Enhanced T_H17 Responses in Patients with IL10 Receptor Deficiency and Infantile-onset IBD

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Abstract

Background IL10 receptor (IL10R) deficiency causes severe infantile-onset IBD. Intact IL10R-dependent signals have been shown to be important for innate and adaptive immune cell function in mice. We have previously reported a key role of IL10 is the generation and function of human anti-inflammatory macrophages. Independent of innate immune cell defects, the aim of the current study was to determine the role of IL10R signaling in regulating human $CD4^+$ T cell function.

Methods: Peripheral blood mononuclear cells and intestinal biopsies cells were collected from IL10/IL10R-deficient patients and controls. Frequencies of $CD4^+$ T cell subsets, naïve T cell proliferation, regulatory T cell (Treg)-mediated suppression and Treg and T_H17 generation were determined by flow cytometry. Transcriptional profiling was performed by NanoString and quantitative real-time PCR. RNA *in situ* hybridization was used to determine the quantities of various transcripts in intestinal mucosa.

Results: Analysis of 16 IL10- and IL10R-deficient patients demonstrated similar frequencies of peripheral blood and intestinal Tregs, compared to control subjects. In addition, *in vitro* Treg suppression of $CD4^+$ T cell proliferation and generation of Treg were not dependent on IL10R signaling. However, IL10R-deficient T naïve cells exhibited significantly higher proliferative capacity, a strong T_H17 signature and an increase in polarization towards T_H17 cells, compared to controls. Moreover, the frequency of T_H17 cells was increased in the colon and ileum of IL10R-deficient patients. Finally, we show that stimulation of IL10R-deficient Tregs in the presence of IL1β leads to enhanced production of IL17A.

Conclusions: IL10R signaling regulates T_H17 polarization and T cell proliferation in humans, but is not required for the generation and *in vitro* suppression of Tregs. Therapies targeting the T_H17 axis might be beneficial for IL10- and IL10R-deficient patients as a bridge to allogeneic hematopoietic stem cell transplantation.

Key words: IL10, IL10R, TH17, T cells, mucosal homeostasis, VEO-IBD

Introduction

IL10 is a key anti-inflammatory cytokine that is secreted by numerous immune and non-immune cells and regulates both innate and adaptive immune populations[1]. The IL10 receptor (IL10R) is a heterotetramer composed of two units of IL10R1 that bind IL10, and two units of IL10R2 that stabilize this complex. Upon ligand binding, JAK1 and TYK2 are recruited to IL10R leading to phosphorylation of STAT3, with its subsequent translocation to the nucleus and initiation of an anti-inflammatory program[1].

A link between aberrant IL10/IL10R signaling and inflammatory bowel diseases (IBD) was first established with the observations that *Il10*$^{-/-}$ and *Il10rb*$^{-/-}$ mice develop spontaneous intestinal inflammation[2, 3]. Patients with deleterious mutations in *IL10, IL10RA* and *IL10RB* have been recently described: They typically present within the first months of life with colitis and perianal disease (abscess, fistulas and/or deep fissures), as well as arthritis and folliculitis[4-7]. Response to conventional IBD medications, including steroids, immunomodulators and anti-TNFα antibodies is poor, and surgical interventions are common[6, 8, 9]. Allogeneic hematopoietic stem cell transplantation (HSCT) has been shown to be curative[4, 8, 9] and is considered the treatment of choice, if a suitable donor can be identified.

IL10 is known to exert anti-inflammatory effects on both innate and adaptive immune cells in mice, yet its role in mucosal homeostasis in mice and humans has been explored only recently. We and others reported that innate immune IL10R signaling is critical in regulating intestinal immune homeostasis and prevention of colitis in mice[10-13]. Loss of IL10R signaling in innate cells impaired their interactions with wild-type (WT) T cells, causing WT T cells to become pathogenic and mediate intestinal inflammation[10]. Moreover, we demonstrated in mice and humans that IL10R-dependent signals are required for the generation and function of anti-inflammatory macrophages[10]. IL10 also suppresses inflammasome-mediated IL1β production, which has a central role in driving the hyper-inflammatory response in IL10R deficiency in mice and humans[14].

IL10R-dependent signals have been shown to be required for both regulatory and effector $CD4^+$ T cell function in mice. Murai and colleagues demonstrated that *Il10rb*$^{-/-}$ Tregs fail to suppress colitis in *Rag1*$^{-/-}$ mice adoptively transferred with WT $CD45Rb^{high}$ cells[15] and similarly, Chaudhry reported that mice with conditional deletion of IL10Rα in $FOXP3^+$ Tregs develop spontaneous colitis, with prominent T_H17-associated responses[16]. In addition, IL10R signaling in murine effector cells enables intact suppression by WT Tregs and prevention of colitis[17, 18].

