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# Intestinal Bacterial Colonization Induces Mutualistic Regulatory T Cell Responses

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## SUMMARY

**Mammals harbor a dense commensal microbiota in the colon. Regulatory T (Treg) cells are known to limit microbe-triggered intestinal inflammation and the CD4$^+$ T cell compartment is shaped by the presence of particular microbes or bacterial compounds. It is, however, difficult to distinguish whether these effects reflect true mutualistic immune adaptation to intestinal colonization or rather idiosyncratic immune responses. To investigate truly mutualistic CD4$^+$ T cell adaptation, we used the altered Schaedler flora (ASF). Intestinal colonization resulted in activation and de novo generation of colonic Treg cells. Failure to activate Treg cells resulted in the induction of T helper 17 (Th17) and Th1 cell responses, which was reversed by wild-type Treg cells. Efficient Treg cell induction was also required to maintain intestinal homeostasis upon dextran sulfate sodium-mediated damage in the colon. Thus, microbiota colonization-induced Treg cell responses are a fundamental intrinsic mechanism to induce and maintain host-intestinal microbial T cell mutualism.**

## INTRODUCTION

The human colon is home to an extremely dense bacterial microflora that can reach densities of up to $10^{12}$ bacteria per gram (Whitman et al., 1998). Colonization of this rich habitat with a commensal bacterial microbiota starts immediately at birth and continues through childhood until a stable commensal flora is established (Tannock, 2007). It is known that the presence of intestinal bacteria is beneficial to the host; it provides colonization resistance for pathogens, enhances our ability to harness energy from otherwise inaccessible food components, and is an important stimulus for the maturation of the mucosal and systemic immune systems (Macpherson et al., 2005; Smith et al., 2007).

It is believed that a breakdown in intestinal immune homeostasis is a major cause of chronic inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis (Sartor, 1997). However, because of the diversity of the commensal flora, the immunological adaptations that occur in response to intestinal colonization are complex and only poorly understood. This is partly due to the fact that it is difficult to determine which bacterial species within the population behave as commensals, mutualistic, or opportunistic pathogens. The CD4$^+$ T cell compartment, including T helper 1 (Th1), Th17, and Foxp3$^+$ regulatory T (Treg) cells, is important for intestinal immune homeostasis in the presence of a nonpathogenic commensal flora (Barnes and Powrie, 2009). Th17 (as well as Th1) cells are constitutively present in the face of a complex flora but are absent in germ-free mice (Ivanov et al., 2006, 2008). Furthermore, individual bacterial species such as segmented filamentous bacteria (SFB) are potent inducers of intestinal Th17 cell responses (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Foxp3$^+$ Treg cells are also constitutively present in the intestinal mucosa and gut-associated lymphoid tissues (GALT) and are critical for keeping inflammatory responses at bay (Izcue et al., 2009). There is evidence that mucosal Treg cells are continuously generated through the action of specialized dendritic cell (DC) subsets via retinoic acid (Benson et al., 2007; Coombes et al., 2007; Iwata, 2009; Jaensson et al., 2008; Sun et al., 2007). In addition, indigenous Clostridia are potent inducers of mucosal and systemic Treg cell responses (Atarashi et al., 2011). However, the complexity and diversity of the commensal microflora in current mouse models makes it extremely difficult to identify truly mutualistic immunological adaptations that induce and maintain intestinal CD4$^+$ T cell homeostasis during and after colonization. The use of single isolated species such as SFB (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Suzuki et al., 2004) or investigating the effects of single bacterial components such as polysaccharide A (PSA) from *B. fragilis* (Mazmanian et al., 2008; Round and Mazmanian, 2010) as well as focusing purely on an unbalanced Clostridia flora (Atarashi et al., 2011) might reveal only mechanisms that are important in the context of these idiosyncratic responses and are therefore limited in their capacity to model true commensal mutualism.

We show here that colonization of germ-free mice with clearly defined and absolutely benign intestinal commensal altered Schaedler flora (ASF) species (Dewhirst et al., 1999) resulted in the compartmentalized expansion, activation, and de novo generation of mucosal Treg cells specifically in the colon lamina propria (cLP). Induction of Treg cells was required for intestinal CD4$^+$ T cell homeostasis as reflected by the absence of mucosal Th17 or Th1 cell responses. Efficient Treg cell activation required the Toll-like receptor (TLR) adaptor molecules MyD88 and

Ticam-1. In addition, successful establishment of intestinal $CD4^+$ T cell homeostasis was dependent on interleukin-10 receptor (IL-10R) signaling whereas induction and activation of Treg cells was not affected by blocking IL-10R signaling. The commensal-induced Treg cell response was functional because the lack of Treg cell activation resulted in mucosal immune deviation toward Th1 and Th17 cell responses, which could be rescued by transfer of wild-type Treg cells. The composition of the ASF and its benign noninvasive properties were identical in the presence and absence of functional Treg cell activation. This finding shows that, in the absence of a mutualistic Treg cell response, deviation toward a Th17 cell response occurs even in the absence of SFB. Commensal induction of an intestinal Treg cell response is therefore an important intrinsic mechanism for the establishment of immune homeostasis after colonization that lays the foundation upon which all subsequent immune responses build.

## RESULTS

### Intestinal Colonization Results in Proliferation and Activation of Mucosal Treg Cells

Although previous studies have reported that the Treg cell population does not differ between germ-free mice and mice colonized with a diverse flora (Chinen et al., 2010; Min et al., 2007), we found that intestinal colonization of germ-free mice resulted in expansion of the $CD4^+CD25^+Foxp3^+$ Treg cell population in the cLP, but not in the spleen, mesenteric lymph nodes (MLN), Peyer's patches (PP), small intestinal lamina propria, or thoracic duct lymph (Figure 1; Figures S1A–S1C available online). Increased proportions of Treg cells upon intestinal colonization were observed in five different inbred or outbred wild-type mouse strains, indicating that Treg cells are selectively and generally induced in the intestinal mucosa regardless of murine genetic wild-type background (Figure 1A). All animals were colonized with a mixture of bacterial species, referred to as altered Schaedler flora (ASF) (Dewhirst et al., 1999), which is a precisely defined and completely benign model commensal microbiota. Analysis of the expanded Treg cell population showed a high proportion of activated integrin $\alpha_E$ (CD103)-expressing effector Treg cells (Figure 1B), which are known to be particularly potent in inhibiting immune responses (Huehn et al., 2004; Lehmann et al., 2002; Siewert et al., 2008; Stephens et al., 2007; Zhao et al., 2008). Until recently, thymic-derived natural Treg (nTreg) cells could not be easily distinguished from de novo generated induced Treg (iTreg) cells via phenotypic markers (Barnes and Powrie, 2009; Curotto de Lafaille and Lafaille, 2009). However, the transcription factor Helios now seems a likely marker for the distinction of nTreg cells and iTreg cells because its expression appears to be restricted to thymic Treg cells (Thornton et al., 2010). We found that the proportion of $Helios^-$ Treg cells in cLP increased upon colonization, indicating proliferation or de novo generation of $Helios^-$ Treg cells (Figure 1C). However, the population of $CD103^+$ effector Treg cells increased upon colonization in both the $Helios^-$ and $Helios^+$ populations, indicating that a proportion of both populations acquired an activated phenotype upon colonization (Figure 1D). Analyzing the expression of other Treg cell markers such as GITR, PD-1, or CD127 revealed that CD103 was the only reliable Treg cell activation marker upon colonization (Figure S1D).

Induction of colonic Treg cell populations in response to ASF colonization correlated with the absence of effector $CD4^+$ T cell differentiation to Th1 or Th17 cell subsets (Figure S1E), suggesting that Treg cell activation was required to establish intestinal $CD4^+$ T cell homeostasis. We know that colonization of germ-free mice with ASF bacteria occurs without inflammatory alterations such as neutrophil infiltration, protein-losing enteropathy, or increases in paracellular permeability (Macpherson and Uhr, 2004; Slack et al., 2009). It is also clear that colonization induces secretion of immunoglobulin (Ig) A and almost no other isotype in the mucosa (Hapfelmeier et al., 2010). Therefore, increases in colonic Treg cells appears to be a further characteristic adaptation of the mucosa to immune mutualism with an ASF microbiota, and activated Treg cells accumulate selectively in the colonic lamina propria in response to colonization with a benign mutualistic commensal flora.

