RESEARCH ARTICLE

Induced Treg Cells Augment the Th17-Mediated Intestinal Inflammatory Response in a CTLA4-Dependent Manner

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Abstract

Th17 cells and Foxp3+ regulatory T cells (Tregs) are thought to promote and suppress inflammatory responses, respectively. However, whether they counteract each other or synergize in regulating immune reactions remains controversial. To determine their interactions, we describe the results of experiments employing mouse models of intestinal inflammation by transferring antigen-specific Th cells (Th1, Th2, and Th17) differentiated in vitro followed by the administration of the cognate antigen via enema. We show that cotransfer of induced Tregs (iTregs) suppressed Th1- and Th2-mediated colon inflammation. In contrast, colon inflammation induced by transfer of Th17 cells, was augmented by the cotransfer of iTregs. Furthermore, oral delivery of antigen potentiated Th17-mediated colon inflammation. Administration of a blocking antibody against cytotoxic T lymphocyte-associated antigen 4 (CTLA4) abrogated the effects of cotransfer of iTregs, while the injection of a soluble recombinant immunoglobulin (Ig) fusion protein, CTLA4-Ig substituted for the cotransfer of iTregs. These results suggest that antigen-specific activation of iTregs in a local environment stimulates the Th17-mediated inflammatory response in a CTLA4-dependent manner.

Introduction

Accumulating evidence indicates that CD4+ helper T cells play a central role in eliciting normal immune responses and in inducing inappropriate reactions leading to allergy and autoimmune diseases [1]. For example, CD4+ regulatory T cells (Tregs) that express the transcription factor FoxP3 represent a distinct cell population with immunosuppressive function [1–3]. In contrast, effector CD4+ helper T cells are classified mainly into Th1, Th2, and Th17 subsets that induce physiological immune responses depending on the infectious pathogens. Unless attenuated after elimination of pathogens, or maintained tolerance to self or innocuous antigens, activation of these effector subsets initiates allergic or inflammatory disorders. The idea that an aberrant Th2-type immune response induces allergy and is regulated by FoxP3+ Tregs is consistent with the results of studies on humans and numerous mouse models [4–6]. In contrast,
the pathogenic role of Th17 cells on the development of autoimmune and inflammatory disorders remains controversial although the vast majority of recent findings from genome-wide studies of humans and mouse models support the intimate involvement of this subset in promoting the diseases [7–9]. This ambiguity may be explained as follows. First, most studies employ mouse models, including spontaneous occurrence of the diseases, which are driven by combinations of various T cell subsets, resembling human disease [10], which impedes the evaluation of the contribution of Th17 cells to pathogenesis. Second, the properties of Th17 cells are diverse and highly plastic in terms of immunological functions, including immune suppression under certain conditions [11–13]. Therefore, whether Th17-type immunity is susceptible to immunological tolerance or suppression mediated by FoxP3+ Tregs remains largely unknown. Moreover, evidence indicates that Tregs support the development of Th17 cells or promote Th17-mediated immunological responses [14–18] by secreting TGF-beta [19] or by consumption of IL-2 [17, 18]. Irrespective of the outcomes of interactions between Th17 cells and Tregs, the role of antigen specificity must be considered. Therefore, to delineate the outcomes caused by one-to-one interactions between iTregs and each effector T cells from otherwise complex immunological responses, we employed a model in which antigen-specific CD4+ T cells are adoptively transferred in combination followed by antigen delivery. We show here that the differential effects of iTregs depending on the effector subsets, and that CTLA4 is critically involved in both processes, inhibition of Th1/Th2-mediated colon inflammation and stimulation of Th17-mediated colon inflammation.

Results and Discussion

Antigen-specific effector cells induce colon thickening

CD4+ T cells were obtained from spleen and mesenteric lymph nodes of DO11.10 transgenic mice with a Rag2-deficient background (DO11.10+:Rag2 KO). Cells were differentiated in vitro in a mutually exclusive manner to an interferon (IFN)-gamma-producing Th1 subset, an interleukin (IL)-4-producing Th2 subset, and an IL-17A-producing Th17 subset under each polarizing condition described in Materials and Methods (S1A Fig) [20]. Approximately 2 × 10⁷ viable cells of each subset were intravenously transferred to wild-type BALB/c mice, and ovalbumin (OVA) was administered via enema once a week for 4 weeks. One day after the last administration of OVA, colon length and weight were measured and the length-to-weight ratio (colon thickness index, CTI), which correlates with histological scores [21], was calculated. Regardless of the Th subset (Th1, Th2, Th17) derived in vitro, all effector subsets increased CTI values, suggesting that each possesses intrinsic activities that induce an inflammatory response in the intestinal tract (Fig 1A and 1B). Furthermore, the induction of colon thickening depended on transfer of cells and delivery of antigen (S2 Fig).

