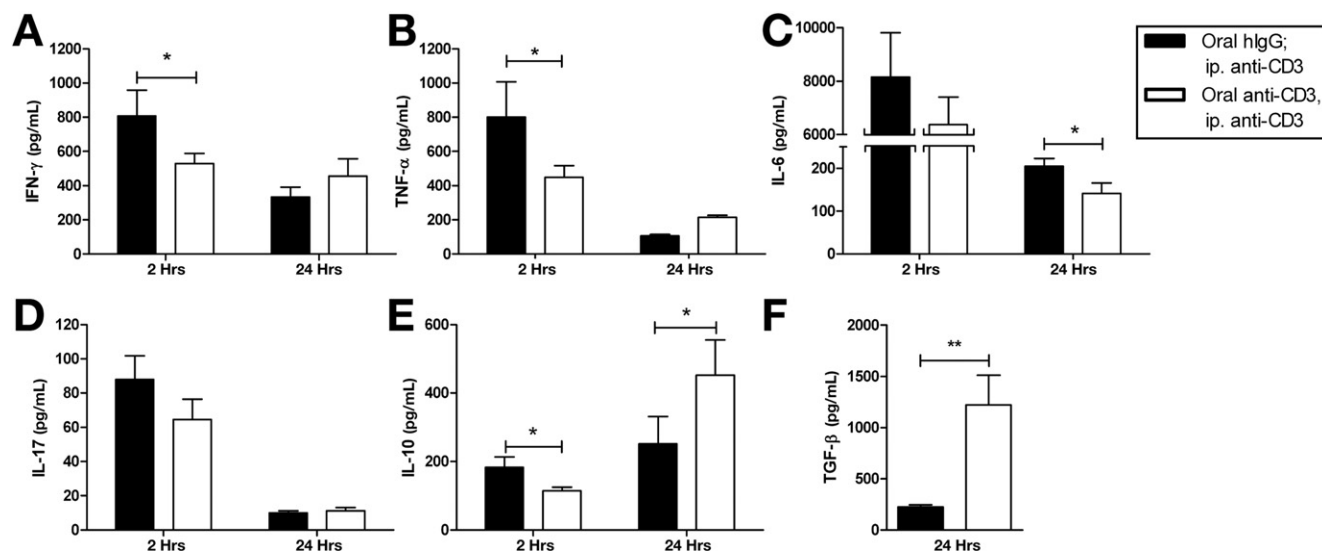


Figure 5. Oral anti-CD3 increases IL-10 and TGF- β and reduces proinflammatory cytokines. Sera from BALB/c mice as described in Figure 4 were obtained at 2 or 24 hours after injection and analyzed for tumor necrosis factor (TNF)- α , interferon (IFN)- γ , IL-6, IL-17, and IL-10. (A–E) Cytokine concentrations in pg/mL are shown as mean $\pm$ standard error of the mean at 2 and 24 hours after challenge (* P < .05, 1-way ANOVA). (F) TGF- β at 24 hours after challenge is expressed as mean $\pm$ standard error of the mean in pg/mL (** P < .01, 1-way ANOVA).