### Establishment of Intestinal Homeostasis during Colonization Is IL-10 Dependent

We next investigated the role of the regulatory cytokine IL-10 in the establishment of successful intestinal $CD4^+$ T cell homeostasis during colonization with a benign noninflammatory commensal flora. We found that induction of *Il10* expression after intestinal colonization with ASF was strictly compartmentalized and induced only in $CD4^+$ T cells from cLP, but not in $CD4^+$ T cells from SPL, MLN, or PP (Figure 2A). This was in contrast to the systemic effects on IL-10 expression observed after colonization with a mixture of 46 Clostridia strains (Atarashi et al., 2011). We next addressed the requirement for intact IL-10R signaling in the establishment of immune homeostasis. Groups of mice were treated with either a blocking IL-10R antibody or an isotype control (35.61) during 4 weeks of commensal colonization. Blocking IL-10R signaling, but not treatment with an isotype control, resulted in intestinal T cell immune deviation and development of Th1 and Th17 cell responses in cLP (Figure 2B). These responses were flora dependent because treating germ-free mice with IL-10R antibody did not induce Th1 or Th17 cell responses (Figure 2B). Thus, IL-10 is an essential cytokine for successful establishment of host-microbial mutualism. IL-10R blockade during colonization did not alter the generation of $CD103^+$ or $Helios^-$ Treg cells (Figure 2B).

### Treg Cell Proliferation and Activation Are Required to Prevent Mucosal Immune Deviation

It has been reported that IL-10 from $CD4^+$ cLP T cells is mostly derived from $Foxp3^+$ Treg cells (Maynard et al., 2007), so we therefore experimentally addressed the role of $Foxp3^+$ Treg cells in the induction of intestinal $CD4^+$ T cell homeostasis after colonization. From the generality of the Treg cell adaptation in the mucosal immune system as mice acquire a benign intestinal microbiota, we hypothesized that the bacterial-induced Treg cells were likely to be essential to establish noninflammatory mucosal immune mutualism. The key question was whether the observed activation and expansion of intestinal Treg cells was critical to establish normal homeostasis even in the presence of an absolutely benign commensal microbiota lacking SFB. To address this experimentally, we made use of the T cell receptor (TCR) transgenic SMARTA mouse (Oxenius et al., 1998), where 98%–100% of peripheral $CD4^+$ T cells carry a transgenic $V\alpha2^+$ TCR

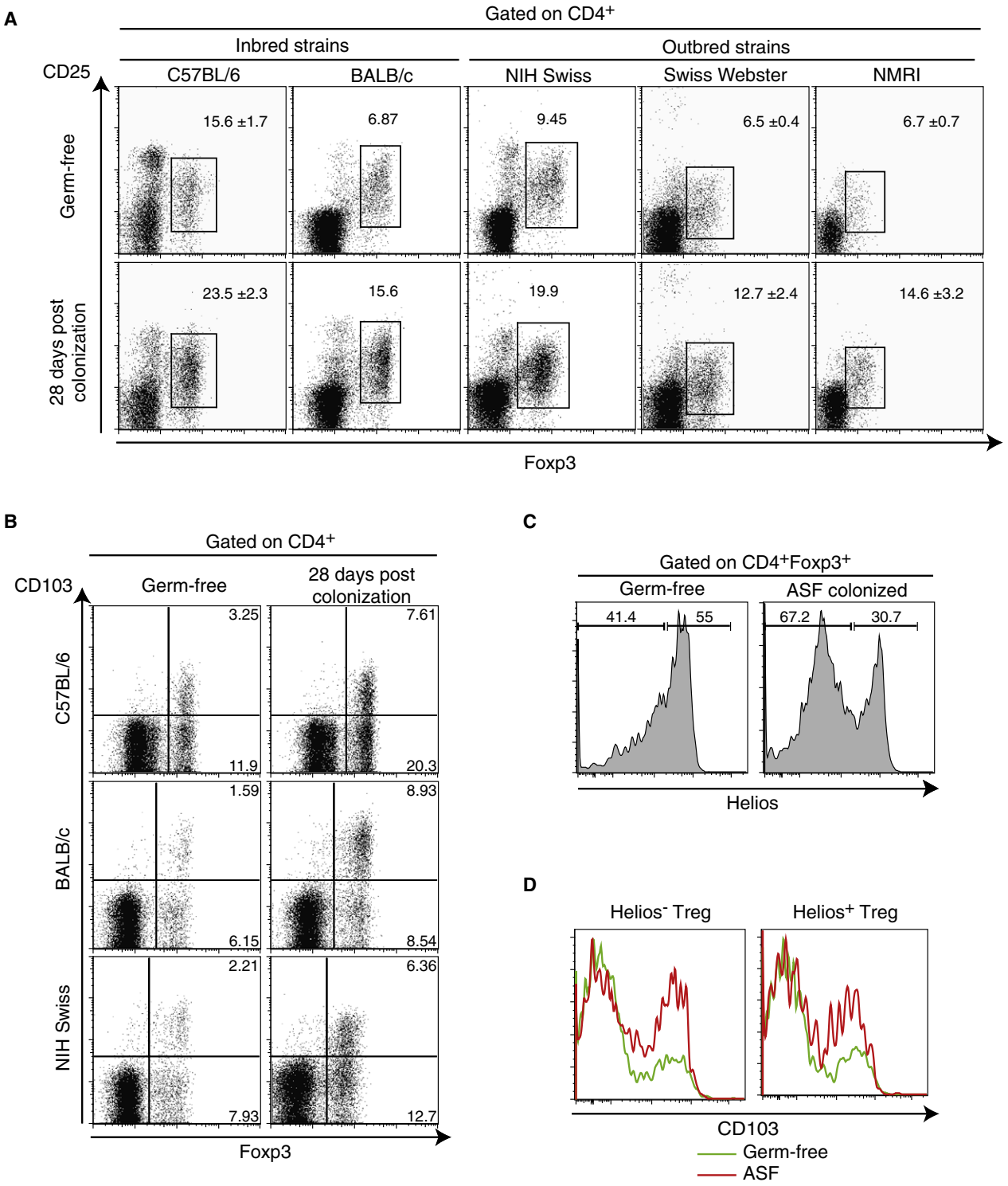


**Figure 1. Colonization of Germ-free Mice Induces a Regulatory T Cell Response Selectively in the Colon Lamina Propria**

(A and B) Flow cytometry for Treg cells (A) and CD103 expression (B) in cLP of germ-free and 28 days colonized mouse strains. Representative dot plots from pooled animals (n = 3–4) for each strain and time point are shown. Data shown for C57BL/6, Swiss Webster, and NMRI are representative of at least two to three independent experiments. Numbers represent mean ± SD where indicated.

(C) Helios expression of Foxp3$^+$ Treg cells before (germ-free) and after (ASF) colonization of C57BL/6 mice.

(D) Expression of CD103 in Helios$^-$ and Helios$^+$ Treg cell populations before (germ-free) and after (ASF) colonization of C57BL/6 mice.

See also Figure S1.