Accumulation of eosinophils defined as CCR3+CD11b+ mononuclear cells in the colon lamina propria (cLP) was more prominent in mice that received Th2 cells (Fig 1C), which is consistent with the increased production of IL-5 (S1B Fig). CCR3+CD11b+ populations specifically expressed the eosinophil marker Siglec-F (Fig 1D). In contrast, the number of CD11b+Gr-1+ cells, which are thought to be neutrophils involved in Th17-type immunity [22, 23], were slightly increased in the cLP of mice that received Th17 cells compared with those in the cLP of mice that received Th1 and Th2 cells (Fig 1C).

Antigen-specific iTregs stimulate Th17-mediated colon thickening

Next, we examined the susceptibility of effector subsets to the suppressive function of iTregs that recognized the same epitope. For this purpose, OVA323-339 epitope-specific iTregs that expressed FoxP3 predominantly and increased levels of CD25 and CTLA4 were prepared.
Fig 1. Adoptive transfer of effector T cells induces colon thickening. Each effector T cell subset (2 × 10^7 viable cells) was intravenously transferred to wild-type BALB/c mice, and OVA protein was administered via enema once a week for 4 weeks. The day after the last OVA challenge, the colonic weight-to-length ratio (mg/mm) was calculated as the colon thickness index (CTI) to evaluate the inflammatory response. (A) Three types of effector cells (Th1/Th2/Th17) induced CTI using this model mouse. (B) Representative histological
Data acquired using similar models, that employ cotransfer of antigen-specific iTregs, followed by the administration of an antigen, regardless of target organ such as the respiratory or digestive tract [24–26], suggest that Th2 cells are highly susceptible to iTregs, however, the susceptibility of Th17 cells to the cotransfer of iTregs has been an arguable issue [27]. We show here that iTregs almost completely suppressed Th1 and Th2 cell-mediated colon thickening, although transfer of iTregs alone had no effect on CTI (Fig 2A), consistent with previous reports [24–26]. In contrast, simultaneously prepared iTregs that suppressed Th1 and Th2 cell-mediated colon thickening, stimulated Th17-mediated colon thickening. Accumulating evidence indicates that lineage stability of iTregs in vivo is not as robust as expected [28]. Therefore it might be possible that iTregs co-transferred with Th17 cells, but not with Th1 or Th2 cells may convert to Th17-like population with some effector function. This issue remains to be clarified further.

Oral-administration of OVA stimulates Th17-mediated colon thickening
Oral administration of antigen suppresses not only gut mucosal but also systemic immune responses, particularly Th2-type allergic reactions against challenge with the same antigen (oral tolerance) [29–31]. Moreover, the function(s) of FoxP3+ Tregs are prerequisite for establishing oral tolerance to allergic immune reactions such as allergic diarrhea and ear swelling induced by protein antigens [32]. However, it remains unknown whether immune responses derived solely from Th17 cells are affected by oral tolerization. We addressed this issue considering the stimulatory effects of iTregs on the Th17-mediated colon thickening described above. We applied a standard protocol to induce oral tolerance, by providing a continuous supply of OVA in drinking water for 7 days. This procedure resulted in the increased FoxP3+ ratio of transferred naïve DO+T cells not only prepared from cLP but also from spleen (S3A Fig). However, FoxP3+ ratio of endogenous CD4+T cells was unaltered, presumably because of the scarcity of the OVA-specific CD4+T cells detectable in this analysis (S3B Fig). Consistent with conventional findings, Th2- and Th1-mediated colon thickening were highly susceptible to immune suppression by prior oral administration of OVA, although the Th17-mediated increase in CTI was not inhibited but accelerated by tolerization (Fig 2B). Furthermore, histological analysis indicated the contrasting effects of oral tolerance on Th1,Th2- and Th17-mediated pathology.

