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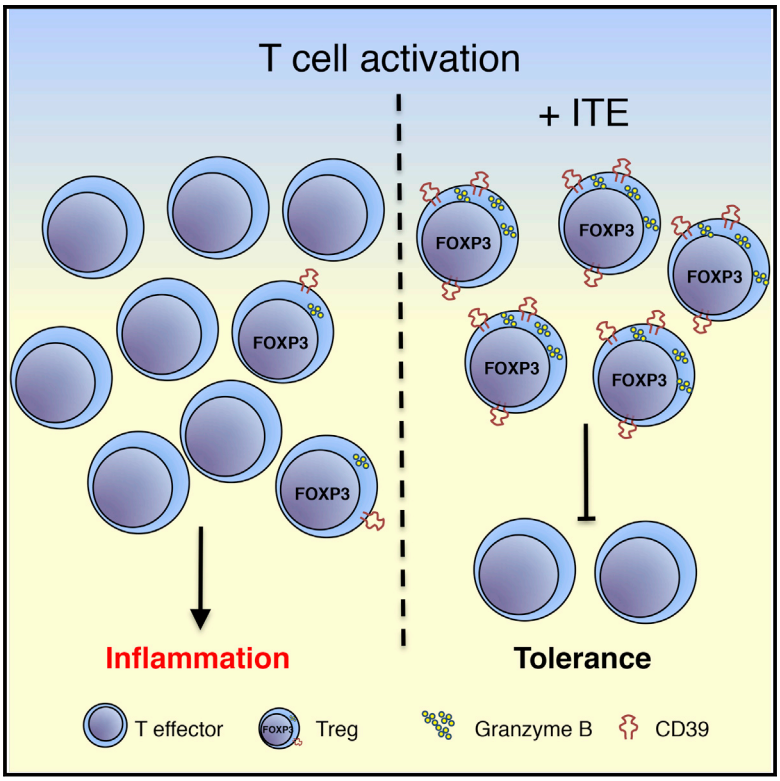
AHR Activation Is Protective against Colitis Driven by T Cells in Humanized Mice

Graphical Abstract



Highlights

- Non-toxic AHR agonist ITE induces human regulatory T cells in vitro
- ITE-mediated in vitro suppression is dependent on CD39 and Granzyme B
- Human CD4 T cells drive TNBS-induced colitis in humanized mice
- ITE protects against TNBS-induced colitis in humanized mice

Authors

Jeremy A. Goettel, Roopali Gandhi, Jessica E. Kenison, ..., Howard L. Weiner, Scott B. Snapper, Francisco J. Quintana

Correspondence

scott.snapper@childrens.harvard.edu (S.B.S.), fquintana@rics.bwh.harvard.edu (F.J.Q.)

In Brief

Therapeutic approaches aimed at expanding regulatory T cells in the gut to promote immune tolerance in patients with inflammatory bowel disease (IBD) are of clinical significance. Goettel et al. establish a humanized murine model of IBD driven by human T cells and find that activation of AHR by the non-toxic agonist ITE can prevent experimental colitis.

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AHR Activation Is Protective against Colitis Driven by T Cells in Humanized Mice

Jeremy A. Goettel,[1,2,8] Roopali Gandhi,[3,4,8] Jessica E. Kenison,[3,4] Ada Yeste,[3,4] Gopal Murugaiyan,[3,4] Sharmila Sambanthamoorthy,[3,4] Alexandra E. Griffith,[1] Bonny Patel,[3,4] Dror S. Shouval,[1,2] Howard L. Weiner,[3,4] Scott B. Snapper,[1,5,6,9,*] and Francisco J. Quintana[3,4,7,*]

[1]Division of Gastroenterology, Hepatology and Nutrition, Boston Children's Hospital, Boston, MA 02115, USA
[2]Department of Pediatrics, Harvard Medical School, Boston, MA 02115, USA
[3]Ann Romney Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA 02115, USA
[4]Department of Neurology, Brigham and Women's Hospital, Boston, MA 02115, USA
[5]Department of Medicine, Harvard Medical School, Boston, MA 02115, USA
[6]Division of Gastroenterology, Brigham and Women's Hospital, Boston, MA 02115, USA
[7]The Broad Institute of MIT and Harvard University, Cambridge, MA 02142, USA
[8]Co-first author
[9]Lead Contact
*Correspondence: scott.snapper@childrens.harvard.edu (S.B.S.), fquintana@rics.bwh.harvard.edu (F.J.Q.)
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SUMMARY

Existing therapies for inflammatory bowel disease that are based on broad suppression of inflammation result in variable clinical benefit and unwanted side effects. A potential therapeutic approach for promoting immune tolerance is the in vivo induction of regulatory T cells (Tregs). Here we report that activation of the aryl hydrocarbon receptor using the non-toxic agonist 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) induces human Tregs in vitro that suppress effector T cells through a mechanism mediated by CD39 and Granzyme B. We then developed a humanized murine system whereby human CD4$^+$ T cells drive colitis upon exposure to 2,4,6-trinitrobenzenesulfonic acid and assessed ITE as a potential therapeutic. ITE administration ameliorated colitis in humanized mice with increased CD39, Granzyme B, and IL10-secreting human Tregs. These results develop an experimental model to investigate human CD4$^+$ T responses in vivo and identify the non-toxic AHR agonist ITE as a potential therapy for promoting immune tolerance in the intestine.

INTRODUCTION

Inflammatory bowel diseases (IBDs) are complex inflammatory disorders of the intestine that are generally associated with defects in mucosal immune regulation (Khor et al., 2011). Dampening the inflammatory response to re-establish immune tolerance is a major therapeutic strategy for IBD treatment. Current clinical approaches often involve broad suppression of the immune system, resulting in limited clinical benefit and concomitant risk for opportunistic infections and other side effects (Beaugerie, 2012; Calabrese, 2006). More recently, the use of biologics such as anti-tumor necrosis factor (TNF) antibodies has proved effective with nearly half of treated patients demonstrating a clinical response (Ben-Horin et al., 2014). However, these beneficial effects are often self-limited and highlight the need for new therapies that promote long-lasting immune tolerance.

Since the initial description that the thymus has critical immunological function (Burnet and Holmes, 1962; Miller, 1961) and subsequent work by many that the thymus has functions independent of elimination of autoreactive T cells (Le Douarin et al., 1996), there has been a concerted effort to understand the mechanisms of immunological tolerance. Extensive experimentation has defined a group of regulatory T cells (Tregs) that are critical for both central and peripheral tolerance. Loss-of-function mutations in the transcription factor forkhead box P3 (FOXP3) cause a fatal autoimmune disorder in humans known as immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome. Similarly, *Foxp3*$^{-/-}$ mice exhibit multi-organ autoinflammatory disease and early mortality (Bennett et al., 2001). In addition, the ability to generate or "induce" Tregs from the pool of helper T cells in the periphery in order to become tolerant to innocuous foreign antigens such as food and commensal microbes at mucosal surfaces is equally important for mucosal immune homeostasis (Atarashi et al., 2013; Hauet-Broere et al., 2003). The mechanisms by which Tregs exert their immunoregulatory function in the intestine are thought to occur via production of soluble mediators and/or direct interactions with other immune cells (Mayne and Williams, 2013). In recent years, a subpopulation of FOXP3$^-$ interleukin (IL)10-secreting iTregs (termed Tr1 cells) has been implicated in the regulation of intestinal inflammation (Groux et al., 1997). This critical role for IL10 signaling in maintaining intestinal immune homeostasis is best exemplified by the observation that loss-of-function mutations in *IL10* or the IL10 receptor cause IBD in both mice and humans (Glocker et al., 2009; Kühn et al., 1993). Because Tregs are thought to play a central role in preventing IBD (Josefowicz et al., 2012; Mayne and Williams, 2013; Sakaguchi et al., 2010), generation

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or expansion of functional Tregs constitutes an attractive therapeutic approach to treat IBD (Canavan et al., 2016), and therapeutic strategies aimed at expanding Tregs in vivo have proved effective in controlling other immune-mediated disorders (Koreth et al., 2011; Saadoun et al., 2011; Desreumaux et al., 2012).