Given the central role of IL10 in adaptive immune cell regulation in mice, we hypothesized that IL10 will have an important regulatory role of T_H17-mediated responses in humans that are essential in mediating intestinal inflammation in IL10R-deficient patients. Aberrant T cell function in IL10R-deficient patients may be secondary to innate immune cell dysfunction, as seen in mice, or may be independent and directly result from loss of IL10R-dependent signals in adaptive immune cells.

Methods

Isolation of peripheral blood mononuclear cells

Blood was collected in EDTA tubes from 12 patients (numbers 1-12 in **Table 1**) with loss-of-function IL10R mutations and control subjects (either a healthy parent or an unrelated healthy donor) in accordance with the local Institutional Review Board and the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS (GE Healthcare) gradient, according to manufacturer's instructions.

Isolation of lamina propria cells

Intestinal biopsies from inflamed rectum of Patient 1 and from normal rectal mucosa of a control subject were obtained during endoscopic procedures. Samples underwent epithelial layer stripping with agitation in 10 mM EDTA at 37^0C twice before digestion in collagenase VIII. Cells were washed twice, stained with different antibodies and analyzed by flow cytometer.

In vitro T cell proliferation and Treg suppression

To assess proliferation, we cultured 5 x 10^4 5mM CFSE-labeled blood-borne FACS-sorted $CD4^+CD25^-CD127^+$ T naïve cells with soluble αCD3/αCD28 coated beads (ThermoFisher Scientific). Treg-mediated suppression of T naïve cell proliferation was evaluated by adding sorted $CD4^+CD25^+CD127^-$ Tregs from patient or control to labeled naïve $CD4^+$ T cells (T naïve:Treg ratio 1:1).

In vitro Treg and T_H17 generation

For Treg generation, sorted T naïve cells from patients and controls were stimulated with αCD3/αCD28 beads and TGFβ 2ng/mL (Peprotech) for 4 days. Cells were then fixed, permeabilized and stained for FOXP3, a hallmark of Tregs. To assess T_H17 polarization naïve $CD4^+$ T cells were cultured with αCD3/αCD28 beads, TGFβ 2 ng/mL, IL6 50ng/mL (Peprotech), anti-IL4 and anti-IFNγ, both 10 μg/mL (BioLegend). After 4 days PMA and ionomycin were added, in the presence of GolgiStop, and then cells were fixed, permeabilized and stained for

IL17A.

IL1 stimulation assay of Tregs

Tregs were sorted using MACS beads (Miltenyi) and plated in a 96-well plate ($4x10^5$ cells/well) in the presence of soluble αCD3/αCD28 coated beads (ThermoFisher Scientific) and IL2 10 ng/mL (Peprotech), with our without IL1β 10 ng/mL (Peprotech). After 4 days IL17A production was measure by ELISA in supernatants (Biolegend).

Flow cytometry

Cells were acquired using FACS Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo. For intra-cellular staining cells were stimulated for 4 hours with 500 ng/mL ionomycin and 50 ng/mL PMA in the presence of 1 µg/mL of Golgistop. Cell were then fixed, permeabilized (BD Bioscience) and stained for intracellular proteins. For FOXP3 staining fixation/permeabilization kit by eBioscience was used. The following antibodies were used: CD3 clone SK7 (BioLegend); CD4 clone RPA-T4 (BD Bioscience); CD25 clone 2A3 (BD Bioscience); CD127 clone HIL-7R-M21 (BD Bioscience); IFNγ clone 4S.B3 (BioLegend); IL17A clone N49-653 (BD Bioscience); FOXP3 clone PCH101 (eBioscience).

RNA analysis

$CD4^+$ T naïve cells and Tregs from two IL10R-deficient subjects (patients 1 and 2) and three controls were sorted into Trizol (Invitrogen). 100ng of extracted RNA were used for each subject. NanoString was performed according to the nCounter Gene Expression Assay User Manual. The nCounter CX Human Immunology code set was used. The data was analyzed in accordance to nCounter expression data analysis guide using the nSolverAnalysisSoftware. Negative probes A-H were used for background subtraction and the data set was normalized to the geometric mean of the samples. Genes that were up or down regulated by more than two folds when compared to controls are represented by the heat map for both naïve and regulatory T cells. These genes were then subjected to the Ingenuity Pathway Analysis (IPA, Qiagen) and

predictive pathways were generated. For selected transcripts quantitative real-time PCR (qRT-PCR) was performed. Complementary DNA was reverse transcribed from RNA with iScript Select cDNA SynthesisKit (Bio-Rad). qRT-PCR was performed using Fast Taqman reagents (Life Technologies), 18S was used as the control probe.