Colon thickening is induced less efficiently by IL17A-deficient Th17 cells
IL-17A, which is expressed specifically by Th17 cells, plays a pivotal role in Th17-type immune responses, particularly in the recruitment of neutrophils to inflamed sites [33]. In fact, there are a couple of reports showing that IL-17A deficiency abrogated the immunopathology driven by Th17 cells [34]. Therefore, we determined the relative contribution of IL-17A to the Th17-mediated immune response in our experimental settings. For this purpose, we employed eosinophil-deficient mice as recipients to evaluate the accumulation of CD11b+Gr-1+ cells in the cLP in addition to the induction of colon thickening. Th17 cells derived and differentiated from IL17A-deficient mice mediated these processes, although to a diminished but significant...
extent (Fig 3A). Splenomegaly was present not only in mice engrafted with IL17A-sufficient Th17 cells but also in mice engrafted with IL17A-deficient Th17 cells (Fig 3A). The expression of IL-17F, which is most closely related to IL-17A, and the lineage-restricted transcription factor RORgammat were comparable to those of IL17A-sufficient Th17 cells in vitro (Figs 2, 3B and 3C). Moreover, migration to the cLP and the probability of survival were unaltered in vivo
Fig 3. *Il17a*-deficient Th17 cells induce a diminished but significant inflammatory responses. (A) Th17
cells were differentiated in vitro from naïve CD4+ T cells derived from *Il17a*-deficient (*Il17a*−/−:DO11.10+;Rag2-KO) or *Il17a*-sufficient (*Il17a*+/−:DO11.10+;Rag2-KO) mice. Eosinophil-deficient deltadblGATA mice
were engrafted and treated with OVA as described above. Spleen weights were measured and analyzed
(n = 4). The weight-to-length ratio of the colon was calculated and expressed as CTI. Mononuclear cells of the

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**A**

- Spleen weight (mg)
  - P = 0.001
  - P = 0.01
  - P < 0.001
  - P < 0.001

- Colon thickness index
  - P = 0.03
  - P < 0.001

**B**

- IL-17A (ng/mL) vs. IL-17F (ng/mL)
  - NS
  - P < 0.01
  - P < 0.05

- IL-2 (ng/mL)
  - NS
  - P < 0.01

**C**

- IL-21 (ng/mL) vs. GM-CSF (ng/mL)
  - P < 0.05

**D**

- Lamina propria
  - KJ1.26+ CD4+ viable cells
  - P = 0.001
  - P = 0.001

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*Il17a*-deficient Th17 cells were differentiated in vitro from naïve CD4+ T cells derived from *Il17a*-deficient (*Il17a*−/−:DO11.10+;Rag2-KO) or *Il17a*-sufficient (*Il17a*+/−:DO11.10+;Rag2-KO) mice. Eosinophil-deficient deltadblGATA mice
were engrafted and treated with OVA as described above. Spleen weights were measured and analyzed
(n = 4). The weight-to-length ratio of the colon was calculated and expressed as CTI. Mononuclear cells of the
Therefore, the induction of colon thickening and accumulation of CD11b⁺Gr-1⁺ cells by engrafting Th17 cells may be due to the redundant functional sums of the activities of IL-17A and other Th17-related cytokines. In fact, a couple of cytokines other than IL-17F, which are reported to be involved in Th17-mediated pathology, were more expressed in IL17A-deficient Th17 cells at least in vitro (Fig 3B), suggesting the intrinsic effect of IL-17A on the cytokine profile produced by Th17 cells. Thus the pathology via transferring IL17A-deficient Th17 cells might be driven by these cytokines.

Cotransfer of iTregs inhibits Th2-mediated colon thickening and stimulates Th17-mediated colon thickening using eosinophil-deficient mice as recipients

Transfer of effector T cells, particularly Th2 cells, led to the accumulation of eosinophils in the cLP, which is considered an inflammatory response of the intestinal tract (Fig 1). Therefore, we attempted to evaluate the involvement of eosinophils in the induction of CTI using eosinophil-deficient mice as recipients. Both Th17 cells and Th2 cells induced colon thickening in eosinophil-deficient recipients (Fig 4A), indicating that eosinophils residing in the cLP were dispensable for the induction of CTI or that other granulocytic populations may compensate in eosinophil-deficient mice. Moreover, the accumulation of CD11b⁺Gr-1⁺ cells was increased in eosinophil-deficient mice engrafted with Th17, but not Th2 cells (Fig 4B). These observations indicate that the accumulation of neither eosinophils nor neutrophils directly leads to colon thickening, although subset-specific immune reactions may occur. In contrast, differential effects of iTregs on Th2 and Th17 cell-mediated colon thickening were reconfirmed using eosinophil-deficient mice as recipients (Fig 4A). Furthermore, CD11b⁺Gr-1⁺ cells were much more abundant in mice that received iTreg and Th17 cells together compared with those that received Th17 cells alone (Fig 4B), indicating that iTregs play a dominant role in determining the stimulatory or inhibitory effect on colon thickening, irrespective of the type of granulocytes residing in the cLP.