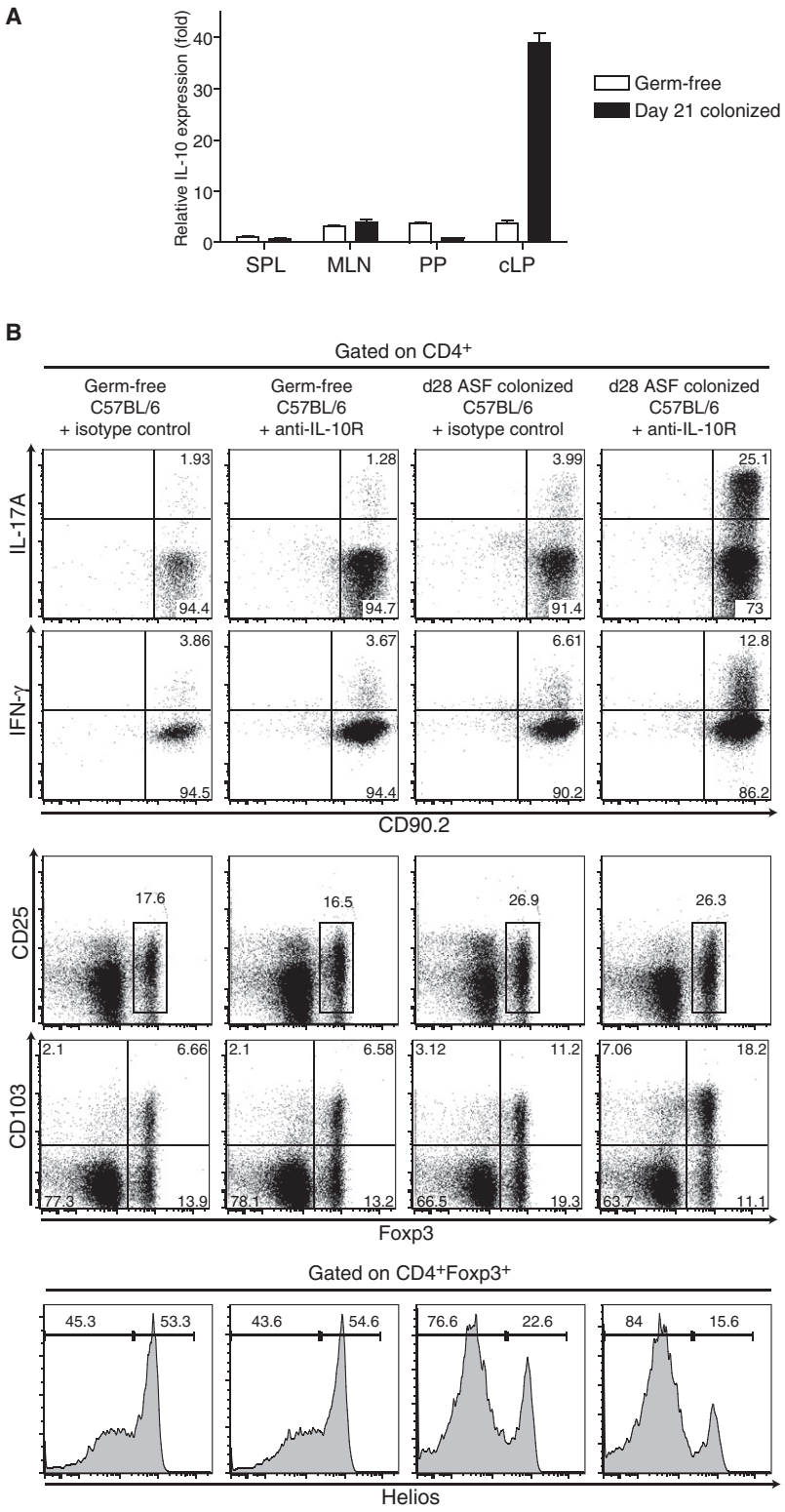


**Figure 2. Establishment of Intestinal CD4⁺ T Cell Homeostasis Is IL-10 Dependent**

(A) *Il10* expression levels were normalized to *Gapdh* and are shown relative to germ-free SPL, which is set to 1. Data were obtained from RNA of pooled purified CD4⁺ cells from germ-free (n = 8) or 21 days colonized (n = 10) C57BL/6 mice. Error bars show the mean ± SD of triplicates. One of two independent experiments is shown.

(B) Indicated groups of mice treated twice weekly with 500 μg of an IL-10R blocking antibody (clone 1B1-2) or Rat IgG1, κ isotype control (clone 35.61) intraperitoneally (i.p.) for 4 weeks. Th1, Th17, and Treg cell populations were analyzed by flow cytometry. Representative dot plots from cLP of pooled animals (n = 3–4) per group are shown. One representative of two similar independent experiments is shown.

specific for the lymphocytic choriomeningitis virus (LCMV) glycoprotein-derived peptide GP61. We had two important rationales for selecting this strain; first, these mice have dramatically reduced proportions of Treg cells in SPL and MLN (Figures S2A and S2B), and second, SMARTA T cells do not nonspecifically proliferate in response to commensal microbial stimulation (Ramsey et al., 2008). When housed under standard specific-pathogen-free (SPF) or conventional conditions, it had been observed both in Zürich (Switzerland) and Hamilton (Ontario, Canada) that a small proportion of these mice developed spontaneous rectal prolapse (data not shown), further suggesting that commensal-host immune homeostasis might be defective in these mice.

Germ-free SMARTA mice were healthy and had no excess Th1 or Th17 cells in cLP (Figure S2C) compared with germ-free C57BL/6 mice. Although no pathological changes were observed by histology at the level of the intestinal mucosa after ASF colonization of germ-free SMARTA mice (Figure S2D), they failed to activate and expand Foxp3⁺ Treg cells after colonization (Figure 3A; Figure S2C). Interestingly, in contrast to C57BL/6 mice, SMARTA mice also failed to generate Helios⁻ Treg cells upon colonization (Figure 3A). Importantly, Th17 and Th1 effector cells were readily detectable in the colonic mucosa of SMARTA mice upon colonization (Figure 3B; Figure S2C).

It is important to note that the TCR-transgenic SMARTA mice used here were on a wild-type C57BL/6 background and therefore although having an almost monoclonal TCR repertoire with specificity for the irrelevant GP61, endogenous polyclonal dual TCR expression might explain why only a fraction of the Vα2⁺ population differentiated into Th17 or Th1 cells (Figure 3B; Figure S2C). Antigen-specific stimulation with DC pulsed with an ASF extract did not result in production of IL-17A or IFN-γ (Figure S2E). Despite the fact that the SMARTA Treg cell population might also express a dual TCR with polyclonal specificity, generation of Helios⁻ Treg cells after colonization was almost completely abrogated in SMARTA mice (Figure 3A), suggesting that the stringency of Treg cell induction is