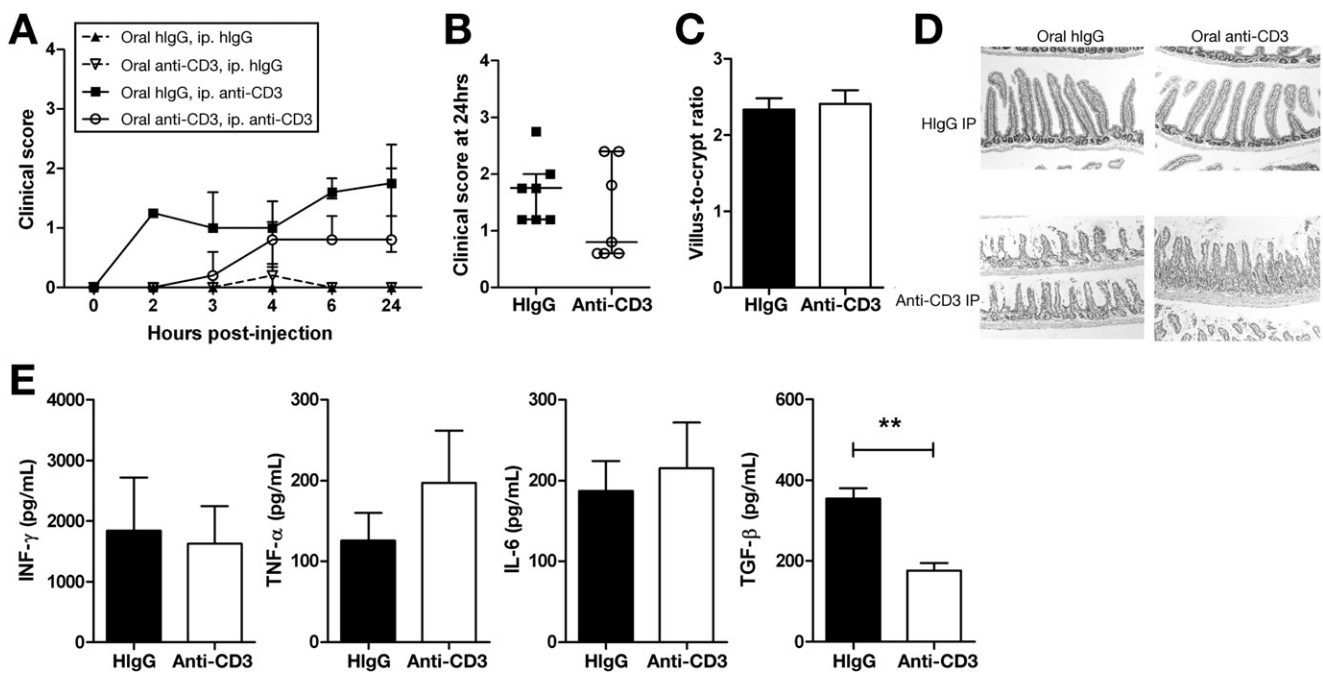


Figure 6. Oral anti-CD3 fails to prevent T-cell-induced enteropathy and does not induce TGF- β or reduce proinflammatory cytokines in IL-10-deficient mice. *IL10*^{-/-} mice were fed 5 μ g of anti-CD3 or control hlgG daily for 5 days and enteropathy was induced by IP injection of a single 33- μ g dose of anti-CD3 or hlgG control. Mice were monitored and scored at 2, 4, 8, and 24 hours post-injection. (A) Depicted is the median disease score and interquartile range for each time point. (B) Average clinical score for orally treated and anti-CD3-injected mice at 24 hours post-injection ($n = 7$ mice over 2 experiments). (C) Columns and error bars represent mean $\pm$ standard error of the mean of villus-to-crypt ratios of the small bowel. (D) Representative photographs, 20 $\times$ magnification of H&E-stained sections from the small bowel harvested 24 hours post-injection. (E) Sera were obtained at 24 hours post-injection and analyzed for interferon (IFN)- γ , tumor necrosis factor (TNF)- α , IL-6, and TGF- β . Cytokine concentrations in pg/mL are shown as mean $\pm$ standard error of the mean (** $P < .01$, Student t test).

fed mice compared with hlgG-fed controls (Figure 7A). In small-bowel LPLs, we observed a trend toward lower IL-17A in CD4⁺ cells and a significantly lower percentage in CD8⁺ cells (Figure 7B). There were no significant differences between the oral treatment groups in other compartments of the gut-associated lymphoid tissue (ie, MLNs, PPs, or colon) (Supplementary Figure 6).

Discussion

There is a need to develop treatments for IBD that will alter the natural history of the disease, possibly by reprogramming the immune system or inducing long-lasting immune tolerance to a triggering Ag. We showed that oral anti-CD3 treatment protected mice from developing colitis in the T-cell transfer model. Subsequently, using a model of T-cell-induced enteropathy, we showed that feeding anti-CD3 attenuated the cytokine storm and associated signs of impaired well-being in mice challenged by polyclonal T-cell activation in vivo. Oral anti-CD3 protection was not the result of deletion or limited expansion of T cells.⁸ In both models, feeding anti-CD3 did not lead to induction or expansion of Foxp3⁺ regulatory T cells, as can be observed in oral tolerance.⁷ We found that cytokine responses in IEL were attenuated by oral anti-CD3. In addition, cytokine analysis in the enteropathy model revealed that feeding anti-CD3 reduced proinflammatory cytokines and increased anti-inflammatory cytokines in the face of systemic T-cell activation in

vivo. These findings suggest that oral anti-CD3 alters T-cell responses to T-cell receptor (TCR) stimulation locally as well as systemically. Consistent with previous studies in other models of inflammation,^{8,14–16,18} we showed that oral anti-CD3 induced IL-10 expression and secretion. We further showed that the effect of feeding anti-CD3 on the prevention of enteropathy was dependent on IL-10 because feeding anti-CD3 to IL-10-deficient mice did not prevent T-cell-induced enteropathy, and failed to induce TGF- β as observed in wild-type mice.

Previous work from our laboratory indicated that feeding specific antigen-induced regulatory T cells, consequently, prevented colitis in the T-cell transfer model,¹⁰ similar to oral myelin basic protein preventing experimental allergic encephalomyelitis.^{19–29} Another group showed that ex vivo generated and in vivo activated antigen-specific regulatory cells prevented colitis in a bystander fashion.³⁰ Studies by Weiner's group have shown that oral anti-CD3 treatment induced antigen-nonspecific tolerance and thus prevented, or reversed, disease in mouse models of autoimmune diseases, such as multiple sclerosis,⁸ diabetes,^{14,18} rheumatoid arthritis,¹⁶ and systemic lupus erythematosus.¹⁵ Similar to our findings, Ochi et al showed that the effects observed with oral anti-CD3 are dose dependent, with the highest efficacy at 5 μ g/dose/day. However, compared with a 5-day regimen, feeding anti-CD3 at 5 μ g/day for 10 days was more effective.