Though several polymorphisms have been associated with altered risk for IBD, surprisingly, only one-third of the disease is explained by genetics, suggesting that environmental triggers play an important role. The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that senses certain environmental chemicals and has been shown to exert significant effects on the immune response. Previous work from our group and others demonstrates a role for AHR in the differentiation and function of Tregs and effector T cells by controlling the production of IL10 and IL22 (Apetoh et al., 2010; Gandhi et al., 2010; Quintana et al., 2008; Yeste et al., 2014; Mascanfroni et al., 2015). In mice, activation of AHR suppresses experimental colitis, and although no current therapies target AHR in humans, the expression of AHR is increased in IBD lesions (Arsenescu et al., 2011; Benson and Shepherd, 2011; Chinen et al., 2015; Fukumoto et al., 2014; Furumatsu et al., 2011; Monteleone et al., 2011). Given the importance of Tregs in intestinal homeostasis, coupled with the immunomodulatory effects of IL10 and IL22 downstream of AHR activation (Mayne and Williams, 2013; Sonnenberg et al., 2011), AHR is an attractive therapeutic target. The exogenous small molecule 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been shown to activate AHR; however, toxicity prevents the use of TCDD for therapeutic intervention in patients. Thus, there is a need not only for non-toxic AHR agonists but also for the establishment of new experimental systems to evaluate the effects of AHR activation on human cells in the context of intestinal inflammation in vivo. In this report, we investigated the effects of the mucosal non-toxic AHR agonist 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) (Song et al., 2002) on human T cells in vitro and developed a new experimental model of IBD driven by human $CD4^+$ T cells in humanized mice to assess the efficacy of AHR activation by ITE in vivo. We found that AHR activation by ITE induces suppressive human Tregs expressing Granzyme B (GZMB), CD39, IL10, and FOXP3 and prevents T-cell-driven colitis in humanized mice. Future studies will address whether ITE is effective in treating established disease, which will be an important step to determine whether ITE would be an attractive candidate for the therapeutic induction of Tregs to treat patients with established IBD.

RESULTS

ITE Induces Tregs through a Mechanism Mediated by AHR

The induction of Tregs and the re-establishment of immune tolerance is a potential approach for the long-term treatment of IBD and other inflammatory diseases and may minimize the deleterious side effects associated with immunosuppressive approaches now in use (Chatenoud, 2015). We and others have shown that AHR activation induces Tregs that suppress the development of experimental autoimmunity and inflammation (Apetoh et al., 2010; Kerkvliet et al., 2009; Quintana et al., 2008). Although AHR activation with TCDD induces functional human Tregs, toxicity concerns exclude its use as therapeutic agent (Gandhi et al., 2010). We therefore investigated the effects of the non-toxic AHR agonist ITE isolated from mucosal tissue (Song et al., 2002). Naive $CD4^+$ T cells from peripheral blood mononuclear cells (PBMCs) of healthy donors were sorted based on $CD4^+CD62L^+CD45RO^-$ (Figure S1) and co-cultured with autologous unfractionated $CD4^+$ T cells, previously treated with ITE, TCDD, or vehicle control, in the presence of anti-CD3/anti-CD28 antibodies with IL2. ITE treatment induced suppressive activity in human T cells in vitro that was comparable to the suppressive activity observed using TCDD as a positive control (Figure 1A). We confirmed the activation of AHR by ITE using quantitative real-time PCR (qPCR) to detect an increase in the expression of the AHR transcriptional target *CYP1A1* (Figure 1B). To test whether the induction of suppressive T cells by ITE was mediated by AHR, we targeted *AHR* using small interfering RNA (siRNA) that abrogated the suppressive effect of ITE and then corroborated these findings using a selective AHR antagonist CH223191 that also blocked ITE-mediated suppression in vitro (Figure 1C).

To further investigate activation of AHR by ITE in human T cells, we analyzed the expression of cytokines and lineage-specific molecules and transcription factors associated with different $CD4^+$ T cell subpopulations by qPCR. ITE treatment decreased expression of *TBX21*, *RORC*, and *IL23R* but not *GATA3* (Figures 1D and S2), which correlates with reduced effector T cell subsets. In contrast, T cell activation in the presence of ITE led to significant upregulation of Treg-associated gene transcripts, including *FOXP3*, *IL10*, *GZMB*, *ENTPD1*, and *IKZF3* (Figures 1E and S2) (Groux et al., 1997; Quintana et al., 2012; Sakaguchi et al., 2010). ITE treatment had a modest or no effect on the expression of *MAF*, *BCL6*, *BLIMP*, *IL12RB1*, or *IL12RB2* (Figure S2). Based on the transcriptional analysis, we then profiled ITE-treated $CD4^+$ T cells to assess protein expression for TBX21, RORC, GATA3, FOXP3, IL10, GZMB, and CD39 (encoded by *ENTPD1*) by flow cytometry and found it to be consistent with the transcriptional analysis (Figures 1F and 1G). Although many of these markers are consistent with traditional $Foxp3^-$ $IL10^+$ Tr1 regulatory cells, a large percentage of ITE-treated T cells expressing CD39 were also positive for $FOXP3^+$, suggesting that ITE induces a mixed population of regulatory cells, with only some possessing a conventional Tr1 phenotype (Figure S3).

The ability of Tregs to control the activity of effector T cells can occur via several mechanisms (Josefowicz et al., 2012; Sakaguchi et al., 2010). Specifically, GZMB, CD39, and IL10 are known to contribute to the suppressive activity of Tr1 cells (Gandhi et al., 2010; Grossman et al., 2004; Mascanfroni et al., 2015). To determine whether the suppressive effect of ITE was mediated by GZMB, we first performed suppression assays in the presence of the GZMB inhibitor benzyloxycarbonyl-Ala-Ala-Asp-chloromethylketone (AAD-CMK) and found that ITE- and TCDD-mediated suppression was abrogated (Figure 1H).