RNA In Situ Hybridization

In Situ Hybridization was performed on formalin fixed colonic and ileal paraffin embedded tissues from IL10R-deficient patient 1 and 11, respectively. These samples were compared to tissue obtained from subjects without IBD (control) matched to the location (ileal when compared to patient 11 and colonic when compared to patient 1) as well as to inflamed colonic tissue obtained from patients with active Crohn's Disease. A total of 3 Crohn's disease control patients and 3-5 healthy controls were included per comparison. One slide per patient was stained and five areas per slide were counted. The slides were cut to 0.4 micron thickness as previously described[19]. RNAScope 2.5 Duplex Detection Kit (chromogenic) was used according to the manufacturers instructions. *FOXP3*, *IL10, IL4 and IFNG* probes were used in C1 and *RORC and CD3* probes was used in C2. All probes and reagents were obtained from Advanced Cell Diagnostics.

Immunochemistry and fluorescence microscopy

Formalin-fixed paraffin-embedded biopsies from two IL10-deficient patients, two IL10RB-deficient patients and two very early-onset IBD patients without an identifiable genetic defect were used for multicolor fluorescence staining as described previously[20]. We applied a sequential tyramide amplification staining procedure: incubation with anti-FOXP3 (clone 236A/E7; Abcam) followed by donkey anti-mouse POD and tyramide FITC amplification (PerkinElmer). POD was inhibited using H_2O_2 and sodium azide. Sections were subsequently incubated with anti-CD3 (polyclonal rabbit; 1:50; Dako) and donkey anti-rabbit POD (Dianova) followed by tyramide Cy5 amplification (PerkinElmer). Dapi (4', 6'-diamidino-2-phenylindole)

counterstaining was performed to visualize the tissue structure and to confirm the nuclear localization of the signals. We analyzed images at 200x magnification. At least 3 representative images were analyzed per tissue section. On average 980 $CD3^+$ cells were analyzed per patient (range from 124 to 3165 $CD3^+$ cells). Analysis was performed blinded to the groups.

Statistical Analysis

All the results were analyzed by Student's t test or ANOVA using GraphPad Prism version 7.0 (GraphPad software) and presented as mean±SEM. For T cell proliferation and Treg generation assays we used paired t test assays. A *p* value of less then 0.05 was considered statistically significant. * P value < 0.05; ** P value < 0.01; *** P value <0.001.

Results

Characterization of IL10R-deficient Patients

We obtained blood samples from 12 patients with IL10R loss-of-function mutations, 5 of which had *IL10RA* mutations and 7 with *IL10RB* mutations (numbers 1-12 in **Table 1**). Formalin-fixed paraffin-embedded rectal biopsies were obtained from four additional patients: two with an *IL10* mutation and two with *IL10RB* mutations (numbers 13-16 in **Table 1**). All patients presented with severe colitis in the first months of life that was refractory to multiple immunosuppressive regimens. No differences in clinical presentation were observed between *IL10-, IL10RA*- and *IL10RB*-deficient patients. Two patients developed diffuse large B cell lymphomas, which is known to be associated with IL10R deficiency[21]. At the time of sample collection, none of the patients had undergone HSCT.

Loss of IL10R-dependent signals leads to increased proliferation of T cells and enhanced T_H17 responses

In order to assess whether response to IL10 is required for suppressing *in vitro* T cell proliferation, sorted $CD4^+$ T naïve cells from patients and controls were labeled with CFSE and stimulated through the antigen receptor with αCD3/αCD28-coated beads. In four of five IL10R-deficienct patients that were studied, T cells exhibited increased proliferative capacity compared to control subjects (**Figure 1**), suggesting that autocrine IL10 suppresses proliferation in human T cells. Next, in order to determine whether IL10R-deficeint T naïve cells possess a unique transcriptional profile that might lead to enhanced proliferation we performed extensive RNA analysis on sorted $CD4^+$ T naïve populations from 2 patients and 3 controls employing NanoString technology. We identified several transcripts that were significantly upregulated among patients' cells (**Figure 2A**), including *RORC, MAF, LTB4R* and *ICOSLG,* all known to regulate T_H17 generation and function[22-25]. Interestingly, expression of SOCS3, which is stimulated by IL10R-dependent signals[1], was higher among patients (**Figure 2A**). However, the upregulation of SOCS3 in IL10R-deficient T naïve cells may result from increased signaling of other pro-inflammatory cytokines, such as IL1β[26], which we have previously shown to be

upregulated in these patients[14]. Finally, IPA analysis predicted an increase in T_H17 generation among patients (**Figure 2B**), suggesting a possible role for these effector cells in the pathogenesis of the disease in IL10R deficiency.