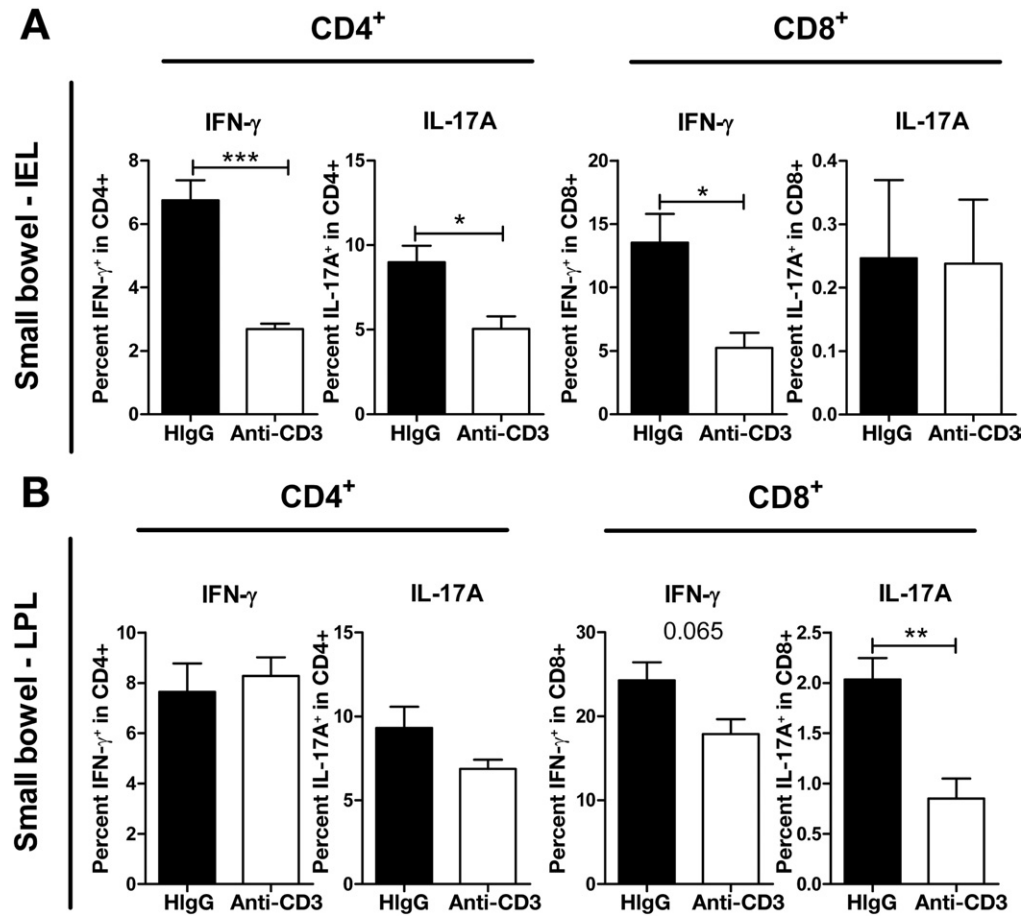


Figure 7. Oral anti-CD3 leads to reduced expression of interferon (IFN)- γ and IL-17A in intestinal lymphocytes. IELs and LPLs were isolated from the small bowel of mice fed 5 μ g of anti-CD3 or hlgG for 3 days and stimulated in the presence of phorbol-12-myristate-13-acetate, ionomycin, and monensin before intracellular cytokine staining. Depicted are mean $\pm$ standard error of the mean of the percentages of cells expressing each cytokine within gated CD4⁺ or CD8⁺ cells (n = 5 mice over 2 experiments, * P < .05, ** P < .01, *** P < .001, Student t test).

Therefore, increasing the length of the treatment interval boosted treatment efficacy.

In contrast to studies on oral tolerance, we and others⁸ did not observe an induction or expansion of Foxp3 expressing regulatory T cells in response to oral anti-CD3. We also confirmed that T-cell depletion or limited expansion is not involved in the mechanism leading to oral anti-CD3-mediated protection.⁸ An oral anti-CD3-induced decrease of proinflammatory cytokines has been reported in response to oral anti-CD3 treatment in other models,^{8,14–16,18} although not always consistently owing to differing overall cytokine profiles in these respective models.

IL-10 and TGF- β are anti-inflammatory cytokines critical in the maintenance of mucosal homeostasis.^{31,32} Both anti-inflammatory cytokines inhibit proinflammatory cytokine expression and T-cell proliferation.^{31,32} In accordance with previous studies, we found that oral anti-CD3 treatment increased TGF- β and IL-10 production. Ochi et al showed that TGF- β is required for oral anti-CD3-mediated protection in vivo and in vitro: however, IL-10 was shown to be dispensable in vitro once tolerance induction was complete. Our data showed that feeding anti-CD3 to *IL10*^{-/-} mice failed to prevent T-cell-induced enteropathy, suggesting that IL-10 is required for protection when feeding anti-CD3. IL-10 dependence of inducible tolerance, as shown here with oral anti-CD3, was

described previously in a uveitis model.³³ In contrast, others have reported that low-dose oral tolerance was independent of IL-10.³⁴ It is not clear why the reports in the low-dose oral tolerance models differ, although the investigators suggest differences in Ag, feeding regimens, and susceptibility to disease as possible causes.³⁴

To date, a number of different Treg cell subsets have been defined. Naturally occurring Treg develops in the thymus and express Foxp3 (reviewed by Vignali et al³⁵), as well as helios.³⁶ Inducible regulatory T cells are a very heterogeneous group consisting of different subtypes. Type 3 T cells were identified during development of oral tolerance.^{21,37} Oida et al³⁸ described a CD4⁺CD25⁻Foxp3⁻LAP⁺, TGF- β -dependent, regulatory cell type within the CD45RB^{low} subset that prevented disease in CD4⁺CD45RB^{high} induced colitis. LAP is the amino terminal domain of TGF- β precursor peptide; remaining covalently bound after cleavage, it forms a TGF- β complex with TGF- β peptide.⁸ Upon TCR activation, type 3 T cells secrete TGF- β and, depending on the milieu, may become CD4⁺CD25⁺Foxp3⁺LAP⁺. Inducible Foxp3⁺ regulatory cells develop in the gut-associated lymphoid tissue in response to bacterial and food antigens in the presence of IL-2 and TGF- β . Tr1 cells are regulatory CD4⁺ T cells, which are characterized by production of high amounts of IL-10 and moderate amounts of TGF- β .³⁹ Tr1 cells are thought to be antigen-specific, inducible T cells that, once activated via their TCR, exert

their suppressor function in an antigen-nonspecific bystander fashion.³⁰ Tr1 cells lack Foxp3 expression and, to date, specific markers to identify this cell population reliably have not been found.³⁹ Similar to Foxp3⁺ Treg, Tr1 cells are anergic in vitro but can be expanded under certain culture conditions.^{39–45} It also has been shown that neutralization of IL-10 inhibits the development of Tr1 cells.^{39,44} Given these findings it is reasonable to speculate that oral anti-CD3 induces Tr1 T cells as described in a recent study showing that nasal administration of anti-CD3 induced Tr1 cells.⁴⁶

In summary, feeding anti-CD3 mAb induced changes in the mucosal immune responses that prevented colitis independent of specific Ag and attenuated T-cell-induced enteropathy in an IL-10-dependent manner. These findings may be of clinical relevance in treating IBD when the triggering Ag is not known. Feeding anti-CD3 could provide a new strategy to induce changes in the immune system for the prevention or treatment of colitis that is independent of specific Ag. Thus, oral anti-CD3 may prove to be a new avenue in the treatment of inflammatory bowel disease.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2012.07.019>.