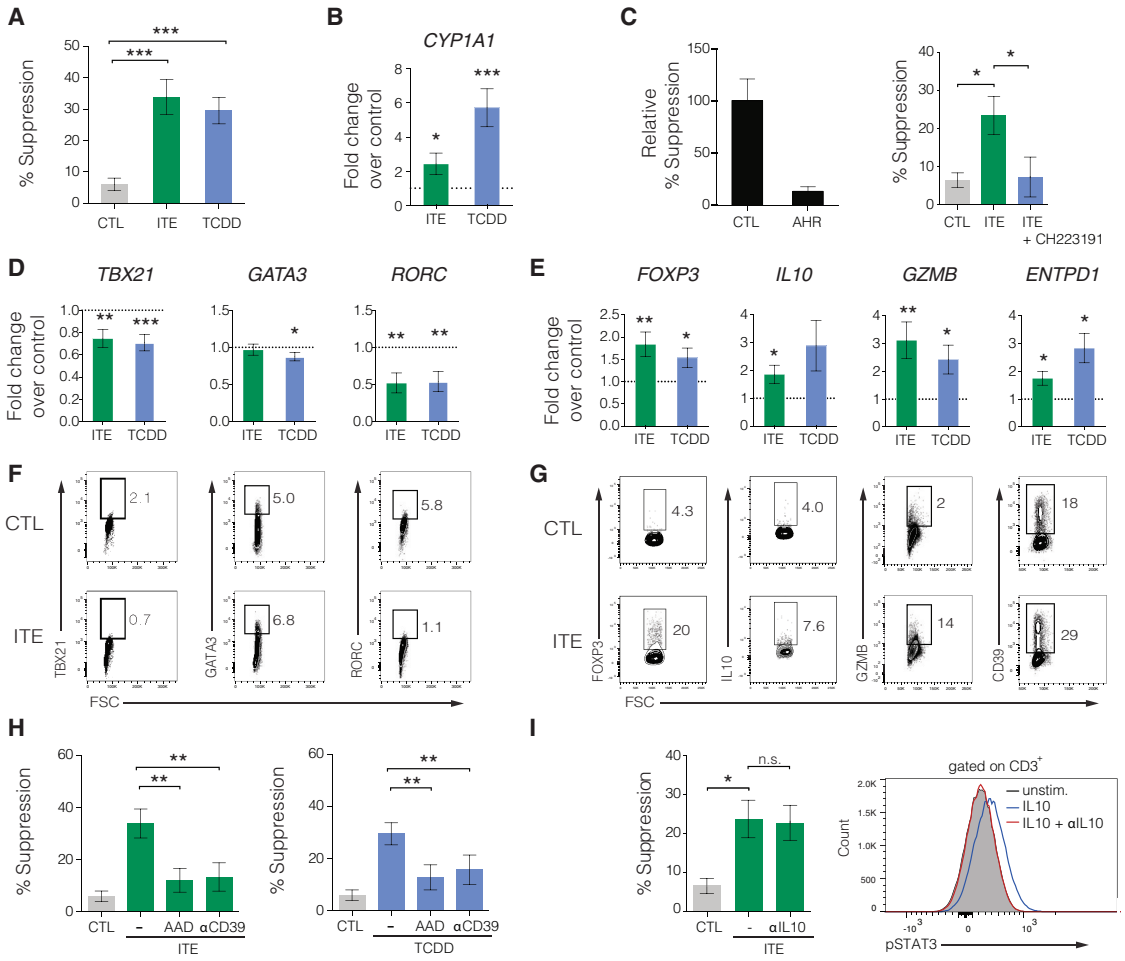

Figure 1. ITE Induces Suppressive Human Tregs through a Mechanism Mediated by AHR

(A) Suppressive activity of human naive $CD4^+$ T cells activated with plate-bound αCD3/αCD28 and IL-2 in the presence of vehicle (CTL), ITE, or TCDD. Percent suppression is depicted as the mean ± SEM pooled from at least three independent experiments (n = 21).

(B) Relative expression of the AHR target *CYP1A1* following stimulation with αCD3/αCD28 and IL-2 in the presence of ITE or TCDD and compared to PBS vehicle control. Data are depicted as the mean ± SEM (n = 16).

(C) Requirement for AHR for the suppressive effect of ITE. Relative suppression following siRNA-mediated knockdown of *AHR* compared to scrambled siRNA as control (left). Percent T cell suppression by ITE in the presence of 10 μM CH223191, a selective antagonist of AHR, compared to DMSO as vehicle control. Results are depicted as the mean ± SEM pooled from two independent experiments.

(D and E) qPCR analysis of RNA isolated from T cells stimulated with αCD3/αCD28 and IL-2 in the presence of vehicle, ITE, or TCDD. Target fold change was calculated using vehicle control, with the dashed line representing the normalized control value of 1 and the mean depicted as ±SEM pooled from three independent experiments ($n \geq 7$).

(F and G) Representative flow cytometric analysis of TBX21, GATA3, RORC, FOXP3, IL10, GZMB, and CD39 protein expression following T cell activation using αCD3/αCD28 and IL-2 in the presence or absence of ITE for 6 days. Each flow panel is representative of at least two independent experiments.

(H) Requirement for GZMB and CD39 for the suppressive activity of ITE using a blocking antibody against CD39 or the GZMB inhibitor AAD-CMK (left) and showing the requirement for GZMB and CD39 for TCDD-mediated suppression as a positive control (right). n = 21.

(I) Role of IL10 in ITE-mediated T cell suppression using 2.5 μg mL^{-1} of αIL10 blocking antibody shown as percent suppression (left) and the ability of αIL10 antibody to inhibit STAT3 phosphorylation in human T cells following a 20 min stimulation with 20 ng mL^{-1} of IL10 (right).

*p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant.

Because CD39 can also participate in the suppression of effector T cell responses, we inhibited CD39 using neutralizing antibodies in ITE-treated T cells. Similar to AAD-CMK, treatment with anti-CD39 blocked ITE- and TCDD-mediated suppressive activity in vitro (Figure 1H). Although IL10 was upregulated by ITE by qPCR, we tested whether antibody-mediated blockade of IL10 would inhibit suppression. While the anti-IL10 antibody effectively blocked the phosphorylation of STAT3 in response to IL10 (confirming a block in IL10R [interleukin-10 receptor] signaling), this had no impact on the suppressive effect of ITE (Figure 1I). Collectively, these results demonstrate that AHR activation by ITE induces functional human Tregs that suppress effector T cell proliferation in a GZMB- and CD39-dependent manner.

ITE Induces Functional Human FOXP3$^+$ Tregs in the Presence of TGF-β1

Transforming growth factor β1 (TGF-β1) is expressed by both immune and non-immune cells in the intestinal lamina propria and regulates the immune response through several mechanisms, one of which consists of the differentiation, maintenance, and function of FOXP3$^+$ Tregs (Rubtsov and Rudensky, 2007). Although TGF-β1 promotes the differentiation of functional FOXP3$^+$ Tregs in mice (Chen et al., 2003), TGF-β1-induced FOXP3$^+$ human T cells do not exhibit suppressive activity (Tran et al., 2007). To mimic the TGF-β1-enriched microenvironment of the intestine, we previously showed that AHR activation by TCDD could cooperate with TGF-β1-induced signaling to induce suppressive FOXP3$^+$ human Tregs in vitro (Gandhi et al., 2010). Therefore, we investigated whether human Tregs induced using TGF-β1 in the presence of ITE would mimic the suppressive effects of TCDD. Similar to our findings with TCDD, human naive CD4$^+$ T cells activated in the presence of TGF-β1 and ITE were suppressive in vitro (Figure 2A). Because we previously observed decreased expression of CD4$^+$ T cell linage-specific transcription factors following ITE treatment (Figure 1D), we tested whether these molecules were downregulated under Treg-inducing conditions. CD4$^+$ T cells were treated with vehicle, TGF-β1, or TGF-β1 in the presence of ITE or TCDD. Although TGF-β1 significantly reduced expression of *TBX21* and *GATA3*, no further decrease was observed if ITE or TCDD was present (Figure 2B). TGF-β1 treatment increased *RORC* expression that was not further enhanced in the presence of ITE and TCDD; however, these data did not achieve statistical significance (Figure 2B). The expression of *IL23R* was elevated over control when stimulated with TGF-β1 and ITE, which was not observed in T cells treated with TGF-β1 alone or in combination with TCDD (Figure 2B). We then evaluated protein expression of these qPCR targets by flow cytometry in T cells activated in the presence of vehicle control, TGF-β1, or TGF-β1 and ITE and observed a reduction in GATA3, increased expression of RORC, and a slight increase in TBX21 over control only in the TGF-β1- and ITE-treated cells (Figure 2C).

Activation of AHR also upregulates the expression of the Ikaros family transcription factor AIOLOS (encoded by *IKZF3*) that silences the *IL2* promoter and, along with IL10 and FOXP3, is associated with suppressive human Tregs (Gandhi et al., 2010; Quintana et al., 2012; Sakaguchi et al., 2010). We analyzed Tregs induced with TGF-β1 alone or in the presence of ITE or TCDD and quantified the expression of *IKZF3*, *IL10*, and *FOXP3*. Although *FOXP3* was elevated in all treatment conditions compared to control, *IL10* and *IKZF3* were upregulated by TGF-β1 and ITE (Figures 2D and S4). Flow cytometric analysis largely supported the transcriptional analysis, whereby increases in FOXP3, IL10, and CD39 were also observed (Figure 2E).

Because CD39 and GZMB were required for ITE-induced AHR-dependent T cell suppression in vitro in the absence of TGF-β1 (Figure 1H), we determined whether *GZMB* and *ENTPD1* were also upregulated following AHR activation with ITE or TCDD under Treg-inducing conditions with TGF-β1. While there was no increase in *GZMB* transcript or protein in vitro (Figures 2D and 2E), *ENTPD1* transcript levels trended higher (Figure 2D), although cell surface expression of CD39 was increased (Figure 2E). We then tested whether CD39 was required for the suppressive effect of ITE in the presence of TGF-β1. Neutralizing CD39 abrogated the suppressive activity of T cells treated with TGF-β1 and ITE, similar to that observed for TGF-β1 and TCDD and consistent with a role for CD39 in this suppressive function (Figure 2F). Altogether, these data demonstrate that AHR activation with ITE in the presence of TGF-β1 induces functional human Tregs that suppress effector T cells in a CD39-dependent manner in vitro.