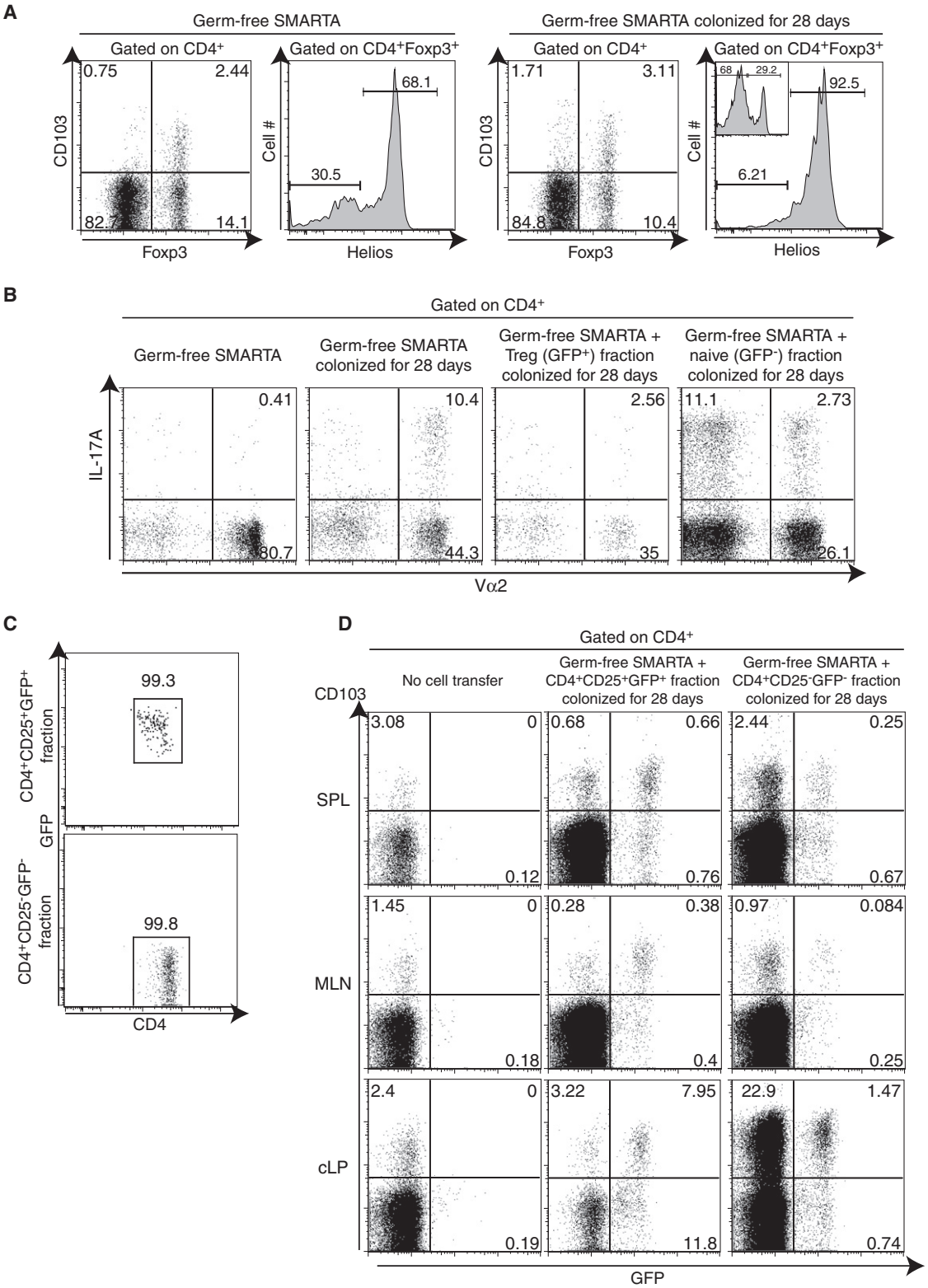


**Figure 3. Activation of Foxp3⁺ Treg Cells Is Required for Induction and Establishment of Intestinal Immune Homeostasis upon Colonization**

(A) Flow cytometry for Foxp3⁺ Treg cells and Helios expression in cLP Treg cells of germ-free or 28 days colonized SMARTA mice. The inset shows Helios expression in Treg cells of 28 days colonized wild-type controls.

(B) IL-17 production by Th17 cells was measured by flow cytometry after PMA+ionomycin stimulation.

(C) Purities of flow cytometry-sorted $CD4^+CD25^+GFP^+$ Treg cells and $CD4^+CD25^-GFP^-$ naive T cells.

greater than that for Th17 or Th1 cells in the face of this very restricted TCR repertoire.

The aberrant expansion of Th17 and Th1 cells in the colonic mucosa in the absence of an induced Treg cell response suggested that Treg cell function in the intestinal mucosa was critical to preserve host microbial mutualism and the mucosal $CD4^+$ Th17 and Th1 cell subset balance even in the presence of a benign commensal microbiota.

## Transfer of Wild-Type Treg Cells Protects SMARTA Mice from Mucosal Immune Deviation

To address whether the defect in establishing intestinal $CD4^+$ T cell homeostasis upon colonization of germ-free SMARTA mice was indeed due to a Treg cell defect, rather than an increased intrinsic differentiation of Th1 and Th17 cells, we next performed transfer experiments of wild-type Treg cells into germ-free SMARTA mice. If mucosal Treg cells are functionally required to establish mucosal $CD4^+$ T cell subset mutualism in the presence of intestinal commensal microbes, we hypothesized that it should be possible to rescue SMARTA mice from immune deviation upon colonization by transferring wild-type Treg cells. We transferred flow cytometry-sorted wild-type $CD4^+CD25^+GFP^+$ Treg cells (Figure 3C) from Foxp3-GFP reporter mice (Fontenot et al., 2005) into germ-free SMARTA mice prior to colonization. After 28 days of colonization, we could detect homing and activation of $GFP^+$ Treg cells in cLP (Figure 3D). Transferred wild-type Treg cells were predominantly present in cLP rather than in secondary lymphoid structures such as SPL and MLN and about half of these were $CD103^+$ effector Treg cells (Figure 3D). Accumulation of these wild-type Treg cells in cLP restored homeostatic mutualism as the induction of Th1 or Th17 cells was abolished (Figure 3B). Identical results were obtained after transfer of wild-type Treg cells from congenic $CD45.1^+$ donor mice (Figures S2C and S2F). Therefore, Treg cell function is required to limit $CD4^+$ T cell deviation in the cLP under conditions of benign intestinal bacterial colonization.

Because of the Helios results indicating that iTreg cells are induced upon colonization and the finding that SMARTA mice have a defect in the generation of $Helios^-$ Treg cells, we also used Foxp3-GFP reporter mice to ask whether iTreg cells are de novo induced upon colonization with intestinal commensal bacteria. After transfer of naive $CD4^+CD25^-GFP^-$ T cells from Foxp3-GFP reporter mice (Figure 3C), $GFP^+$ Treg cells were detected in the cLP and more than 50% of these had an activated $CD103^+$ phenotype (Figure 3D). From the purity of the transferred $GFP^-$ population (Figure 3C, >99%), this suggested that these were truly de novo generated in vivo converted iTreg cells. Because only negligible populations of $GFP^+$ iTreg cells could be detected in MLN and SPL (Figure 3D) and no $CD103^+$ Treg cells could be found in the thoracic duct lymph after colonization (Figure S1C), it is likely that de novo generation of iTreg cells and their activation to $CD103^+$ status mainly occurred in cLP. However, because only a small fraction of the transferred $GFP^-$ population was converted into $GFP^+$ iTreg cells resulting in a high proportion of $CD4^+GFP^-CD103^+$ donor effector T cells in the recipient mice (Figure 3D), there was no protective effect because the $CD103^+GFP^-$ effector population substantially contributed to the $CD4^+V\alpha2^-$ Th17 cell response observed in cLP (Figure 3B). These findings demonstrate directly in vivo that activation and proliferation of Treg cells is required to prevent immune deviation of the mucosal immune system toward Th1 and Th17 cells and that de novo generated iTreg cells may contribute to the expanding Treg cell population in cLP upon colonization of germ-free mice.

## Commensal Flora-Induced Treg Cell Activation Is an Intrinsic Mechanism for Homeostasis

We next addressed the minimal requirements for Treg cell activation and expansion during colonization. Specifically, we wished to determine whether control of immune deviation of the mucosal immune system toward Th1 and Th17 cells is a genuinely intrinsic function of mucosal immune regulation in the presence of a benign microbiota rather than an idiosyncratic result of stimulation with particular microbial species. This is important because the composition of the microflora can have dramatic extrinsic effects on the induction of Th1 and Th17 cell responses, as well as on the proportion of Treg cells within the intestine (Atarashi et al., 2011; Gaboriau-Routhiau et al., 2009; Ivanov et al., 2008, 2009). Although both C57BL/6 and SMARTA germ-free mice were colonized with the same ASF sentinel to ensure a common microbiota, it is possible that the lack of Treg cell induction in SMARTA mice could allow for differential colonization leading to qualitative or quantitative changes in the composition of the microbiota. However, analysis of the flora in both mouse strains by 16S rDNA sequencing 28 days after colonization revealed no differences (Figure 4A). In addition, specific PCR confirmed the absence of SFB (Figure S3A), which has been shown to be a potent inducer of Th17 cell responses (Ivanov et al., 2009). We also performed electron microscopy on colon samples from both 28 days colonized C57BL/6 and SMARTA mice and could not observe an increased propensity of the flora in SMARTA mice to associate with the host epithelia or invade intestinal tissue in the absence of a functional Treg cell response (Figure 4B). Therefore, Th17 cell induction in ASF-colonized SMARTA mice was independent of the flora composition and did not require the presence of SFB (Figure S3B). In addition, Th17 cell induction was not due to a more invasive behavior of the ASF microbiota in SMARTA mice, suggesting a Treg cell-intrinsic effect.