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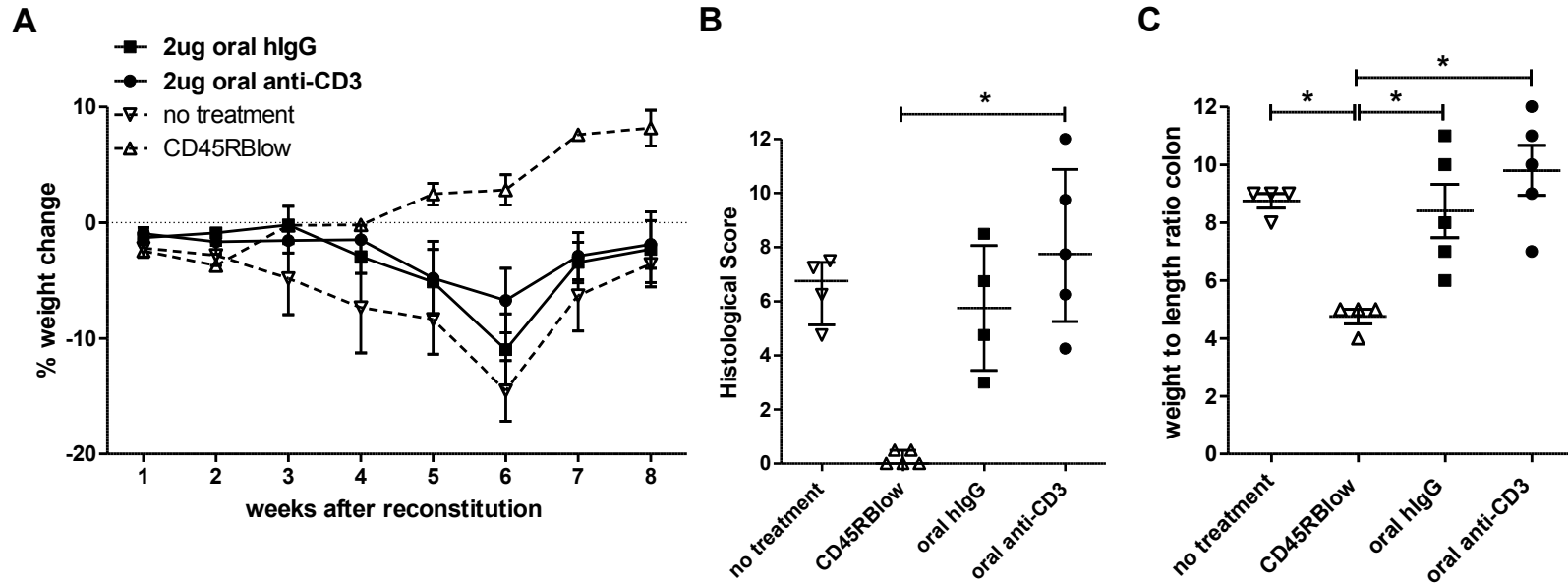
Conflicts of interest

The authors disclose no conflicts.

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Supplementary Figures



Supplementary Figure S1. 2 μ g per day oral Anti-CD3 does not prevent wasting and pathology in T cell transfer model of colitis. SCID recipients of CD4⁺CD45RB^{high} T cells were fed 2ug of anti-CD3 or hlgG starting at week 4 post reconstitution. Colitis controls received colitogenic CD4⁺CD45RB^{high} T cells alone; healthy controls 1.5x10⁵ CD4⁺CD45RB^{low} along with colitogenic T cells. (A) Depicted are percentages (mean $\pm$ SEM) of changes in weights in all groups of mice treated for 5 days. (B) Depicted are colitis scores of SCID recipients. Data points represent individual mice, bars the median and interquartile range for each group (* P <0.05, 1-way ANOVA - Kruskal Wallis). (C) Depicted are weight to length ratios of colons as mean $\pm$ SEM (* P <0.05; 1-way ANOVA).