T-Cell-Dependent Colitis Model in Humanized Mice

Most experimental immunotherapies successful in treating experimental autoimmunity in animal models show limited success in human clinical trials (Hay et al., 2014; Persidis, 1999). One important contributor to the limited translational application of experimental findings in mice into successful therapies for human autoimmunity is the lack of models to study the human immune system in vivo. Over the past decade, immunodeficient mice have been developed that are capable of engrafting human immune cells (Rongvaux et al., 2014; Shultz et al., 2005; Traggiai et al., 2004). Although sub-optimal adaptive immune responses are often observed in many humanized murine systems, we described non-obese diabetic (NOD).*Prkdc*^{scid}.*Il2rg*$^{-/-}$ (NSG) mice that lack murine major histocompatibility complex (MHC) class II and instead express human leukocyte antigen (HLA) DR1 under the control of the murine MHC class II promoter (NSGAb$^\circ$DR1 mice) (Goettel et al., 2015). These mice intrinsically lack murine lymphocytes, as well as natural killer (NK) cells, and when made immune replete using human CD34$^+$ hematopoietic stem cells (HSCs), the mice displayed improved human CD4$^+$ T cell responses (Goettel et al., 2015). To specifically evaluate CD4$^+$ T cell responses, we used a reductionist approach by reconstituting NSGAb$^\circ$DR1 mice with human CD4$^+$ T cells isolated from allelically matched HLA-DR1$^+$ donors. To evaluate the effects of candidate drugs on human CD4$^+$ T cells in vivo, we adapted an established experimental model of intestinal inflammation using the hapten 2,4,6-trinitrobenzenesulfonic acid (TNBS) that is largely mediated by T cells (Neurath et al., 1995). Reconstituted NSGAb$^\circ$DR1 mice were sensitized with TNBS to prime antigen-specific T cells, and 1 week post-sensitization mice were administered a single rectal enema containing 0.25 mg of TNBS in 50% ethanol (EtOH) or 50% EtOH as a vehicle control (Figure 3A). Although reconstituted NSGAb$^\circ$DR1 mice challenged with TNBS exhibited significant weight loss 3 days post-challenge, weight loss was not readily observed in reconstituted mice challenged with EtOH or in non-reconstituted mice challenged with TNBS (Figure 3B). Consistent with these observations, blinded histological evaluation of colonic sections stained with H&E revealed extensive crypt and goblet cell loss with edema, fibrosis, and transmural inflammation in TNBS-treated NSGAb$^\circ$DR1 mice reconstituted with human CD4$^+$ T but not in control mice (Figure 3C), with a histological colitis score that was significantly higher in TNBS-treated reconstituted NSGAb$^\circ$DR1 mice compared to controls (Figure 3D). This correlated with an increase in CD4$^+$ T cells

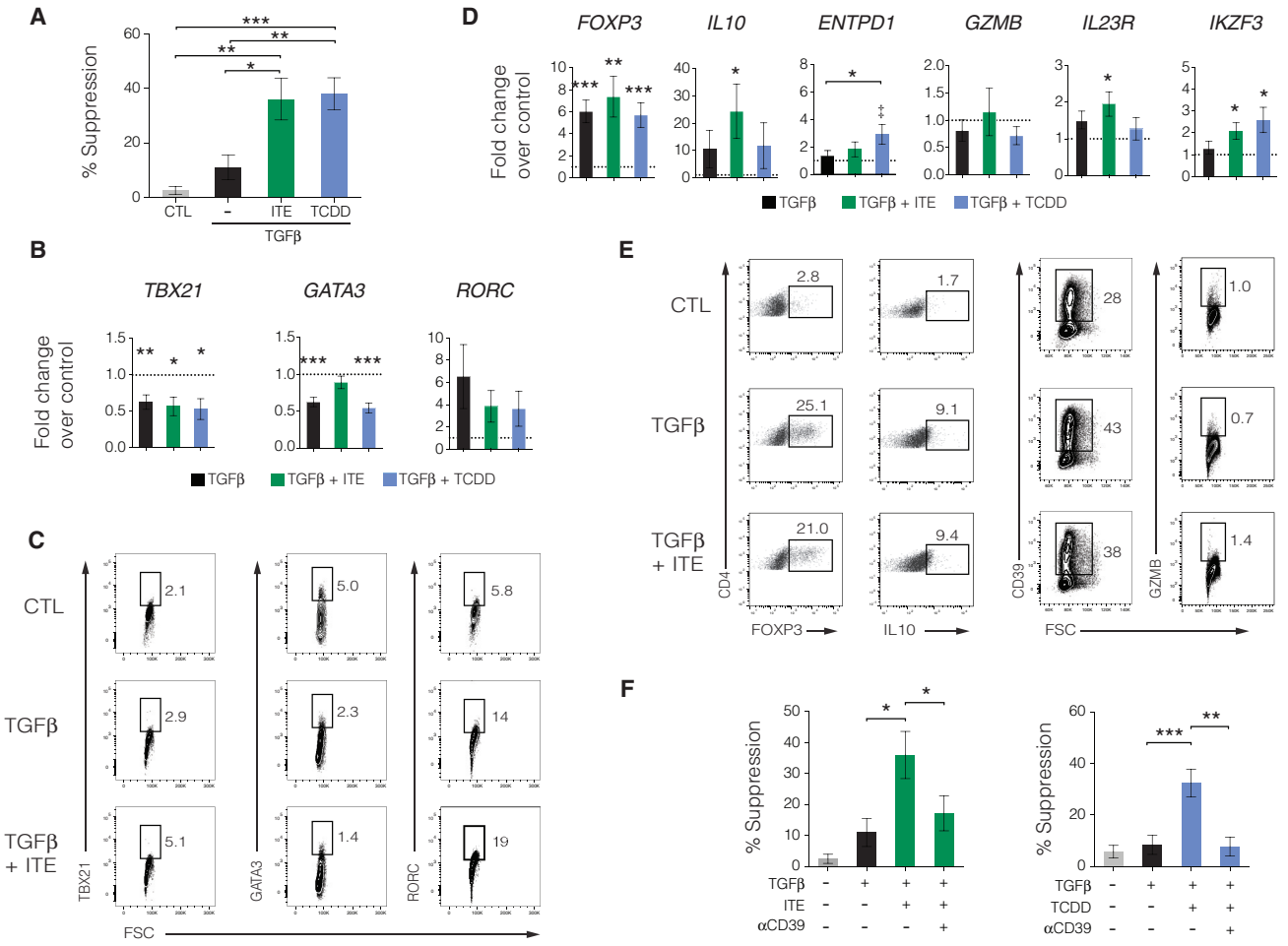

Figure 2. ITE Induces Functional Human FOXP3$^+$ Tregs in the Presence of TGF-β1

(A) Suppressive activity of human naive CD4$^+$ T cells activated with αCD3/αCD28 and TGF-β1 in the presence of CTL, ITE, or TCDD. Percent suppression is depicted as the mean ± SEM pooled from at least two independent experiments with ten unique donors.

(B) qPCR analysis on RNA isolated from T cells stimulated with αCD3/αCD28 and IL-2 in the presence of CTL, TGF-β1, TGF-β1 + ITE, or TGF-β1 + TCDD. Target fold change was calculated using vehicle control, with the dashed line representing the normalized control value of 1 and relative expression shown as the pooled mean ± SEM ($n \geq 10$).

(C) Representative flow cytometric analysis of TBX21, GATA3, and RORC protein expression in T cells following activation using αCD3/αCD28 and IL-2 in the presence of CTL, TGF-β1, or TGF-β1 + ITE from three independent experiments.

(D) qPCR analysis on RNA isolated from T cells stimulated with αCD3/αCD28 in the presence of CTL, TGF-β1, TGF-β1 + ITE, or TGF-β1 + TCDD. Target fold change was calculated using vehicle control, with the dashed line representing the normalized control value of 1 and relative expression shown as the pooled mean ± SEM ($n \geq 7$). ‡$p < 0.05$ compared to control.