It is likely that SFB-mediated induction of intestinal Th17 cell responses is linked to the close interaction of SFB with the epithelium (Ivanov et al., 2009). Given the clear adherence and epithelial damage by SFB in electron micrographs (Figure 4C), this bacterial species cannot be considered as entirely benign and therefore the mucosal immune response generated in response to SFB does not reflect a truly mutualistic adaptation. We did not detect any bacterial interactions with the intestinal

(D) Flow cytometry to detect homing of $GFP^+$ Treg cells and conversion of $GFP^-$ into $GFP^+$ Treg cells after intravenous transfer of the $GFP^+$ or $GFP^-$ population into germ-free SMARTA mice and colonization for 28 days. Data shown are from 3–4 pooled mice per group. One representative of two independent experiments is shown.

See also Figure S2.

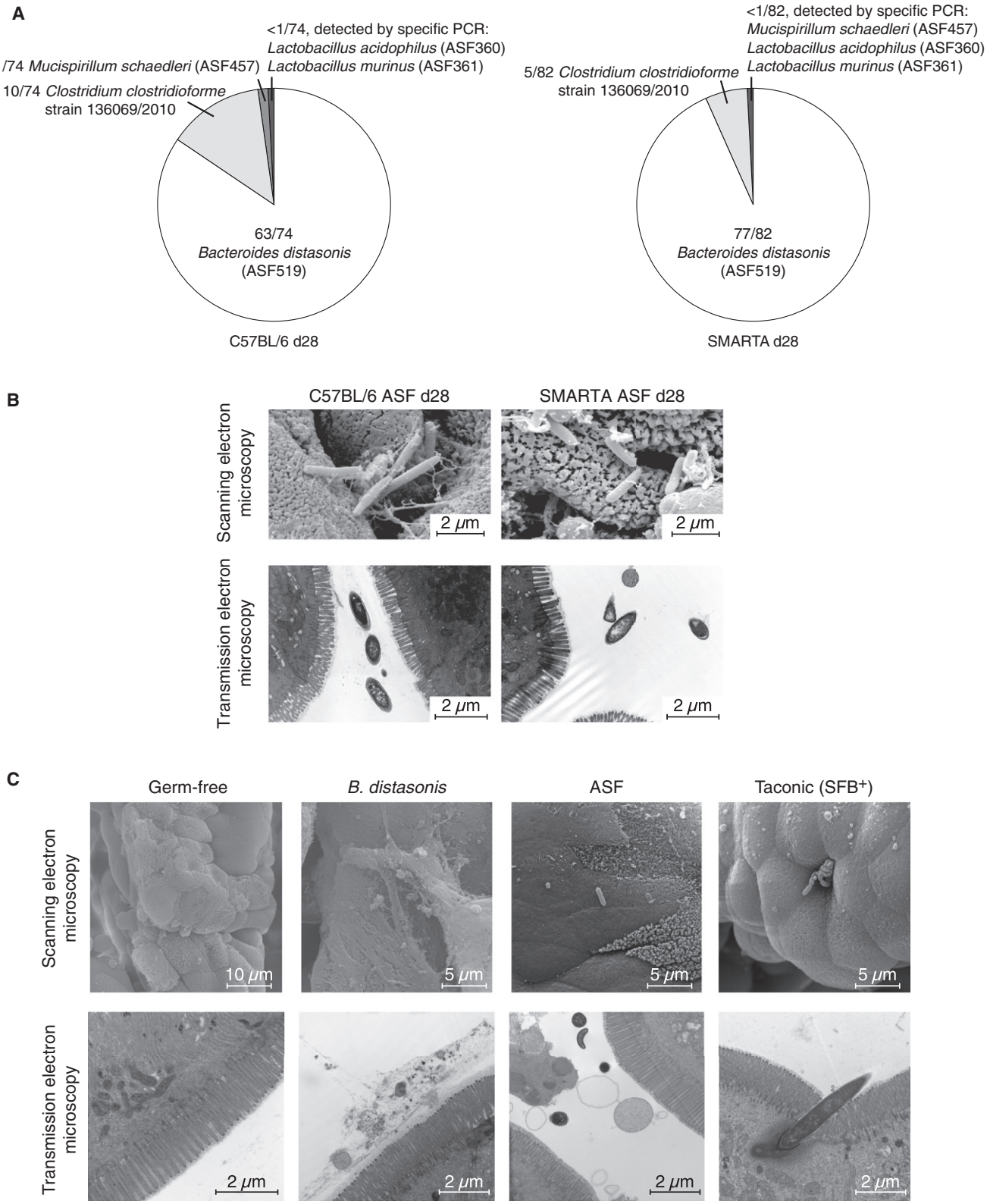


**Figure 4. Induction of Th17 Cells in SMARTA Mice Is Independent of the Flora Composition and the Presence of SFB**

(A) 16S rDNA sequences were amplified from DNA isolated from caecal contents with universal 16S-specific primers, subcloned into pGEM-T, and sequenced. The presence of additional members of the altered Schaedler flora (ASF) below detection limit of 16S amplicon sequencing was confirmed by specific PCR. The pie charts show the relative abundance of each ASF species present.

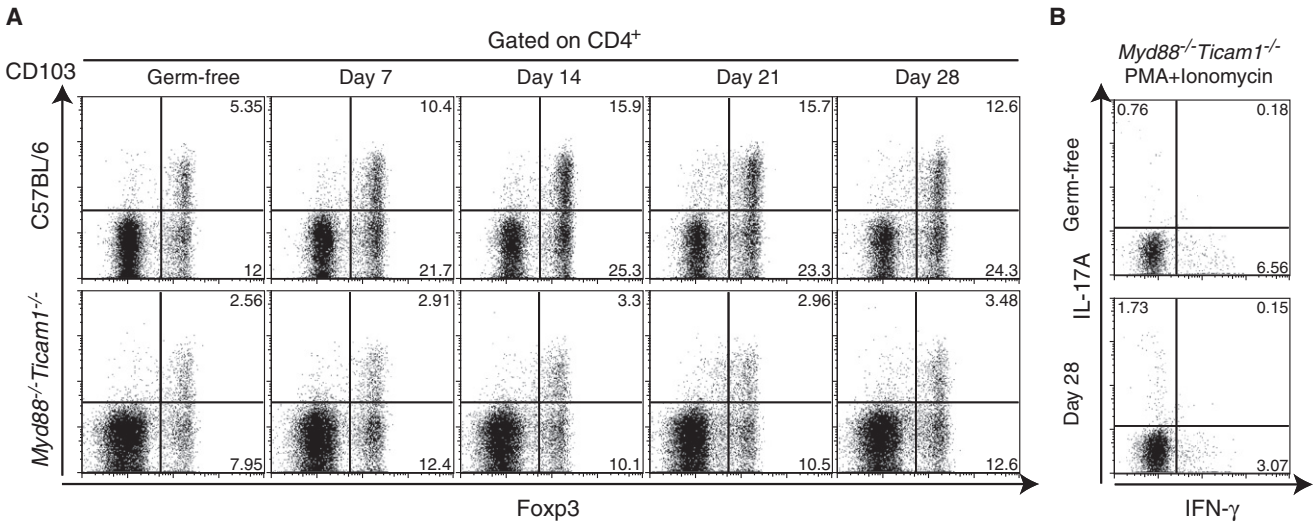


**Figure 5. Activation and Expansion of Colonic Treg Cells Is TLR Dependent**

(A) Germ-free C57BL/6 and *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ were colonized and the activation and expansion of Treg cells in cLP over time was assessed. Representative dot plots of n = 3–4 pooled mice per time point, and one representative of three independent experiments is shown. *Ticam1*$^{-/-}$ mice expanded their cLP Treg cell after 21 days (from 7.92% to >11.1%) to the same degree as wild-type controls (from 9.28% to >12.1%), whereas *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ did not (from 8.94% to >7.94%).

(B) Th1 and Th17 effector cells in cLP were measured by flow cytometry after PMA+ionomycin stimulation. Dot plots are from n = 3–4 pooled mice per group, and one representative of three independent experiments is shown.

epithelium by electron microscopy either in mice mono-associated with *B. distasonis* or colonized with ASF in the absence of SFB contamination (Figures 4B and 4C).