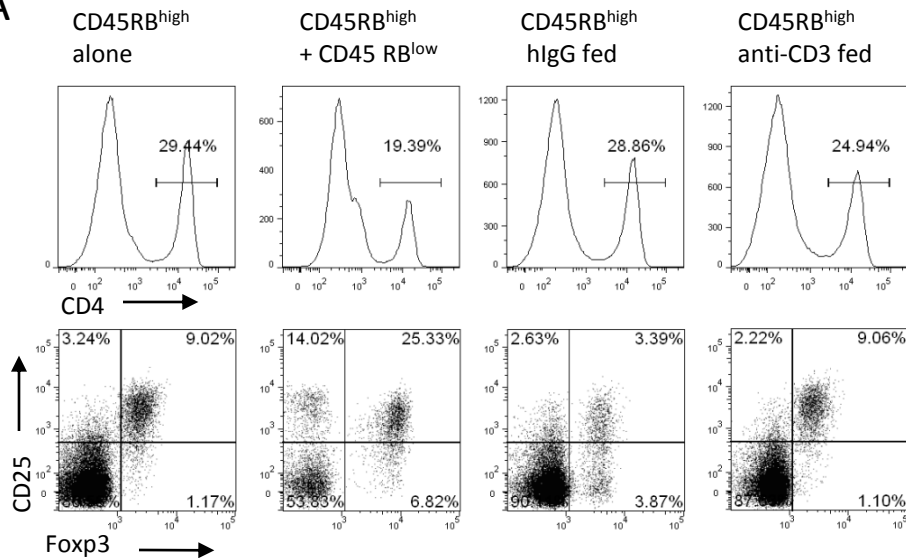
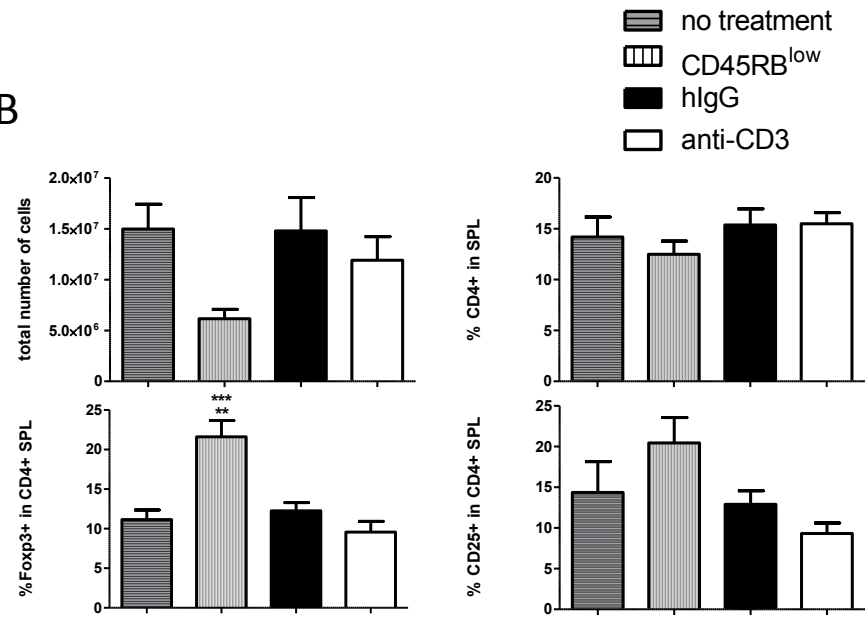
A**B**

Figure S2 Oral anti-CD3 does not alter percentages of CD4⁺ T cells nor induce regulatory phenotype T cells. Cells harvested from spleens of SCID recipients treated for 5 days as described in Figure 1, were analyzed for expression of CD4, CD25 and Foxp3. (A) Depicted are representative histograms and dot plots of SPL in all treatment groups for expression of CD4 (histogram), CD25 and Foxp3 (dot plot). (B) Columns represent mean SEM of the number of cells recovered from the spleen, the percentage of CD4⁺ T cells in the spleen or the percentage of Foxp3⁺ and CD25⁺ cells within CD4⁺ gated SPL (** $P < 0.01$, CD45RB^{low} vs. no treatment; *** $P < 0.001$, CD45RB^{low} vs. oral anti-CD3 and oral hlgG, 1-way ANOVA).