(E) Representative flow cytometric analysis of FOXP3, IL10, CD39, and GZMB protein expression in T cells following activation using αCD3/αCD28 and IL-2 in the presence of CTL, TGF-β1, or TGF-β1 + ITE from two independent experiments.

(F) Effect of CD39 blockade using CD39 blocking antibodies on the suppressive activity of TGF + ITE (left panel) or TGF + TCDD (right panel), with the mean depicted as ±SEM ($n \geq 12$).

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

infiltrating in the colonic lamina propria in TNBS-treated, but not EtOH-treated, mice (Figure 3E).

In the standard model, TNBS-induced colitis leads to the up-regulation of several pro-inflammatory cytokines including *Tnf*, *Il2*, *Il12a*, and *Ifng* (Hollenbach et al., 2005; Neurath et al., 1997). We analyzed the expression of human cytokines in the colons of TNBS- or EtOH-treated NSGAb°DR1 mice by qPCR and found increased expression of *TNF*, *IFNG*, *IL2*, *IL4*, and *IL17A* (Figure 3F). Moreover, human T cells recovered from the colonic lamina propria of TNBS-treated mice showed increased production of TNF and interferon γ (IFN-γ) following ex vivo stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Figure 3G). Collectively, these data show that human CD4$^+$ T cells mediate disease pathology in TNBS-induced colitis in humanized NSGAb°DR1 mice. Moreover, these data support the use of this humanized model to assess the efficacy of therapeutics

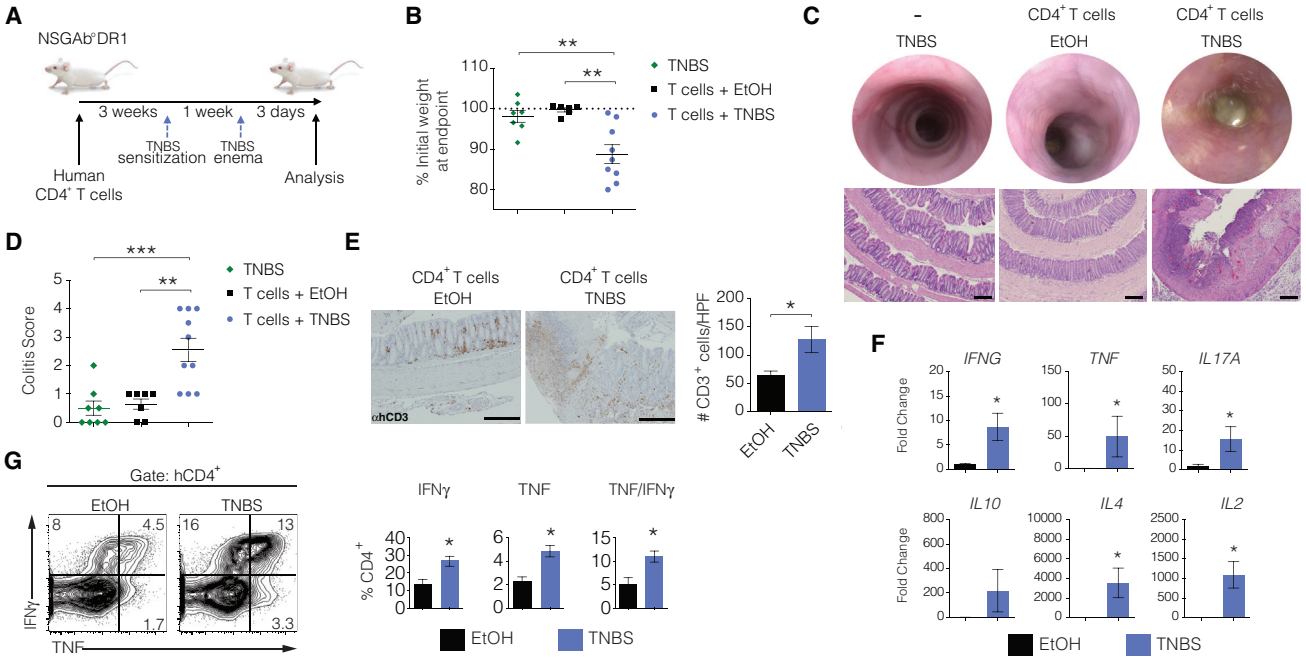

Figure 3. TNBS-Induced Colitis Mediated by Human CD4+ T Cells

(A) Schematic of human CD4+ T cell reconstitution and TNBS experimental colitis in NSGAb°DR1 mice.

(B) Change in body weight in NSGAb°DR1 mice reconstituted with human CD4+ T cells 3 days following rectal administration of EtOH as a vehicle control (n = 6), TNBS (n = 9), or NSGAb°DR1 mice without human T cells administered TNBS (n = 7). Each dot represents an individual animal, with the mean depicted as ±SEM. Data shown are pooled from three independent experiments and are representative of more than six experiments with similar results.

(C) Endoscopic images of representative mice 3 days following TNBS or EtOH challenge (top), with representative H&E-stained colonic sections (bottom) from three independent experiments (10× magnification).

(D) Colitis scoring of H&E-stained colon sections 3 days following the TNBS or EtOH rectal challenge. Scores for each mouse are shown, with the mean for each group depicted as ±SEM.

(E) Representative microscopic images of formalin-fixed, paraffin-embedded colonic sections stained for human CD3 from NSGAb°DR1 mice previously reconstituted with human CD4+ T cells treated with EtOH or TNBS (n = 4) (left) and quantified (right) with bars representing the mean ± SEM (20× magnification).

(F) qPCR analysis on RNA isolated from colonic tissue of reconstituted NSGAb°DR1 mice treated with TNBS or EtOH (n = 6). Human cytokines were normalized to hypoxanthine phosphoribosyltransferase (*HPRT*), and the fold change was compared to a pooled human RNA control sample using the formula $2^{-(Ct(target)-Ct(HPRT))}$. Bars represent the mean ± SEM.

(G) Representative flow cytometric analysis of human CD4+ T cells isolated from the colonic lamina propria of NSGAb°DR1 mice stimulated with PMA and ionomycin for 4 hr in the presence of GolgiStop and then stained for intracellular TNF and IFN-γ (top) and quantified (bottom) from three independent experiments ($n \geq 5$).

Scale bars, 200 μm. *p < 0.05, **p < 0.01, ***p < 0.001.

that target human CD4+ T cells in promoting intestinal immune homeostasis. Consistent with our results, recent studies have showed that the homology of MAdCAM-1 between mice and humans permits binding of human α4β7 integrin to murine MAdCAM-1 and that human CD4+ T cells injected into DSS-treated NSG mice migrate to the colon (Fischer et al., 2016).

ITE Prevents T-Cell-Driven Experimental Colitis in Humanized Mice

To investigate the effects of ITE on human T cells in vivo, we used the humanized mouse model described earlier, and 1 week following engraftment of human CD4+ T cells, mice were administered daily injections of ITE or PBS as a vehicle control for 5 days. The day following the last ITE or PBS injection, mice were sensitized to TNBS and 1 week later administered a single TNBS rectal challenge (Figure 4A). The administration of ITE to humanized mice followed by the TNBS challenge resulted in a trend in protection against weight loss compared to control (Figure 4B). Histological evaluation of H&E-stained colonic sections showed a significant reduction in the severity of inflammation in mice receiving ITE that corresponded to a reduction in colitis score (Figures 4C). This reduction in colitis by ITE correlated with an increase in the frequency of regulatory human T cells in the colon based on increased expression of GZMB, CD39, IL10, and FOXP3 by flow cytometry (Figures 4D). These data are highly similar to the effects of ITE on human CD4+ T cells observed in vitro (Figure 1). We further investigated the consequences of ITE treatment on human T cells in vivo by analyzing the transcriptional profile of CD4+ T cells isolated from the spleens of ITE- or PBS-treated mice using Nanostring nCounter arrays. ITE treatment upregulated the expression of several transcription factors and molecules linked to anti-inflammatory pathways in human T cells, including *IL10*, *IL21*, *IL22*, *IL32*, *GZMB*, *IKZF2*, and *IKZF3* (Figure S4A) (Evans et al., 2014; Gandhi