Several kinds of molecular apparatus have been proposed to account for the immunomodulatory function of FoxP3⁺ Tregs [2, 35]. Of these, we tested the contribution of IL-10 expressed intrinsically in CD4⁺ T cell to colon thickening. The Il10-deficiency of Th2 cells induced a hyper-Th2 phenotype, that produced massive amounts of Th2-related cytokines in vitro (S4A and S4B Fig) and a higher abundance of eosinophils in the cLP in vivo (S4C Fig). However, II10-deficient iTregs suppressed the colon thickening mediated by Il10-deficient Th2 cells with efficiency comparable to that of Il10-sufficient iTregs (S4D Fig). The expression levels of FoxP3, CD25, and CTLA4 in iTregs differentiated in vitro were equivalent, irrespective of the presence of II10 (S4E Fig). Therefore, we next focused on the role of CTLA4 in this model system.
Anti-CTLA4 antibody abrogates the effects of iTregs and a CTLA4-Ig fusion protein mimics iTreg function

Although effector T cells other than Tregs express CTLA4 after stimulation [36], FoxP3⁺ cell-restricted deletion of Ctla4 leads to a sub-lethal multifocal inflammatory disorder similar to that caused by systemic deletion of Ctla4, albeit with a later age of onset [37]. This finding indicates that FoxP3⁺ Tregs require CTLA4 to restrain immune responses. We first employed an anti-CTLA4 antibody to evaluate the contribution of CTLA4 to iTreg-mediated modulation of colon thickening. Administration of the anti-CTLA4 antibody simultaneously cell transfer abrogated the effects of the iTregs, i.e., suppression of Th2-mediated colon thickening and enhancement of Th17-mediated colon thickening (Fig 5A). Histological observations were consistent with CTI values. It might be possible that anti-CTLA4 antibody directly targets Th17 cells or Th2 cells since CTLA4 is induced after activation of the effector T cells. However, administration of anti-CTLA4 antibody does not have significant effect on Th2- or Th17-mediated CTI induction in the absence of iTregs (Fig 5B). In addition, anti-CTLA4 antibody shows no effect on viability of engrafted iTregs or status of FoxP3 expression in vivo (S5 Fig). Therefore, it is likely that anti-CTLA4 antibody hampers the function of CTLA4 expressed on iTregs as well as, if any, endogenous Tregs. In this context, we were intrigued that FoxP3⁺ cell-specific deletion of Ctla4 resulted in an increase of the number of IFN-gamma⁺ or IL-4⁺ cells, but not that of IL-17⁺ cells [37], suggesting CTLA4 expressed on FoxP3⁺ cells plays a less prominent role in regulating the Th17-type response, yet apparent functional role in suppressing Th1- and Th2-type immune responses. Furthermore, deletion of Stat3 from FoxP3⁺ cells induces hyperactivation of Th17 cells in vivo, while Stat3-deficient Tregs express higher levels of Ctla4 mRNA compared with
Fig 5. Anti-CTLA4 antibody abrogates the effects of iTregs, and soluble CTLA4 alone mimics the effects of iTregs. (A) Effector T cells (Th2 or Th17) were intravenously transferred with or without iTregs in the presence or absence of anti-CTLA4 antibody (20 microg) and mice were treated as described in Fig 1. CTI values were calculated and are shown as mean and standard error (SE). Independent experimental sets were designed for histological analysis, and representative images are shown. (B) iTregs or effector T cells (Th2 or Th17) were intravenously transferred in the presence of anti-CTLA4 antibody (20 microg) or control antibody (20 microg, indicated as minus) and mice were treated as described in Fig 1. (C) Effector T cells (Th2 or Th17) were intravenously transferred in the presence or absence of an indicated amount of CTLA4-Ig, and mice were treated as described in Fig 1. CTI values were calculated and are shown as mean and SE.

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Stat3-sufficient Tregs do [38], suggesting that CTLA4 expressed by FoxP3+ cells may have little effect on suppressing Th17-type immunity or otherwise have a function to promote Th17-type immunity.