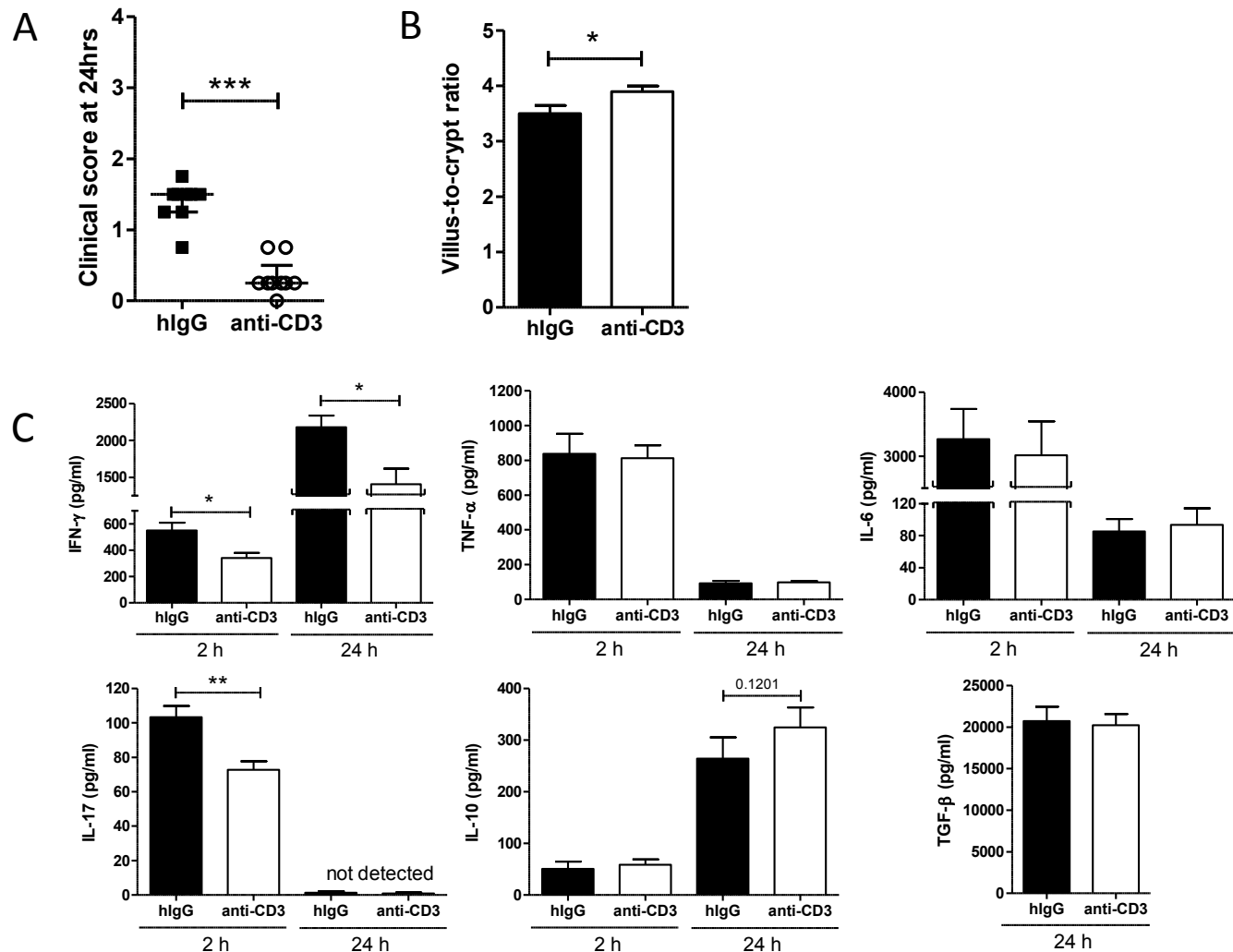


Figure S3 Oral anti CD-3 ameliorates T cell induced enteropathy. C57BL/6 mice were fed 5ug of anti-CD3 or control hlgG daily for five days and enteropathy induced by ip. injection of a single 33 or 50 ug dose of anti-CD3. Mice were monitored and scored at 2, 4, 8, and 24 hours post injection. (A) Clinical scores for each treatment at 24 hours post injection, data points represent individual mice, bars median interquartile range. (2 experiments, *** $P < 0.001$; Mann-Whitney-U-Test). (B) Depicted are villus-to-crypt ratios as mean SEM for oral treated anti-CD3 injected mice (* $P < 0.05$; Student t test) (C) Cytokine concentrations in pg/ml are shown as mean SEM at 2 and 24h post challenge (* $P < 0.05$, ** $P < 0.01$; Student t test).

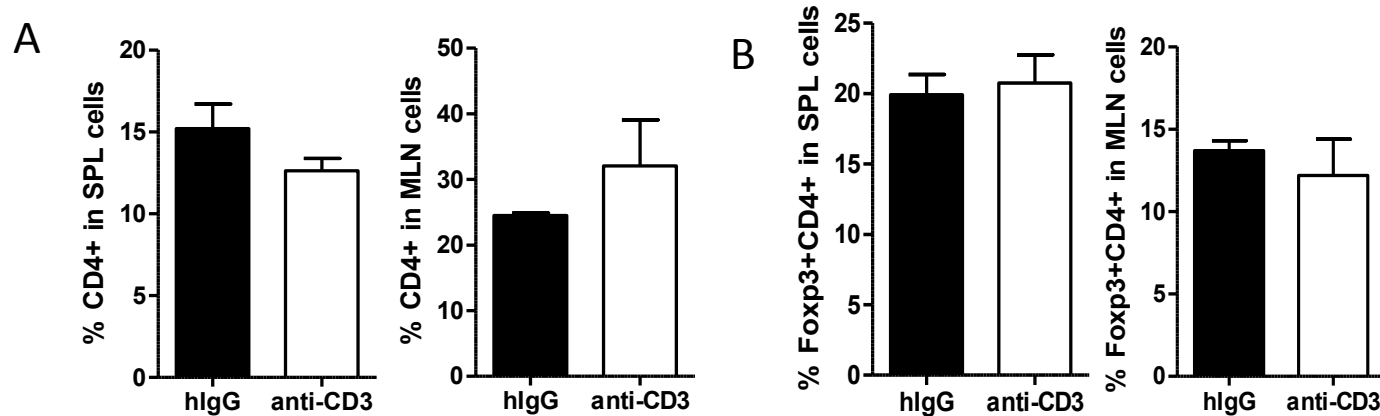


Figure S4 Oral anti-CD3 does not alter percentages of CD4⁺ T cells nor induce regulatory phenotype T cells. *IL10*^{-/-} mice were fed anti-CD3 or hlgG and injected with 33 ug of anti-CD3 ip.. Cells were harvested from SPL and MLN, were analyzed for CD4 and Foxp3 expression. (A) Depicted are mean SEM of the percentages of CD4⁺ cells within gated mononuclear cells in SPL and MLN (B) Shown are mean SEM of the percentages of Foxp3⁺ cells within gated CD4⁺ T cells in SPL and MLN.