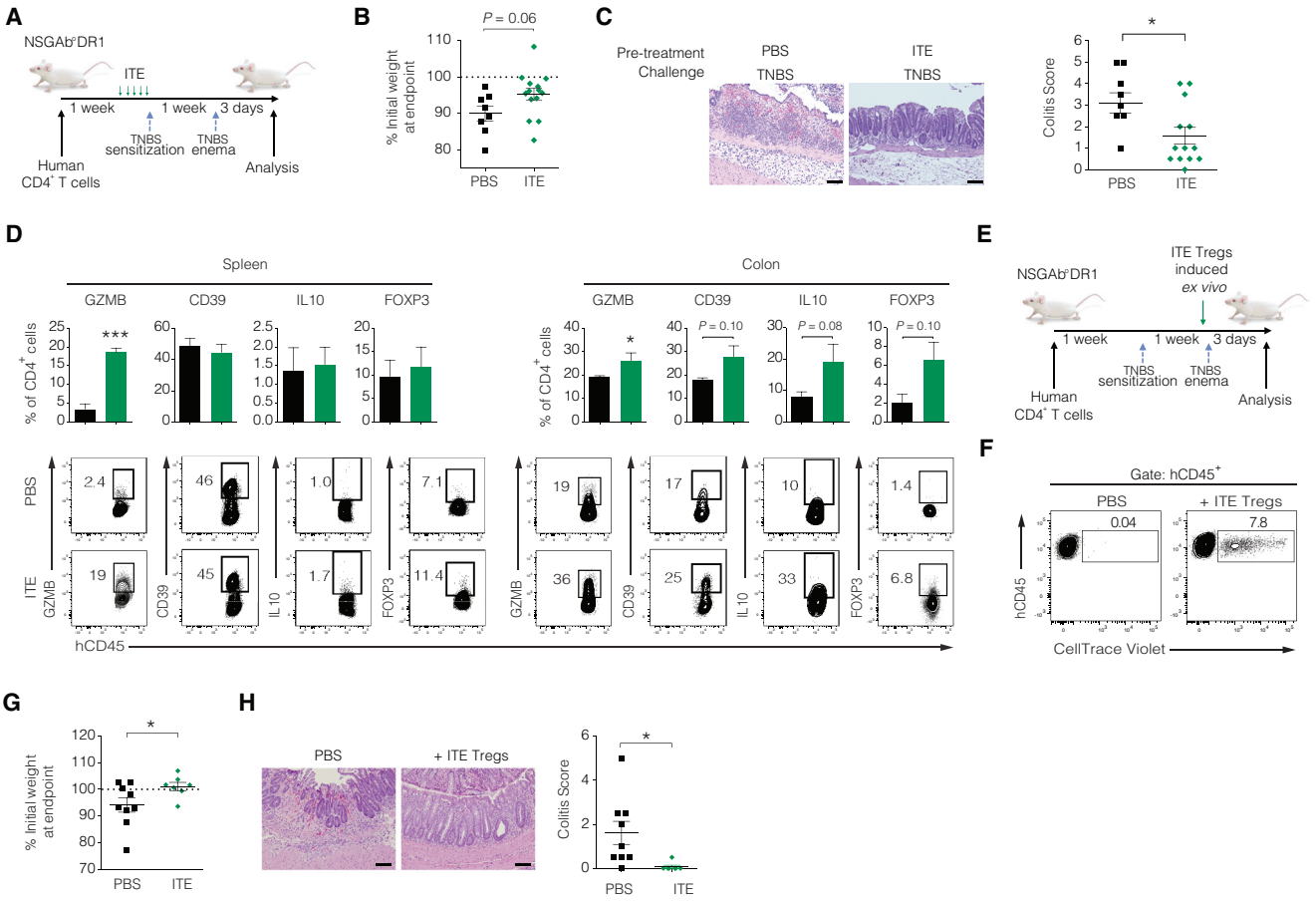

Figure 4. ITE Prevents T-Cell-Driven Experimental Colitis in Humanized Mice

(A) Schematic of ITE administration in TNBS experimental colitis in NSGAb°DR1 mice reconstituted with human CD4+ T cells.

(B) Body weight change 3 days following TNBS rectal challenge in NSGAb°DR1 mice reconstituted with HLA-DR1-matched human CD4+ T cells treated with PBS (n = 8) or ITE (n = 13). Each dot represents an individual mouse, with the mean depicted as ±SEM pooled from three independent experiments using two unique HLA-DR1-matched healthy donor sources.

(C) Representative H&E-stained colonic sections 3 days post-TNBS challenge of reconstituted NSGAb°DR1 mice treated with PBS (n = 8) or ITE (n = 13) (left), and colitis score quantified with the mean for each group depicted as ±SEM pooled from three independent experiments with two unique donor sources (right).

(D) Quantified flow cytometric data of human CD4+ T cells isolated from the spleen and colonic lamina propria of PBS- or ITE-treated NSGAb°DR1 mice stained for human CD45 (hCD45), GZMB, CD39, and FOXP3 (top), with representative dot plots shown below. Intracellular IL10 staining was performed on T cells isolated from spleen and colonic lamina propria and stimulated ex vivo with PMA and ionomycin for 4 hr in the presence of GolgiStop. Bars represent the mean ± SEM pooled from two independent experiments using two unique HLA-DR1-matched healthy donors.

(E) Schematic depicting injection of in vitro-generated autologous ITE-induced suppressive T cells before rectal challenge with TNBS in NSGAb°DR1 mice previously reconstituted with matched donor CD4+ T cells.

(F) Flow cytometry dot plot showing the recovery of in vitro-generated autologous Tregs (labeled with CellTrace violet before intraperitoneal injection of 2×10^6 labeled cells) from spleens of humanized mice.

(G) Body weight change 3 days following TNBS rectal challenge in NSGAb°DR1 mice reconstituted with HLA-DR1-matched human CD4+ T cells and injected with PBS (n = 9) or autologous in vitro ITE-generated suppressive cells (n = 7) 1 day before TNBS rectal challenge. Each dot represents an individual mouse, with the mean depicted as ±SEM pooled from two independent experiments using two unique HLA-DR1-matched healthy donor sources.

(H) Representative H&E-stained colonic sections 3 days post-TNBS challenge of reconstituted NSGAb°DR1 mice injected with PBS (n = 9) or CellTrace violet-labeled autologous ITE-induced Tregs (n = 7) (left), and colitis score quantified with the mean for each group depicted as ±SEM pooled from two independent experiments (right).

Images are 20× magnification. Scale bars, 100 μm. *p < 0.05.

et al., 2010; Quintana et al., 2012; Sakaguchi et al., 2010). Although ITE induced cytokine transcripts known to be both pro- and anti-inflammatory, pathway analysis and functional gene clustering determined that the most affected gene sets were linked to several pathways relevant for IBD pathogenesis with significant downregulation in TNFR1, death receptor signaling, and nuclear factor κB (NF-κB) signaling (Figure S4B). Conversely, ITE treatment led to an upregulation of IL22 (Figure S4B), a known target of AHR linked to the protection of the intestinal epithelium (Sonnenberg et al., 2011; Yeste et al., 2014).

Expression of AHR is not restricted to immune cells, and although our data suggest that the suppressive effects of ITE in vivo were likely mediated by the effects on human $CD4^+$ T cells, we cannot exclude the possibility that the therapeutic benefit of ITE in TNBS-induced colitis is mediated by the activation of AHR in murine cells. To directly test whether AHR activation by ITE in human T cells was sufficient to attenuate TNBS colitis in humanized mice, we first reconstituted NSGAb°DR1 mice with HLA-matched donor $CD4^+$ T cells. We then initiated in vitro cultures of autologous human $CD4^+$ T cells as before to induce suppressive T cells. After 6 days of culture, we labeled the autologous ITE-treated T cells with CellTrace violet and injected 2×10^6 cells per mouse. We administered the rectal TNBS challenge the next day (Figure 4E). We euthanized the mice 3 days post-TNBS challenge and confirmed the presence of the in vitro-cultured Tregs based on CellTrace violet staining (Figure 4F). Humanized mice receiving ITE-treated cells more readily recovered to their initial body weight with less colonic inflammation and a significantly improved colitis score (Figures 4G and 4H). Thus, autologous Tregs generated in vitro were sufficient to protect against TNBS-mediated colitis in humanized mice.