In contrast, recent reports show that Th17 cells acquired a follicular helper T cell (TFH cell) phenotype in vivo to induce an antigen-specific IgA production [39] or ectopic lymphoid follicles [40]. Consistent with these findings, transfer of Th17 cells increased OVA-specific IgA production, although Th1 and Th2 cells exhibited enhancement of OVA-specific IgA production compared with the control (S6A Fig). Th17-driven OVA-specific IgA secretion, however, was diminished by cotransfer of iTregs and abrogated again by simultaneous administration of the anti-CTLA4 antibody (S6B Fig). This suggests that the versatile functions of Th17 cells in vivo are distinct in terms of susceptibility to the iTreg-CTLA4 axis.

CTLA4 is regarded as an immunosuppressive molecule and numerous studies elucidating its molecular action [41, 42] have advanced the development of therapeutic applications for various immune-related disorders as well as anti-tumor immunotherapy [36, 43]. For example, the soluble fusion protein CTLA4-immunoglobulin (CTLA4-Ig) suppresses immune reactions in vitro and in vivo, presumably by binding to costimulatory ligands expressed by antigen presenting cells to block CD28 signaling in effector T cells [44]. In fact, CTLA4-Ig is effectively used to treat autoimmune and inflammatory diseases such as rheumatoid arthritis [45, 46]. Therefore, we addressed the effect of CTLA4-Ig on Th2- or Th17-driven intestinal immune responses. Th2 and Th17-mediated colon thickening responded differentially to the administration of CTLA4-Ig, in other words, Th2-mediated colon thickening was inhibited and at the same time and doses, Th17-mediated colon thickening was accelerated (Fig 5C). In addition, the frequency of DO+Th17 cells, but not of DO+Th2 cells among CD4+T cells was slightly increased in mice administered CTLA4-Ig (S7 Fig). These results indicate that restriction of costimulatory ligand availability and following inhibition of CD28 signaling, both of which are induced by CTLA4-Ig, lead to the opposite outcomes depending on the effector T cell subsets. This finding seems to be compatible with that of study showing that the differentiation of Th17 cells is blocked by an anti-CD28 antibody, indicating the adverse effect of CD28 signaling on the development of Th17 cells, at least in vitro [47]. In fact, we showed that differentiation of DO+Th17 cells as well as iTregs from naïve CD4+T cells was accelerated in the presence of CTLA4-Ig in terms of IL-17A+ ratio and FoxP3+ ratio, respectively (Fig 6A and S8 Fig), which is consistent with a report mentioned above [47]. Moreover, we found that even in the re-stimulation phase the addition of CTLA4-Ig maintains or rather augments the frequency of IL-17A-producing cells (Fig 6B). These results might indicate that administration of CTLA4-Ig favor the differentiation of Th17 cells through curbing of CD28 signaling, which is reminiscent of the function of TGF-beta in this process [48]. In this context, we examined the effect of anti-TGF-beta antibody in conjunction with CTLA4-Ig in our in vitro system. As a result, the addition of CTLA4-Ig increased the frequency of IL17A+producing cells even in the presence of anti-TGF-beta antibody in both process, differentiation from naïve to Th17 cells (Fig 6C) and re-stimulation of Th17 cells (Fig 6D). These observations seem to be consistent with the scenario that both immunomodulatory molecules, CTLA4 and TGF-beta act independently on the cell surface but following events merge to inhibit CD28-signaling cascade within the cells.

In this context, we were intrigued that mice administered an agonistic anti-CD3 epsilon antibody, which induces TCR stimulation without CD28 signaling, shows preferential accumulation of IL-17A-produing CD4+ T cells in the small intestine [11]. In the aggregate, Th17-type immune responses may favor the signal input delivered via the T-cell receptor under conditions of costimulation blockade. Therefore, targeting the CTLA4/CD28 signaling pathway should be carefully evaluated in relation to the presence of pathogenic effector subsets and target organs. In this respect, it is worth to note that in phase III trials, CTLA4-Ig had no
Fig 6. CTLA4-Ig augments the ratio of IL-17A-producing cells in the presence of anti-TGF-beta antibody. (A) CD4+ T cells prepared from DO11.10×Rag2-KO mice were stimulated under the condition for Th17 cell lineage, namely medium supplemented with IL-6, IL-23, TGF-beta1, IL-1beta and TNF-alpha, in the absence (control) or presence of CTLA4-Ig (+CTLA4-Ig, 20 microg/mL). After 7 days, cells were restimulated with PMA (20 ng/mL) and ionomycin (1 μM) in the presence of monensin for 4 h. Cells were stained with anti-CD4 and treated with FVD and subjected to the analysis for intracellular expression of the indicated cytokines (IL-17A and IL-17F) and FoxP3. (B) Th17 cells were stimulated again using the APCs (irradiated splenocytes derived from Rag2-KO mice) and OVA peptide in the medium without cytokines in the absence (control) or presence of CTLA4-Ig (+CTLA4-Ig, 20 microg/mL). (C) Th17 cells were stimulated using the Th17 recipe without TGF-β1 in the absence (control) or presence of anti-TGF-β1,2,3 antibody (+anti-TGF-β1,2,3, 1 μM). After 7 days, cells were restimulated and analyzed as described above. (D) Th17 cells were stimulated again using the APCs (irradiated splenocytes derived from Rag2-KO mice) and OVA peptide in the medium without cytokines in the absence (control) or presence of anti-TGF-β1,2,3 antibody (+anti-TGF-β1,2,3, 1 μM). After 7 days, cells were restimulated and analyzed as described above.
significant benefit for patients with inflammatory bowel diseases and, on the contrary, exacer-
bated ulcerative colitis [49]. Moreover, it was reported that patients with rheumatoid arthritis
developed ulcerative colitis during the treatment of CTLA4-Ig [50, 51].