Because *B. distasonis* was the dominant species in our ASF flora (Figure 4A) and mono-association experiments are frequently carried out to try and elucidate mechanisms of commensal-host mutualism and immunity, we asked whether a system reduced to a single *Bacteroides* anaerobe is representative of the Treg cell-mediated mutualistic immune adaptation that we observed in the presence of a more complex (but defined) ASF microbiota. Mono-association with either *B. distasonis* or *Bacteroides thetaiotaomicron* did not result in the activation or expansion of Treg cells, and this absence of Treg cell induction did not cause considerable Th1 or Th17 cell responses (Figure S3C). Clostridia species have recently been shown to induce colonic Treg cells when present as a complex mixture of 46 different species (Atarashi et al., 2011). Because we detected *Clostridium clostridioforme* as the only Clostridia species in our flora (Figure 4A), we wanted to address its isolated effect on Treg cell induction. However, we were unable to colonize germ-free mice with this single species of Clostridia alone or with selected ASF members. Taken together, our data indicate that the ASF bacterial species present in our mice represent a functional minimal diverse flora with the capacity to induce truly mutualistic compartmentalized mucosal immune adaptations required for normal intestinal CD4$^+$ T cell host-microbial mutualism.

## TLR Deficiency Results in Impaired Activation of Mucosal Treg Cells during Colonization

We next examined the mechanism of Treg cell activation upon commensal bacterial colonization. We hypothesized that activation and proliferation of Treg cells after colonization was dependent on Toll-like receptor (TLR)-mediated sensing of the commensal flora or their catabolic products. To address this experimentally, we colonized germ-free mice deficient in the TLR adaptor molecules MyD88 and Ticam-1 (Hoebe et al., 2003), which are deficient for all TLR signaling, and followed the activation and proliferation of Foxp3$^+$ Treg cells in the cLP over time compared with wild-type controls. *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ mice displayed reduced Treg cell populations overall and impaired generation of activated CD103$^+$ effector Treg cells in the cLP (Figure 5A). Despite this marked defect in induction and activation of Treg cells, *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ mice maintained intestinal CD4$^+$ T cell homeostasis 28 days after ASF colonization (Figure 5B). This could reflect impaired effector T cell responses in the face of this severe innate immunodeficiency. Nevertheless, our data show that TLR signaling is required for efficient generation of a CD103$^+$ effector Treg cell response during colonization.

## Expansion and Activation of Treg Cells Maintains Homeostasis after Epithelial Damage

Once colonization and immune homeostasis are established, the intestinal epithelial layer in humans is subjected to constant

(B) Scanning and transmission electron microscopy on colon samples of C57BL/6 and SMARTA mice colonized for 28 days with ASF.

(C) Scanning and transmission electron microscopy on small intestinal samples from germ-free, *Bacteroides distasonis*-monocolonized, ASF-colonized, and SFB$^+$ Taconic mice.

See also Figure S3.

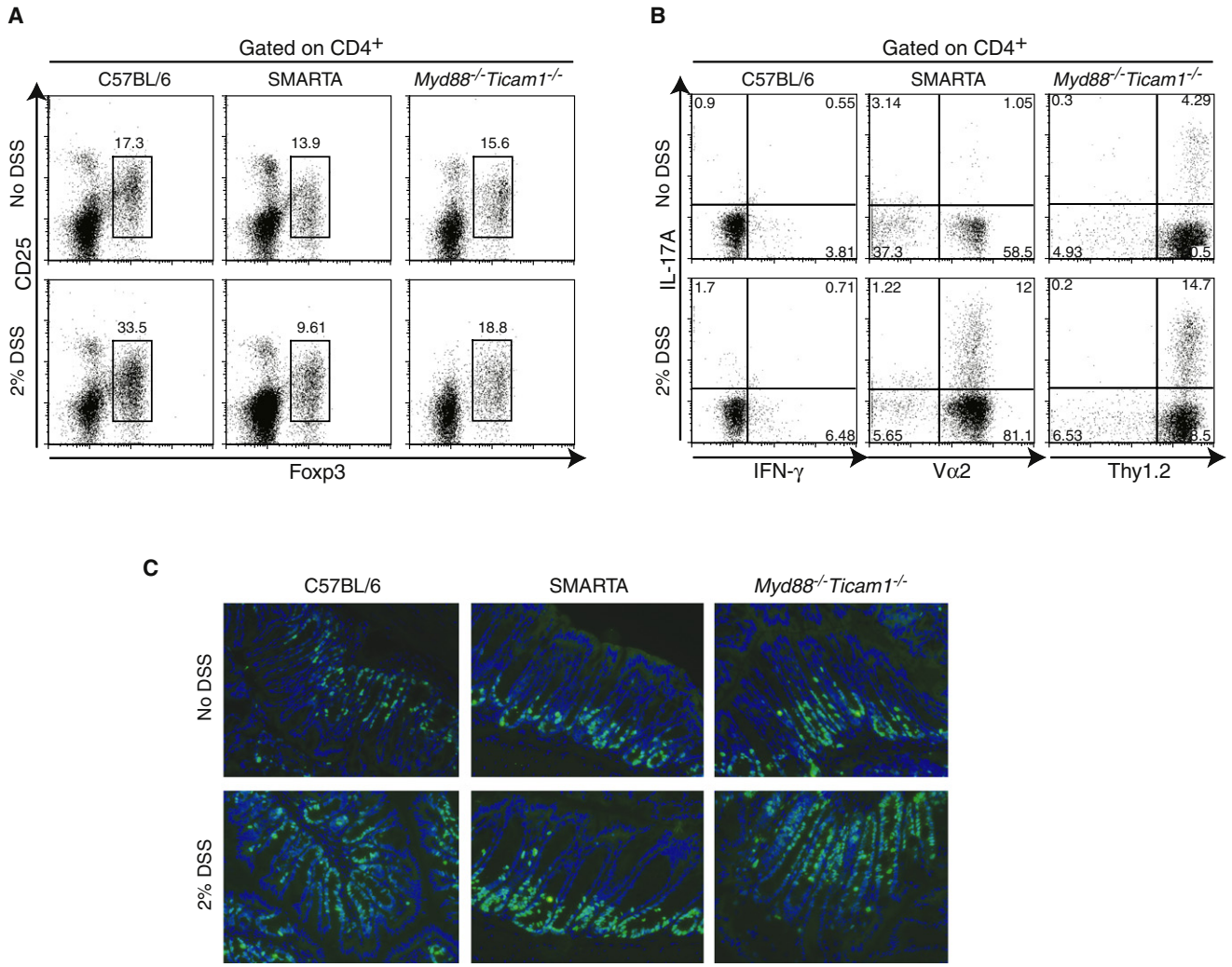


**Figure 6. Activation and Expansion of Colonic Treg Cells Is Required for Maintenance of Homeostasis**

(A and B) ASF C57BL/6, SMARTA, and *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ mice were treated for 7 days with 2% DSS in the drinking water or left untreated. On day 10 Treg cell populations in cLP were measured by flow cytometry (A) and Th1 and Th17 effector cells in cLP were measured after 4 hr of PMA+ionomycin stimulation (B). Dot plots are from n = 3–4 pooled mice per group, and one representative of four independent experiments is shown.

(C) On day 10, immunofluorescent Ki-67 staining was performed on colon cryosections. Representative sections from one of three independent experiments with n = 3–4 mice per group is shown.

See also Figure S4.

attrition from physiological and pharmacological insults, yet these rarely result in prolonged intestinal inflammation or enteropathy. We therefore addressed whether the intrinsic mechanisms we have shown to be responsible for commensal bacteria-mediated induction of homeostasis during colonization are also effective in maintaining mucosal immune homeostasis after colonic epithelial damage. We treated ASF-colonized C57BL/6, "Treg cell-defective" SMARTA, and *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ mice with a low dose (2%) of dextran sulfate sodium (DSS) in the drinking water for 7 days and allowed the animals to recover for 3 days. It is important to note that this was not used as a pathological colitis model but rather as a model of colonic epithelial damage to temporarily disturb intestinal homeostasis in ASF-colonized mice. This treatment consistently led to an increase in the proportion of Treg cells in cLP of C57BL/6 mice (Figure 6A) and maintenance of homeostasis as indicated by the absence of intestinal Th1 and Th17 cells (Figure 6B). Again, induction of Treg cells was restricted to cLP and not observed in SPL or MLN (Figure S4A). In contrast, DSS-treated Treg cell-defective SMARTA mice or *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ mice again failed to expand colonic Treg cells (Figure 6A) resulting in loss of homeostasis and induction of colonic Th17 cell responses (Figure 6B). Histological analysis and assessment of cell proliferation by Ki-67 staining revealed no overt pathology in C57BL/6 or SMARTA mice after DSS treatment, whereas *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ mice failed to recover from DSS-induced epithelial damage by day 10 (Figure 6C; Figure S4B). The pathology and damage observed in *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ mice is most probably attributable to the numerous consequences of the innate signaling defects (Rakoff-Nahoum et al., 2004).