DISCUSSION

Current strategies for the long-term treatment of IBD and other inflammatory diseases often depend on broad immunosuppression that can cause deleterious side effects. Unfortunately, most experimental immunotherapies developed using rodent models have had limited success in human clinical trials (Hay et al., 2014; Persidis, 1999). This is likely due, at least in part, to inherent physiological differences in immune cells between mice and humans. We previously described improved human T cell responses in NSGAb°DR1 mice that express human, but not murine, MHC class II. Moreover, when these mice were reconstituted using HSCs isolated from an IPEX patient lacking functional FOXP3 and Tregs (Goettel et al., 2015), the mice developed a multi-organ inflammatory syndrome, with the development of autoantibodies analogous to patients. We expanded upon this model and demonstrated that in the presence of normal human T cells, TNBS administration to NSGAb°DR1 mice resulted in severe intestinal inflammation. Because the re-establishment of immune tolerance in immune-mediated diseases has shown early promise in the clinical setting through the induction or expansion of autologous Tregs (Desreumaux et al., 2012; Koreth et al., 2011), we investigated whether an endogenous ligand to AHR could induce human Tregs and have a therapeutic effect in a humanized mouse model of intestinal inflammation. We and others have previously shown that AHR activation induces Tregs that suppress the development of inflammation and experimental autoimmunity in mice (Apetoh et al., 2010; Kerkvliet et al., 2009; Mascanfroni et al., 2015; Quintana et al., 2008, 2010; Vogel et al., 2008; Wu et al., 2011; Yeste et al., 2012, 2014; Zhang et al., 2009). Although AHR activation with TCDD induces functional human Tregs in vitro, toxicity prevents the use of TCDD as a therapeutic in humans (Gandhi et al., 2010). Here we demonstrate that the non-toxic AHR agonist ITE induces functional human Tregs that suppress effector T cell proliferation in a CD39- and GZMB-dependent manner in vitro. Furthermore, we employed the TNBS human $CD4^+$ T cell mouse model of colitis described earlier and demonstrated that ITE promoted mucosal immune homeostasis and was protective against colitis development.

For many inflammatory diseases, the effector cytokine profile of $CD4^+$ T cells can be informative and, in some cases, indicative of the T cell subset or subsets involved in disease pathogenesis. The polarization of $CD4^+$ T cells into these specific subsets is highly regulated by transcription factors that drive Th1, Th2, and Th17 differentiation, namely, TBX21, GATA3, and RORC, respectively. We found that ITE, like the toxic AHR ligand TCDD (Gandhi et al., 2010), downregulated Th1 and Th17 transcription factors *TBX21* and *RORC*, although the expressions of molecules associated with Treg suppressive function, including *IL10*, *FOXP3*, *GZMB*, and *ENTPD1*, were increased. This suggests that the suppression of effector T cells by ITE likely occurs by restricting differentiation of Th1 and Th17 effector subsets, in addition to inducing immunoregulatory molecules associated with Tregs. Because IL10, GZMB, and CD39 are known to participate in T cell suppression by Tregs, we tested whether the suppressive effects of ITE were mediated by any of these three molecules. ITE-mediated suppression in the absence of TGF-β1 was dependent on GZMB and CD39 as pharmacological or antibody-mediated inhibition blocked suppression. Under Treg-inducing conditions with TGFβ, ITE did not alter the expression of *GZMB* or *ENTPD1*, whereas protein levels of CD39 were increased at the cell surface. This suggests that AHR activation by ITE may mobilize intracellular pools of CD39 to the plasma membrane. Although CD39 is an important molecule in Tr1 cell differentiation and function (Mascanfroni et al., 2015) and was involved in the suppressive effect of ITE in vitro, it appears that the suppressive cells induced by ITE may be a mixed population of $FOXP3^-$ Tr1 cells expressing traditional markers CD39, GZMB, and IL10, as well as $FOXP3^+$ Tregs. We also observed a population of $CD39^+$ cells that were positive for FOXP3 and may constitute a Tr1-like cell population previously described to possess a regulatory phenotype (Borsellino et al., 2007; Deaglio et al., 2007; Moncrieffe et al., 2010).

Although IBD is a complex disorder triggered by genetic, environmental, and microbial factors, genome-wide association studies have identified more than 160 polymorphisms associated with altered risk for IBD (Jostins et al., 2012). Many of these mutations are known to regulate immune responses, with several being enriched in immune cells, in particular $CD4^+$ T cells and dendritic cells (Jostins et al., 2012). Although polymorphisms in AHR have not yet been associated with IBD, AHR is known to play a central role in the regulation of intestinal inflammation and is upregulated in the inflamed gut (Arsenescu et al., 2011; Benson and Shepherd, 2011; Chinen et al., 2015; Fukumoto et al., 2014; Furumatsu et al., 2011; Huang et al., 2013; Ji et al., 2015; Mascanfroni et al., 2015; Monteleone et al., 2011; Qiu et al., 2013; Quintana et al., 2012; Singh et al., 2011; Takamura et al., 2010, 2011; Yeste et al., 2014). We and others previously showed that in T cells, AHR controls the production of IL10 (Apetoh et al., 2010; Gandhi et al., 2010; Mascanfroni et al., 2015; Wu et al., 2011) and IL-22 (Quintana et al., 2008; Veldhoen et al., 2008, 2009; Yeste et al., 2014) to modulate and promote

immunological tolerance in the intestine. Consistent with these previous findings, our present data demonstrate that AHR activation by ITE induces a transcriptional program in human $CD4^+$ T cells that promotes *IL22* and *IL10* expression in vivo. Moreover, ITE treatment led to an increase in GZMB, CD39, IL10, and FOXP3, particularly in the colon of humanized mice, and attenuated intestinal inflammation induced by TNBS. Although our in vivo data are consistent with in vitro findings for ITE inducing suppressive $CD4^+$ T cells, we cannot exclude the possibility that some of the protective effects of ITE in vivo could also be mediated by other cells types that express AHR, including intestinal epithelial cells and murine innate immune cells (Esser and Rannug, 2015). Nevertheless, we showed that in vitro-generated autologous ITE-treated T cells could be administered just before the TNBS challenge and protect against TNBS-induced colitis in humanized mice. Further investigations on ITE using human $CD4^+$ T cell reconstituted NSGAb°DR1 mice with established TNBS-induced colitis are warranted prior to clinical application aimed at treating IBD.

In conclusion, we have demonstrated that AHR activation with the non-toxic agonist ITE induces, in the presence or absence of TGF-β1, human Tregs in vitro that can suppress effector T cell proliferation in a CD39- and GZMB-dependent manner. Given the inability to readily assess therapeutics in patients, we developed a humanized murine system to directly assess the ability of ITE, as well as other agents, to modulate human $CD4^+$ T cell responses in vivo. We showed that TNBS-induced colitis in humanized mice requires human $CD4^+$ T cells and that ITE attenuated colitis development, promoting immunological tolerance. This model has the advantage that it does not require full human immune reconstitution using HSCs and can be established using T cells from healthy controls, as well as patient cells. The use of this model will facilitate evaluation of potential treatments for IBD and, more importantly, investigations into the inflammatory response of human $CD4^+$ T cells from patients with mutations in loci associated with altered risk for IBD, including *IL10R* or *IL23R* (Duerr et al., 2006; Glocker et al., 2009; Sarin et al., 2011). This may enable clinicians to stratify patients and distinguish responders versus non-responders, leading to tailored therapeutics.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

The following flow cytometry antibodies were obtained through BioLegend: FOXP3, clone 259D; CD39, clone A1; CD45, clone HI30; CD4, clone OKT4; GZMB, clone GB11; TNF, clone MAb11; and IFNG, clone 4S.B3. The following flow cytometry antibodies were obtained through eBioscience: GATA3, clone TWAJ; TBX21, clone eBio4B10; and RORC, clone AFKJS-9. IL10, clone JES3-19F1, was obtained through BD Biosciences. Anti-CD3, clone OKT3, and anti-CD28, clone CD28.6, were used for in vitro stimulation and purchased through eBioscience. TGF-β1 was obtained from R&D Systems. Human recombinant IL-2 was obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (NIAID). Annexin V-PE/FITC and 7-AAD were obtained from BD Biosciences.