The relevance of Th17-driven colon inflammatory responses described in the present study
must be considered from the perspective of the pathogenesis of inflammatory bowel disease
in the future. Taken together, the results of the present study demand reconsideration of
Treg/CTLA4-based immunological modulation to suppress or treat autoimmune diseases, par-
ticularly in patients with Th17-driven intestinal inflammation.

Materials and Methods

Mice

Balb/c mice were purchased from Nihon SLC (Shizuoka, Japan), DO11.10×Rag2-KO mice,
Il10-KO mice, and eosinophil-deficient (deltadblGATA) mice were obtained from The Jackson
Laboratory (Bar Harbor, ME). Il17A-KO mice were provided by Dr. Iwakura. Il10-KO mice
and Il17A-KO mice were backcrossed with Balb/c genetic background more than 10 times in
our facility to produce Il10-KO or Il17A-KO×DO11.10×Rag2-KO mice. Female mice 8 to 12
weeks older were used and bred in specific pathogen-free facilities at the Tokyo Metropolitan
Institute of Medical Science. This study was carried out in strict accordance with the guidelines
in the Proper Conduct of Animal Experiments, as defined by the Science Council of Japan and
the Animal Care and Use Committees of the Tokyo Metropolitan Institute of Medical Science
approved all experimental procedures (Permit Number:15035and 15036).

Evaluation of colon thickening and histology

The colon, starting from just below the cecum to above the anal, was excised. Connective tis-
ues were removed, cut longitudinally, washed to remove fecal material, following which length
and weight were measured to calculate the length-to-weight ratio. Independent experimental
groups were used to obtain transverse sections of mid-to distal-colons. for histology. Specimens
were formalin-fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Reagents

We purchased OVA from Sigma-Aldrich (St Louis, MO) and OVA peptide (323–339) from
Scrum Inc. (Tokyo, Japan).

Antibodies

eFluor 450-CD11b (M1/70), APC-eFluor 780-Gr-1 (RB6-8C5), FITC- and Pacific Blue-IFN-
gamma (XMG1.2), PE-Cy7-IL-4 (BVD6-24G2), Alexa Fluor 647- and Alexa Fluor 488-IL-17A
(eBio17B7), PerCP-Cy5.5-FoxP3 (FJK-16s), PE-ROGammat (B2D), PE-IL-10 (JES5-16E3),
APC-eFluor 780-CD4 (RM4-5), purified CD16/32 (93), and 7-AAD were purchased from
eBioscience. We purchased fluorescein-CRC3 (#83101) from R&D Systems; PE-Cy5-CD45
(30-F11), PE-Siglec-F (E50-2440), PE-CD152 (UC10-4F10-11), and PE-DO11.10 (KJ1-26, BD)
from BD Biosciences; Alexa Fluor 488-CD25 (PC61) and Alexa Fluor 647-IL-17F (9D3.1C8) from BioLegend; and VioBlue-DO11.10 (KJ1-26) from Miltenyi Biotec. Purified anti-CD152 (CTLA4) antibody (9H10) and isotype control antibody (SHG-1) used for in vivo studies was purchased from BioLegend, and anti-TGF-beta1,2,3 antibody (1D11) was R&D systems. The soluble fusion protein of CTLA4 and immunoglobulin (Ig) G1 Fc region (CTLA4-Ig, Abatacept) was purchased from Bristol-Myers Squibb.

**In vitro differentiation and adoptive transfer of OVA-specific T cells**

Antigen-specific effector T cells were prepared as described previously [20]. Approximately $2 \times 10^7$ viable effector T cells were transferred intravenously with or without $1 \times 10^7$ viable iTregs.