The lack of overt pathology in SMARTA mice indicated that the defective Treg cell response was responsible for the Th1 and Th17 cell immune deviation but did not cause an enteropathy during short-term ASF colonization. Absence of pathology may have also resulted from the induced SMARTA Th1 and Th17 cells not being commensal specific in this system with a highly polarized TCR repertoire (Figure S2E). We were unable to protect SMARTA mice from immune deviation after DSS by transfer of wild-type Treg cells (Figure S4C), although the timing of the DSS experiment may be too short for a protective Treg cell response to develop. Although the transferred Treg cells had 28 days to exert their effector function in the colonization experiments, the DSS experiments lasted only 10 days.

These data suggest that Treg cell responses must develop during colonization for Th1 and Th17 cell homeostasis of the mucosal immune system with the epithelium intact, and developed Treg cell responses continue to be relevant in situations of epithelial damage.

## DISCUSSION

We have shown here that colonization with a benign physiological defined ASF resulted in the compartmentalized activation and induction of intestinal Treg cells essential for successful establishment of intestinal $CD4^+$ T cell homeostasis. This is in contrast to other studies that report no differences between Treg cell populations of germ-free and colonized mice (Chinen et al., 2010; Min et al., 2007). IL-10, most probably derived from $Foxp3^+$ Treg cells (Maynard et al., 2007), was required for induction of effector T cell homeostasis because blocking the IL-10R during colonization resulted in immune deviation. Blocking the IL-10R, however, did not affect the generation of $CD103^+$ and $Helios^-$ Treg cells. Colonization of Treg cell-defective TCR transgenic SMARTA mice did not result in activation and expansion of Treg cells and led to mucosal immune deviation to Th1 and Th17 cell responses. Such immune deviation as a result of failure to induce homeostasis has also been described when intestinal DCs are defective in the Wnt signaling pathway (Manicassamy et al., 2010).

Understanding truly mutualistic $CD4^+$ T cell responses that are part of the immune adaptation that occurs in response to intestinal colonization is important to better understand diseases that result from a breakdown of this mutualism. It is often difficult to distinguish truly mutualistic responses from idiosyncratic, often rather inflammatory, responses to single bacterial species or their products. Although intestinal Th17 cell responses are clearly required to control the presence of SFB (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009), it is not clear whether this reflects true mutualistic adaptation, especially considering the close interaction of SFB with the intestinal epithelium (Ivanov et al., 2009). It is likely that the Th17 cell induction observed in response to SFB reflects a canonical immune response rather than true mutualistic adaptation. The same limitations apply to experiments with *Bacteroides fragilis* and PSA (Mazmanian et al., 2008; Round and Mazmanian, 2010) because it is unclear whether this reflects a good model for true mutualistic adaptation. Although *B. fragilis* is only a minor component of the human intestinal commensal flora (0.5%), it is the most commonly isolated anaerobic pathogen, in part because of its potent virulence factors, which include PSA (Coyne et al., 2001; Polk and Kasper, 1977; Wexler, 2007). *B. fragilis* (and therefore also PSA) are absent from our nonpathogenic benign ASF microbiota and therefore our experiments model true mutualistic adaptation in the absence of any virulence factors. A mixture of 46 different Clostridia species has recently been shown to be a potent inducer of colon lamina propria Treg cells (Atarashi et al., 2011). Although this reflects an unbalanced flora, which might be the reason for lack of compartmentalization and systemic induction of regulatory responses in this system (Atarashi et al., 2011), the ASF flora used in our experiments did contain a Clostridia species. This *Clostridium* colonized only germ-free mice in the presence of all other ASF species. The truly mutualistic Treg cell responses that we report during colonization with ASF are compartmentalized to the colon lamina propria and functionally essential for intestinal Th1 and Th17 cell immune homeostasis.

SMARTA T cells do not nonspecifically proliferate in response to commensal microbial stimulation (Ramsey et al., 2008), suggesting that SMARTA Treg cells are not likely to cross-react with commensal flora antigens. However, in the absence of Treg cell activation and generation of $Helios^-$ Treg cells, $V\alpha2^+$ SMARTA T cells differentiated into Th17 effector cells during bacterial colonization. The fact that transferred wild-type Treg cells protected from the development of a Th1 and Th17 cell response clearly shows the fundamental role of Treg cells in establishing and maintaining $CD4^+$ T cell homeostasis in response to colonization of the gut with a completely benign microflora.

TLR signaling-deficient $Myd88^{-/-}Ticam1^{-/-}$ mice also displayed a defective Treg cell response and did not generate $CD103^+$ effector Treg cells after colonization. It remains unclear whether this is due to defective TLR signaling on Treg cells themselves, on DCs, or even on intestinal innate or epithelial cells. We have previously demonstrated that these mice have also a defect in compartmentalization of the mucosal and systemic immune system, which allows intestinal bacteria to reach systemic sites resulting in systemic hyperreactivity toward the commensal flora (Slack et al., 2009).

Although electron microscopy did not show any epithelial association of ASF bacteria compared with the intimate penetration of epithelial cells by SFB, minor degrees of association of the ASF are not excluded. However, bacterial-derived molecules can penetrate host tissues in very small amounts (Hrncir et al., 2008; Konno et al., 1993), so it is possible that bacterial products and not whole bacteria trigger TLR for induction of Treg cells.

Massive activation and expansion of Treg cells after epithelial damage in the colon suggests that the same mechanism that induces and establishes $CD4^+$ T cell homeostasis after colonization is also involved in the maintenance of homeostasis. After epithelial damage immune deviation occurred in both SMARTA and $Myd88^{-/-}Ticam1^{-/-}$ mice that are unable to activate colonic Treg cells. This result also shows that the limited and defined ASF is not only powerful enough to induce immune mutualism, it can also induce Th17 and Th1 cell subsets in the face of defective Treg cell responses.

It is clear that gut infections drive an inflammatory intestinal $CD4^+$ T cell response with deviation between the different $CD4^+$ T cell subsets (Ivanov et al., 2006; Mangan et al., 2006) and that absent or defective induction of Treg cells in the absence of pathogens can also lead to chronic intestinal

inflammation (Barnes and Powrie, 2009; Izcue et al., 2009). However, an understanding of pure commensal-driven establishment of intestinal homeostasis in the presence of a more complex but benign flora is essential to appreciate host-microbial interactions when they are truly mutualistic. We show directly in vivo that activation and expansion of Treg cells, including de novo generation of iTreg cells upon intestinal colonization, is critical to establish and maintain normal T cell homeostasis in an intestine loaded with benign commensal bacteria. Therefore, in addition to playing a key role in prevention of intestinal inflammation, commensal-driven Treg cell-mediated modulation of T cell homeostasis upon initial intestinal colonization is a fundamental mechanism allowing for host-microbial mutualism and establishes the baseline for all subsequent T cell responses in the gut.