Isolation of Naive T Cells

Blood samples were collected from healthy controls upon informed consent. The institutional review board at Brigham and Women's Hospital approved all procedures described in this work. PBMCs were obtained by Ficoll density gradient. Total $CD4^+$ T cells were purified using Miltenyi Biotec AutoMACS, and $CD62L^{high}$ and $CD45RO^-$ T cells were purified by fluorescence-activated cell sorting (FACS) using FACSAria (BD Biosciences) to typically obtain 96%–98% purity in post-sort analysis.

T Cell Differentiation

After FACS, naive T cells were activated with plate-bound antibodies to CD3 (1 μg mL^{-1}), soluble CD28 (1 μg mL^{-1}), and human recombinant IL-2 (50 U/mL) with or without TGF-β1 (1 ng mL^{-1}), in the presence or absence of 100 nM ITE. After 6 days of differentiation, the cells were resorted to exclude dead cells and were used for suppression assays or analyzed by FACS or qPCR.

Suppression Assays

Responder T cells ($CD4^+$ T cells) were activated with beads coated with antibodies to CD3 and CD28 (1 μg/10^7) for 5 days in the presence of Tregs at a 2:1 (responder to regulatory) ratio. Cells were pulsed with ^{3}H-thymidine (1 μCi/well) for 16–24 hr at the end of the incubation period. Anti-IL10 (clone 25209) was purchased from R&D Systems and used at 2.5 μg mL^{-1}. CH223191 was purchased from Tocris and was used at 10 μM.

Quantitative Real-Time PCR

RNA was extracted with QIAGEN RNAeasy columns; cDNA was prepared following the manufacturer's instructions (Applied Biosystems) and used as a template for real-time PCR. All primers and probes in this work were provided by Applied Biosystems and were used on the GeneAmp 7500 Sequence Detection System (Applied Biosystems). Expression was normalized to the expression of *GAPDH*. All murine qPCR primers and reagents were obtained from Applied Biosystems.

For humanized mouse studies, qPCR was performed with SYBR Green (Bio-Rad) using a CFX96 real-time PCR (Bio-Rad) machine on cDNA generated with the iScript cDNA kit (Bio-Rad) on 1 μg of total RNA isolated from whole tissue homogenized in TRIzol (Life Technologies). Then, 2 μM of each human target primer was used in the reaction and quantified by normalizing the cycle threshold (Ct) of the target gene to the Ct value of *HPRT*, and the fold change was compared to a pooled human RNA control sample using the formula $2^{-(Ct(target)-Ct(HPRT))}$.

Sequences for human targets are as follows:

IFNG: forward, 5′-TCGGTAACTGACTTGAATGTCCA-3′; reverse, 5′-TCGCTTCCCTGTTTTAGCTGC-3′
TNF: forward, 5′-GAGGCCAAGCCCTGGTATG-3′; reverse, 5′-CGGGCCGATTGATCTCAGC-3′
IL10: forward, 5′-GACTTTAAGGGTTACCTGGGTTG-3′; reverse, 5′-TCACATGCGCCTTGATGTCTG-3′
IL17A: forward, 5′-TCCCACGAAATCCAGGATGC-3′; reverse, 5′-GGATGTTCAGGTTGACCATCAC-3′
IL4: forward, 5′-CGGCAACTTTGTCCACGGA-3′; reverse, 5′-TCTGTTACGGTCAACTCGGTG-3′
IL2: forward, 5′-AACTCCTGTCTTGCATTGCAC-3′; reverse, 5′-GCTCCAGTTGTAGCTGTGTTT-3′
HPRT: forward, 5′-CCTGGCGTCGTGATTAGTGAT-3′; reverse, 5′-AGACGTTCAGTCCTGTCCATAA-3′

TNBS-Induced Colitis and ITE Treatment in Humanized Mice

The generation of NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}*H2-Ab1*^{tm1Doi}.Tg(HLA-DRA*0101, HLA-DRB1*0101) mice (NSGAb°DR1) was previously described (Goettel et al., 2015). Mice were maintained in autoclaved cages with autoclaved food and water ad libitum in the specific pathogen-free facility at Boston Children's Hospital. All animal experiments were approved and conducted according to the institutional guidelines at Boston Children's Hospital.

The 6–8 week old NSGAb°DR1 mice were injected intraperitoneally with 2 × 10^6 human $CD4^+$ T cells isolated from a healthy donor that was positive for the HLA-DRB*0101 allele were purchased from STEMCELL Technologies. Then, 2–3 weeks later, mice were bled and screened for human T cell reconstitution and mice exhibiting greater than 10% human chimerism in peripheral blood were selected for experimental groups. Mice were first sensitized by

applying 150 μL of a 2.5% TNBS (Sigma-Aldrich) solution in 50% EtOH to a 1 cm^2 patch of bare skin at the base of the neck. Seven days later, mice were anesthetized via a single intraperitoneal injection of saline containing 100 mg kg^{-1} of ketamine and 10 mg kg^{-1} of xylazine. Anesthetized mice were held inverted by hand, and a sterile lubricated 3.5 F soft silicon catheter inserted into the colon to a distance of 3–4 cm. Mice were given a single enema containing 0.25 mg of TNBS in a 50% EtOH, 50% PBS mixture in a volume of 50 μL. Mice were then held inverted for 30 s and returned to their cage. Mice were weighed daily, and colitis was assessed 3 days after the TNBS rectal challenge. Histological assessment was performed and scored using a modified system as previously described (Scheiffele and Fuss, 2002). For ITE-treated mice, 200 μg of ITE dissolved in PBS was administered intraperitoneally starting 1 week after injection of human $CD4^+$ T cells for 5 consecutive days. Mice were then sensitized and challenged as earlier.

Analysis of Cytokine Production by T Cells in Humanized Mice

Total splenocytes and colonic lamina propria cells were plated at a concentration of 1 × 10^6 cells in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 mM non-essential amino acids, 10 mM HEPES, 55 μM 2-mercaptoethanol, and 100 U/mL penicillin/streptomycin (Life Technologies) (hereafter referred to as T cell media). Cells were stimulated with stimulated with 20 ng mL^{-1} PMA and 1 μg mL^{-1} ionomycin (Sigma-Aldrich) for 4 hr at 37°C in the presence of 10 μg mL^{-1} GolgiStop (BD Biosciences). Cells were collected and washed 2× with FACS buffer (PBS supplemented with 2% FBS and 0.1% NaN_3). Cell surface staining for human CD4 was performed for 30 min at room temperature (RT), washed 2× with FACS buffer, and then fixed using BD Cytofix/Cytoperm (BD Biosciences) following the manufacturer's protocol. Cells were then stained with IFN-γ and TNF for 45 min at RT. Cells were washed 2× with FACS buffer, and intracellular cytokine production was detected using a three-laser FACSCanto II (BD Biosciences) flow cytometer.

Immunohistochemistry

Histopathology was carried out on formalin-fixed, paraffin-embedded colonic tissue sections stained with an anti-human CD3 antibody (cat# A0452, Dako). Images were acquired using an Olympus microscope mounted with an Olympus DP70 digital camera and DP-Manager software (Olympus) and were quantified using ImageJ software (NIH).

Statistical Analysis

Statistical analyses were performed using the Prism software (GraphPad), and t tests were used in Figures 1, 2, 3E–3G, 4B–4D, 4G, 4H, and S2. ANOVA with multiple comparisons was used in Figures 3B and 3D. $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.082.

AUTHOR CONTRIBUTIONS

J.A.G. and A.Y. performed in vivo experiments. R.G., J.E.K., S.S., D.S.S., and G.M. performed in vitro expression and suppression experiments. A.E.G. performed IL10R signaling experiments. B.P. performed bioinformatics analysis. H.L.W. contributed to the initial experimental design. J.A.G., R.G., S.B.S., and F.J.Q. wrote and edited the manuscript. S.B.S. and F.J.Q. supervised the study.

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