**OVA Treatment**

Two hundred microliters of OVA solution (10 mg/mL dissolved in PBS) was injected intra-recally with animal feeding needles (1.5 mm od x 52-mm long, FUCHIGAMI, Kyoto, Japan), such that the tip was 4 cm proximal to the anus. This treatment was repeated five times daily at approximately 10 min intervals for each. To establish oral tolerance, mice were fed with drinking water supplemented with 1 mg/mL OVA for 7 days before adoptive transfer of cells.

**Quantification of cytokines and OVA-specific IgA**

Cells were activated using Dynabeads T-Activator CD3/CD28 (Life Technologies) or by culturing with antigen-presenting cells (irradiated splenocytes derived from Rag2-KO) and OVA (50 mg/mL), and cytokines in the supernatants were measured using ELISA kits for IL-17F, IL-22, IL-21 and GM-CSF (eBioscience) or a multiplex bead array for IL-5, IL-13, IL-4, IL-10, IL-17A, and IL-2 (Millipore). Fecal extracts were obtained by adding weighed pellets to PBS (1 mL/100 mg fecal sample) containing protease inhibitors (P8340; Sigma-Aldrich). The samples were mixed and centrifuged, and the supernatants were collected for assay. OVA-specific IgA titers were determined using an ELISA with OVA as the capture antigen, and immune complexes were detected using horseradish peroxidase-conjugated anti-mouse IgA (Southern Biotech).

**Preparation of cells from the cLP and flow cytometric analysis**

Cells from the cLP were prepared by cutting the large intestine into 1-cm long pieces, and then stirred for 20 min at 37°C in PBS containing 5 mM EDTA and 5 mM EGTA to dissociate epithelial and intraepithelial cells. After washing with PBS three times, the remaining tissue was treated for 50 min at 37°C with RPMI containing 2 mg/mL collagenase D (Roche) and 1 mg/mL DNase I (Roche). Mononuclear cells were isolated using a discontinuous Percoll gradient (40% and 75%) and subjected to flow cytometry (FACScantoII, BD Biosciences). Following stimulation with PMA (20 ng/mL) and ionomycin (1μM) in the presence of monensin for 4h, intracellular staining of the cells was performed using Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience) according to the manufacturer’s instructions.

**Statistical analysis**

Data were analyzed using an unpaired two-tailed Student’s t test. A P-values of <0.05 was considered statistically significant.
Supporting Information

S1 Fig. Flow cytometric profiles of T cell subsets (Th1, Th2, Th17, and iTreg cells). CD4⁺T cells prepared from DO11.10⁺::Rag2-KO mice were polarized under conditions appropriate for each T cell lineage in the presence of APCs and OVA323-339 peptide (0.3μM), as described in Materials and Methods. (A) After 7 days, the cultured cells were restimulated with PMA (20 ng/mL) and ionomycin (1 μM) in the presence of monensin for 4 h. Cells were reacted with anti-CD4, anti-DO11.10 TCR, and anti-CD25 antibodies and treated with Fixable Viability Dye (FVD). The detection of intracellular expression of the indicated cytokines, transcription factors (FoxP3 and RORγt), and CTLA4 is described in Materials and Methods. CD4⁺DO11.10 TCR⁺ FVD⁻ cells were gated for the analysis. (B) Cells (1 x 10⁶) were stimulated in the presence of APCs (1 x 10⁶, irradiated splenocytes derived from Rag2-KO mice) and OVA (50 microg/mL) for 48 h and cytokine levels in culture supernatants were measured using multiplex bead assay.

S2 Fig. Specificity of colon thickness induction. Wild-type mice were engrafted or not engrafted with Il10-deficient Th2 cells and were not treated (none), or challenged with (OVA), or with (BSA) via enema.

S3 Fig. Administration of OVA increased the frequencies of FoxP3⁺ cells among the transferred DO⁺CD4⁺ cells. (A) CD4⁺T cells (1 x 10⁷) prepared from DO11.10⁺::Rag2-KO mice were transferred to wild-type BALB/c mice, and OVA protein was administered in a drinking water (1mg/mL) for a week (OVA-fed) or not (control). Mononuclear cells (MNCs) were isolated from the spleen (SPL) and colon lamina propria (cLP) of mice and subjected to the flow cytometric analysis. Frequencies of CD4⁺DO⁺ cells were shown and gated populations were analyzed for FoxP3 expression and ratio of FoxP3⁺ cells were shown in histograms. (B) Wild-type BALB/c mice were treated as described in (A). MNCs were isolated from the spleen (SPL) and subjected to the flow cytometric analysis as described in (A).