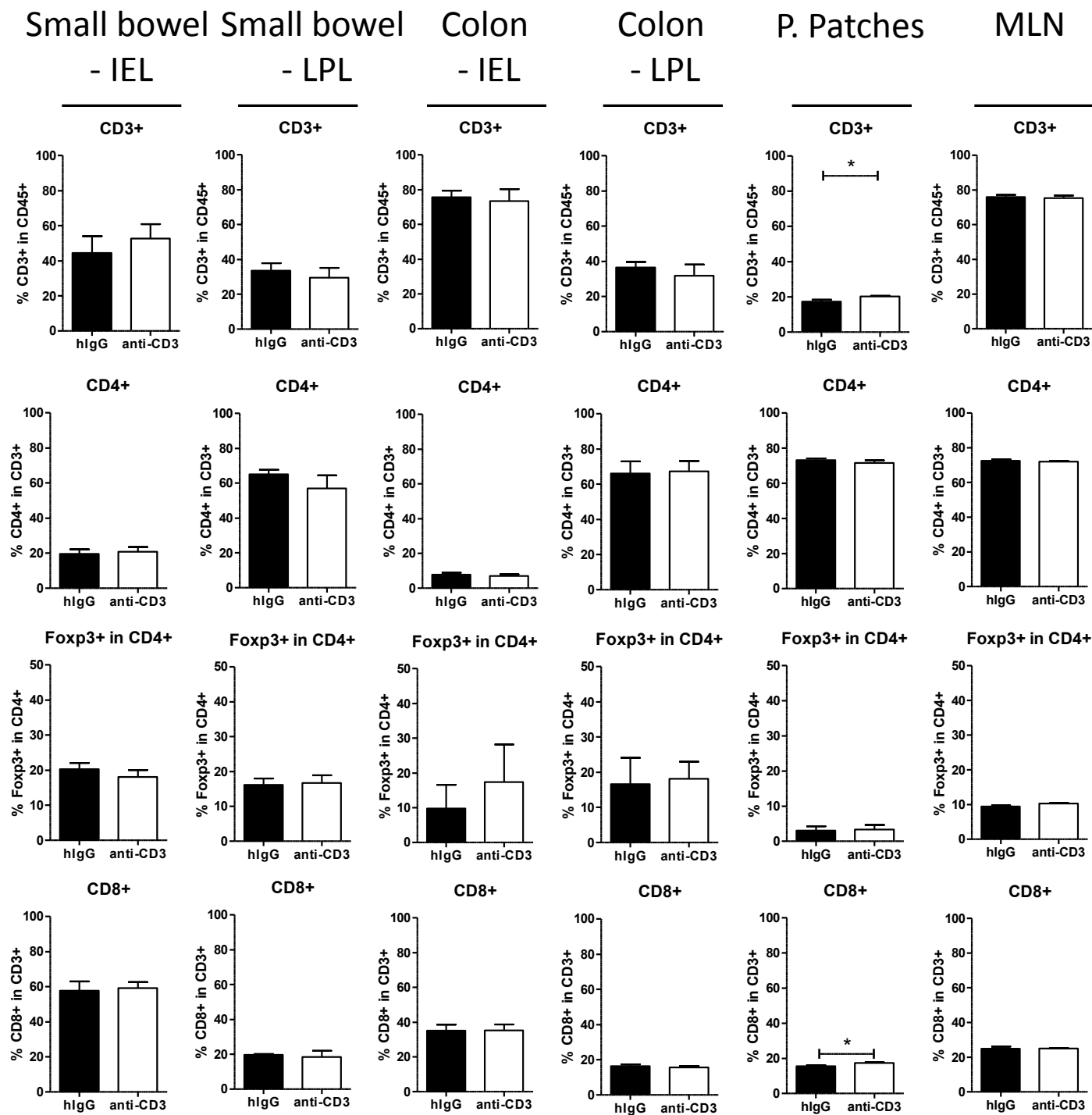


Figure S5. Oral anti-CD3 does not alter percentages of CD3⁺, CD4⁺ or CD8⁺ T cells nor induce Foxp3⁺ CD4⁺ T cells in Gut associates lymphoid tissues. BALB/c mice were fed anti-CD3 or hlgG for 3 days and cells harvested from MLN PP, IEL and LPL 24 hours after the last feeding (A) Depicted are mean SEM of the percentages of CD3⁺ cells within live CD45⁺ cells in small bowel and colon IEL and LPL, PP and MLN (**P*<0.05; Student *t* test) (B) Depicted are mean SEM of the percentages of CD4⁺ cells within gated CD3⁺ in small bowel and colon IEL and LPL, PP and MLN. (C) Depicted are mean SEM of the percentages Foxp3⁺ within CD4⁺ cells in small bowel and colon IEL and LPL, PP and MLN (D) Depicted are mean SEM of the percentages of CD8⁺ cells within gated CD3⁺ cells in small bowel and colon IEL and LPL, PP and MLN (**P*<0.05; Student *t* test).