## EXPERIMENTAL PROCEDURES

### Mice

Germ-free C57BL/6, BALB/c, NIH Swiss, Swiss Webster, NMRI, *MyD88*$^{-/-}$ *Ticam1*$^{-/-}$, SMARTA, and ASF C57BL/6.CD45.1$^{+}$ mice were rederived by axenic two-cell embryo transfer as previously described (Slack et al., 2009). Germ-free mice were bred and maintained in flexible film isolators at the axenic gnotobiotic facility at McMaster University (Hamilton, Canada) or the Clean Mouse Facility of the University of Bern and the Inselspital (Bern, Switzerland). SFB$^{+}$ C57BL/6 were purchased from Taconic. Germ-free status was routinely confirmed by aerobic and anaerobic culture as well as sytox green (Invitrogen) and gram staining (Harleco) of caecal contents to detect unculturable contamination. ASF-colonized NMRI mice were generated by inoculating germ-free NMRI mice with ASF bacteria according to standard protocol. Subsequent ASF colonization of all other strains was then performed by placing an ASF-colonized mouse into a cage of germ-free animals. All experiments were carried out in accordance with the Institute animal utilization protocols, Canadian Council on Animal Care (CCAC) guidelines, and Swiss animal regulations.

### Flora Analysis

DNA was isolated from caecal contents with the QIAamp DNA stool kit (QIAGEN). A 1500 bp segment containing a variable region of 16S rDNA was amplified by PCR (Weisburg et al., 1991) with forward primers fD1 5′-AGAGTTTGATCCTGGCTCAG-3′ and fD2 5′-AGAGTTTGATCATGGCTCAG-3′ in combination with reverse primer rP1 5′-CGGTTACCTTGTTACGACTT-3′. 16S PCR amplicons were then subcloned into pGEM-T (Promega) and sequenced. Species identification was performed with the online database nBLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To demonstrate the absence of SFB, specific PCR with primers SFBfwd 5′-GACGCTGAGGCATGAGAG CAT-3′ and SFBrev 5′-GACGGCACGGATTGTTATTCA-3′ was performed. DNA from caecal contents of Taconic C57BL/6 mice was used for positive control.

### Thoracic Duct Cannulation

Mice were gavaged intragastrically with 1 ml olive oil and 6 hr later lymph was collected from the thoracic duct with a fine glass capillary.

### Colonic Lamina Propria Preparation

Colons from 3–4 mice per group were pooled to obtain sufficient numbers of cells. The excised colon was flushed to remove contents, opened longitudinally, washed thoroughly in $Mg_2Cl_2$- and $CaCl_2$-free DPBS (GIBCO), and then cut into 3–5 mm pieces that were incubated 4–5 times in 25 ml EDTA/HEPES/DPBS solution at 37°C for 20 min to remove the epithelial layer. Intestinal pieces were collagenase-digested for 40 min at 37°C in 25 ml IMDM containing 0.5 mg/ml collagenase type VIII (Sigma), 50 U DNaseI (Roche), and 0.01 M HEPES (GIBCO). The crude cell suspension was loaded onto a 30%/100% percoll (GE Healthcare) gradient and centrifuged at 680 × g for 30 min at room temperature with acceleration and brake turned off. Cells were collected from the 30%/100% interphase.

### Intracellular Cytokine Staining

Single-cell suspensions were stimulated for 4 hr in the presence or absence of 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) and 750 ng/ml ionomycin (Invitrogen) with 10 μg/ml Brefeldin A (Sigma). For ASF-specific stimulation, MACS-sorted splenic CD11c$^{+}$ DC were incubated overnight with a heat-killed ASF preparation and used as antigen-presenting cells for stimulation. GP61-specific stimulation of SMARTA T cells was performed with the GP61 peptide (GLNGPDIYKGVYQFKSVEFD) at a concentration of 2 μg/ml. Intracellular cytokine staining was performed with the BD Cytofix/Cytoperm staining kit (BD).

### Flow Cytometry and Sorting

Antibodies and corresponding clones used for flow cytometry were as follows: IFN-γ-FITC (XMG1.2), IL17A-PE (TC11-18H10), CD4-PerCp (RM4-5), CD103-biotin (M290), Vα2-PE (B20.1), CD25-PE (7D4), CD45.1-PE (A20), and streptavidin-APC were purchased from BD PharMingen. CD4-Pacific blue (RM4-5), CD25 PerCp-Cy5.5 (PC61), GITR-FITC, PD-1-PE, and CD127 PE-Cy7 were purchased from Biolegend. Foxp3-FITC, Foxp3-AlexaFluor700 (FJK-16a, eBioscience), and Helios-PE (22F6, Biolegend) were used in combination with the Foxp3 staining kit (eBioscience). Data were acquired on a FACSCalibur (BD) or an LSRII (BD) and analyzed with FlowJo software (Tree Star, Inc.). For FACS sorting, splenocytes from Foxp3-GFP reporter mice were magnetically pre-enriched for CD4$^{+}$ cells (see below) and then stained for surface CD4 and CD25. Live/dead discrimination was performed with 7-aminoactinomycin (7-AAD). Highly purified GFP$^{+}$ and GFP$^{-}$ CD4$^{+}$ T cell populations were sorted on a FACSVantageSE (BD) at the flow cytometry core facility at McMaster University.

### Blocking IL-10R In Vivo

500 μg blocking IL-10R antibody (clone 1B1-2) or a Rat IgG1, κ isotype control (clone 35.61) were injected i.p. twice per week for 4 weeks.

### MACS Sorting

Single-cell suspensions were prepared by digestion with liberase C1 (Roche). Magnetic cell sorting of CD4$^{+}$ T cells, CD11c$^{+}$ DCs, and regulatory T cells was performed with microbeads specific for CD4, CD11c, or the regulatory T cell isolation kit, respectively, in combination with an octoMACS or autoMACS according to the manufacturer's instructions (Miltenyi Biotech). CD45.1$^{+}$ Treg cells were obtained from pooled SPL and MLN and transferred intravenously into germ-free SMARTA mice at the time of colonization.

### RNA Isolation and Quantitative Real-Time PCR

Total RNA from sorted cell populations was isolated with TRIzol Reagent (Invitrogen). Potential DNA contamination was removed by DNase treatment (DNA-free, Ambion). RNA quantity and integrity after clean-up (RNeasy Mini Kit, QIAGEN) were analyzed with an RNA Nano Chip (Agilent technologies) on an Agilent 2100 Bioanalyzer. Only RNA samples with a high integrity (RIN > 7.5) were used for subsequent cDNA synthesis with Superscript III and random hexamers (Invitrogen). Equal amounts of input RNA were used for each sample. Quantitative real-time PCR was performed on a CFX384 system (BioRad) with the SsoFast EvaGreen Supermix (BioRad). Primers used were as follows: IL-10fwd, 5′-TTTGAATTCCCTGGGTGAGAA-3′; IL-10rev, 5′-GGAGAAATCGATGACAGCGC-3′; GAPDHfwd, 5′-CATCAAGA AGGTGGTGAAGC-3′; and GAPDHrev, 5′-CCTGTTGCTGTAGCCGTATT-3′. The CFX manager software (BioRad) was used to calculate the relative fold change in expression normalized to *gapdh* expression by the $\Delta\Delta C_t$ method. All procedures were performed according to manufacturer's instructions.

### DSS Treatment

Dextran sulfate sodium (DSS; MP Biomedicals Inc.) was dissolved in sterile water (Baxter) to obtain a 2% solution that was then sterile filtered. 2% DSS water was given to the mice ad libitum and the uptake was equal in all experimental groups.

### Histology and Immunofluorescence

Intestinal tissue samples were embedded in Tissue-Tek OCT compound (Sakura Finetek), snap frozen in liquid nitrogen, and stored at −80°C. Cryosections (6 μm) were mounted on glass slides, air-dried for 2 hr at room

temperature, and either stained with hemotoxylin and eosin (H&E) or fixed with fresh PBS-buffered 2% para-formaldehyde (PFA) before staining with rabbit anti-Ki-67-FITC (NeoMarkers) as primary antibody and donkey anti-rabbit-FITC (Jackson ImmunoResearch Laboratories) as secondary antibody. Nuclei were stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich). After the staining procedure, slides were mounted in Vectashield (Vector labs).

### Electron Microscopy

Intestinal biopsies were fixed in 1.5% PFA and 1.5% glutaraldehyde in 0.15 M HEPES. Subsequent sample preparation for transmission and scanning electron microscopy was performed by the electron microscopy core facility at the anatomical institute of the University of Bern or at the electron microscopy core facility at McMaster University.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.immuni.2011.03.021.

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