S4 Fig. Il10-deficient iTregs repress Th2-mediated colon thickening. Th2 and iTregs were differentiated in vitro from CD4⁺ T cells derived from Il10-deficient (Il10⁻/-:DO11.10⁺::Rag2-KO) or Il10-sufficient (Il10⁺/+:DO11.10⁺::Rag2-KO) mice. (A) Il10-sufficient (Il10⁻/WT) and Il10-deficient (Il10⁻/KO) Th2 cells were restimulated with 12-O-Tetradecanoylphorbol-13-acetate (PMA) (20 ng/mL) and ionomycin (1 μM) in the presence of monensin for 4 h. Cells were stained with mAbs against CD4, and the T cell receptor (DO11.10), and treated with Fixable Viability Dye, and subjected to flow cytometric analysis after incubation with antibodies against IL-4 and IL-10. The frequencies of cells expressing IL-4 or IL-10 were determined according to populations gated on CD4⁺DO⁺ cells and colon lamina propria (cLP) of mice engrafted with each combination of cells as indicated and incubated with antibodies against CCR3 and Siglec-F. Representative flow cytometric profiles are shown with the frequencies of CCR3⁺Siglec-F⁺ cells in MNCs isolated from the cLP. (C) CTI values were calculated and are shown as mean and standard error (SE). (E) Il10-sufficient (Il10⁻/WT) and Il10-deficient (Il10⁻/KO) iTregs were restimulated as in (A) and stained with
anti-CD4, anti-DO11.10 TCR, and anti-CD25 mAbs followed by Fixable Viability Dye staining. Cells were subjected to flow cytometry to determine the intracellular expression of IL-4, IL-10, CTLA4, and FoxP3. CD4+DO11.10 TCR+ FVD cells were gated for the analysis.

**S5 Fig. Anti-CTLA4 antibody shows no effect on survival of iTregs.** (A) MNCs were isolated from the spleen and cLP of mice engrafted with iTregs in the presence of anti-CTLA4 antibody (20 microg) or control antibody (20 microg) and treated as described in Fig 1. CD4+ T cells were enriched using anti-CD4 magnetic beads (Miltenyi Biotech) and stained with indicated antibodies. Representative flow cytometric profiles are shown with the frequencies of CD3epsilon+DO+ cells and FoxP3+ cells gated on CD3epsilon+DO+ cells. (B) As a control for FoxP3 staining, Th17 cells were used and analyzed in the same way as described in (A).

**S6 Fig. Increase of IgA secretion by Th17 cells is suppressed by cotransfer of iTregs.** (A) Fecal extracts were prepared the day after the third challenge with OVA, and OVA-specific IgA was detected using ELISA to estimate relative endpoint titers as described in Materials and Methods. (B) Th17 cells with or without iTregs were transferred in the absence or presence of an anti-CTLA4 antibody (20 microg). After the third challenge with OVA, fecal samples were collected, and relative titers of OVA-specific IgA were determined.

**S7 Fig. The effect of CTLA4-Ig on probability of survival of Th17 cells and Th2 cells in vivo.** Effector T cells (Th2 or Th17) were intravenously transferred in the presence or absence of CTLA4-Ig (50 microg), and mice were treated as described in Fig 1. MNCs were isolated from the SPL or cLP of mice engrafted and subjected to the flow cytometric analysis. Frequencies of CD3epsilon+CD4+ cells were shown and gated populations were analyzed for KJ1.26 staining and ratio of DO (KJ1.26)+ cells were shown in histograms.

**S8 Fig. CTLA4-Ig augments the ratio of FoxP3+ cells.** CD4+ T cells prepared from DO11.10+ Rag2-KO mice were stimulated under the condition for iTreg lineage, namely medium supplemented with IL-2, TGF-beta1 and retinoic acid (iTreg recipe), or medium containing IL-2 and TGF-beta1 but not retinoic acid (iTreg recipe without retinoic acid) in the absence (control) or presence of CTLA4-Ig (+CTLA4-Ig, 20 microg/mL). After 7 days, cells were subjected to the analysis as described in S1 Fig.

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**Author Contributions**

Conceived and designed the experiments: NW TH. Performed the experiments: NW OS NK. Analyzed the data: NW OK NK. Contributed reagents/materials/analysis tools: NW OK NK. Wrote the paper: NW TH.
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