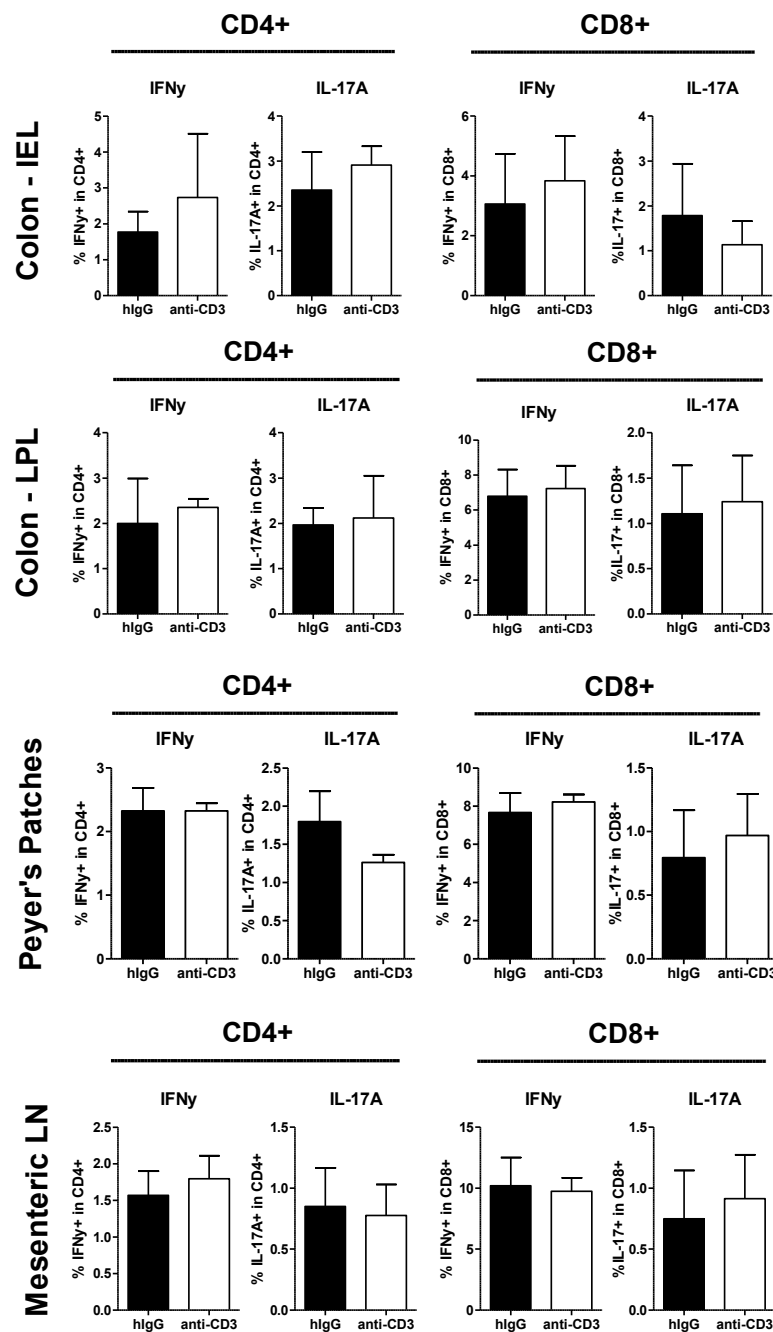


Figure S6. Oral anti-CD3 has no effect on the expression of IFN- γ and IL-17A in colon, PP and MLN. Colonic IEL and LPL, cells from PP and MLN were isolated from mice fed 5 μ g of anti-CD3 or hlgG for 3 days and stimulated in the presence of phorbol-12-myristate-13-acetate, ionomycin and Monensin prior to intracellular cytokine staining. Depicted are mean SEM of the percentages of cells expressing each cytokine within gated CD4 $^{+}$ or CD8 $^{+}$ cells (n=5 mice over 2 experiments).

Supplementary Methods and Materials

Lamina propria leukocytes and intraepithelial leukocyte isolation

Cell isolation was performed as described by Geddes et al.¹³ with some modifications: Small intestines and colons were harvested, washed in Ca^{2+} - Mg^{2+} -free HBSS, cut into 1-cm segments. Samples were incubated three times (37 °C, 15 min) in stripping buffer (Ca^{2+} - Mg^{2+} -free HBSS (Gibco, Grand Island, NY, USA), 10mM HEPES (BioShop, Burlington, ON, Canada), 25mM sodium bicarbonate (Sigma, St. Louis, MO, USA), 1 mM EDTA (BioShop). Following incubation, the buffer containing IEL was filtered through a series of three cell strainers: 100- μm (45 mL into 5mL of FBS (PAA Laboratories Inc., Etobicoke, ON, Canada)), followed by a 70- μm cell strainer and finally 40- μm to remove debris. IELs were pelleted and washed twice in HBSS containing 5% FBS. To obtain LPL, stripped tissue segments were minced and digested in RPMI (Gibco) containing 10% FBS and 1 mg/mL collagenase D (Roche, Mississauga, ON, Canada) for three 45-min incubations at 37 °C. Digested material containing LPL was passed through a series of 100- μm , 70- μm cell and 40- μm cell strainers, cells were pelleted and washed twice in RPMI containing 10% FBS.

Flow Cytometry

For intracellular cytokine staining, cells restimulated *in vitro* for 5 hours in the presence of phorbol-12-myristate-13-acetate (50ng/mL), ionomycin (1 μg /mL) (Sigma, Oakville, ON Canada) and Monensin (eBioscience, San Diego, CA, USA). Dead cells were stained with eFluor450 Fixable Viability Dye (eBioscience), followed by staining for surface antigens. Intracellular staining for cytokines and Foxp3 was performed using a kit as per manufacturer's instructions (#00-5523-00, eBioscience).

The following reagents and mAb's were obtained from eBioscience: PE-conjugated anti-CD45RB (C363 16A); PE- or PE-Cy7-conjugated anti-Foxp3 (FJK-16s); FITC-, APC- or A780-conjugated anti-CD4 (RM4-5, GK1.5); APC- or PE-Cy5.5-conjugated CD25 (PC61.5), APC-conjugated anti-CD8 α , PE-Cy5.5-Conjugated anti-IFN- γ , eF450 Fixable Viability Dye, Monensin. Anti-CD8 α , anti-CD11b, anti-B220 were purchased from BD Biosciences (Mississauga, ON, Canada) BV605-conjugated anti-IL-17A was and

functional grade anti-CD3 ϵ monoclonal antibody (145-2C11) were purchased from Biolegend (Biolegend, San Diego, CA, USA).

Mice

IL10^{-/-} mice have a propensity to develop spontaneous colitis. The B6.129P2-*Il10*^{tm1Cgn}/J animals used in this study were obtained from Jackson (Bar Harbor, Maine). According to the supplier, incidence of colitis in IL-10 deficient mice on the C57B/6 background is low, disease mild and occurring later in life. Small bowel involvement is very rare (incidence of 8% across all strains including more susceptible genetic backgrounds). For more information:

<http://jaxmice.jax.org/strain/002251.html>,

<http://jaxmice.jax.org/jaxnotes/archive/471a.html>