We next assessed whether lack of IL10R signaling alters *in vitro* differentiation of T naïve cells into T_H17 cells. Under polarizing conditions, generation of IL17A-producing $CD4^+$ T cells was significantly increased in IL10R-deficienct $CD4^+$ T naïve cells compared to controls (**Figure 3A-B**). A T_H17 signature was also seen in the gut: Flow cytometry of inflamed colonic sample from an IL10R-deficient patient demonstrated increased frequency of IL17A producing T cells, compared to a healthy control (**Figure 3C**). In addition, Colonic *RORC* RNA expression (upregulated in IL17A producing T cells) was highly expressed in that patient, similar to active Crohn's disease patients with colonic disease, and was also increased in another IL10R-deficient patient with ileal disease (**Figure 3D-E**). However, colonic IFNγ levels were lower compared to Crohn's disease controls while and IL4 levels were comparable (**Supplemental Figure 1**). Overall, our data indicates that IL10R-dependent signals regulate T_H17 differentiation in both peripheral and mucosal compartments.

IL10R signaling is dispensable for Treg generation and *in vitro* suppressive function

Murine data suggests that IL10R-dependent signals in Tregs are required for preserving mucosal homeostasis and prevention of colitis[15, 16]. In addition, Murai and colleagues showed that IL10R-deficient Tregs loose FOXP3 expression over time[15]. There is limited data in humans whether Treg architecture and function are impaired in the setting of IL10R deficiency. The frequency of Tregs in the blood of IL10R-deficient patients was comparable to healthy subjects (**Figure 4A-B**), as was *FOXP3* expression (**Figure 4C**). In addition, the *in vitro* generation of $FOXP3^+$ Tregs from naïve $CD4^+$ T cells under polarizing conditions was also not dependent on IL10R-dependent signals in T naïve cells (**Figure 4D-E).** Moreover, the frequency of $FOXP3^+$ Tregs was also not decreased in mucosal samples from several patients with IL10 and IL10R deficiency compared to healthy controls and very early-onset IBD patients without an identifiable

genetic cause (**Figure 5A-C, Supplemental Figure 2**). Interestingly, in our cohort of 3 Crohn's disease patients with colonic disease mucosal FOXP3 expression was very low. IL10 expression was decreased in the inflamed rectum of a single IL10R-deficient patient as well as in active Crohn's disease patients vs. healthy subjects. However, in an inflamed ileal tissue from a different IL10R-deficient patient IL10 expression was comparable to healthy controls (**Figure 5B-C**).

We next sought to determine whether IL10R-dependent signals are required to facilitate Treg suppression *in vitro*. IL10R-deficient Tregs exhibited similar suppressive capacity of control T naïve cell proliferation, compared to Tregs from control subjects (**Figure 6A)**. In addition, control and IL10R-deficient Tregs were equally competent to suppress T naïve cells proliferation of patients with IL10R deficiency (**Figure 6B)**. Together, our data indicates that IL10R-dependent signals are not required for the generation and *in vitro* suppressive function of Tregs in humans.

Upregulation of IL1 signaling in IL10R-deficient Tregs leading to increased production of IL17A.

To further characterize Treg function in IL10R-deficient patients vs. controls we performed transcriptional profiling of sorted Tregs, using NanoString technology. Although there were some differences in gene expression, the Treg signature was similar (e.g., *FOXP3, CTLA4, IL10, TGFB)* between the groups (**Figure 7A**; data not shown). However, using IPA, IL10R-deficient Tregs are predicted to upregulate the downstream signaling cascade of *IL1A* and *IL1B* (**Figure 7B**).

Several groups have reported that stimulation of Tregs in the presence of IL1β converts them into T_H17 cells that produce IL17[27-29]. We have recently demonstrated that IL1β is highly upregulated in human IL10R-deficient patients[14]. Therefore, we hypothesized that as a result of high IL1β production in IL10R deficiency Tregs may cross-differentiate into T_H17 cells and acquire a pro-inflammatory phenotype. Unstimulated control and IL10R-deficient Tregs did not produce IL17A; however, following αCD3/αCD28 and IL2 stimulation, Tregs from a single

IL10R-deficient patient produced higher quantities of IL17A than Tregs from a control subject (**Figure 7C**). Importantly, addition of IL1β to stimulated Tregs markedly enhanced IL17A production in the IL10R-deficient patient, and to a much lesser extent in the control subject (**Figure 7C**). Overall, our data supports the hypothesis that IL1β, which is upregulated among IL10R-deficeint patients, leads to enhanced cross-differentiation of Tregs into T_H17 cells.

Discussion

IL10- and IL10R-deficient patients develop a severe form of IBD manifesting very early in life. Our previous work demonstrated that intact IL10R-dependent signals in humans are required for the generation and function of anti-inflammatory macrophages and suppression of pro-inflammatory responses[10]. In addition, data from IL10R-deficient mouse models suggested that loss of IL10R signaling alters the function of effector and regulatory $CD4^+$ T cells[15-18]. Here we studied the role of IL10 in regulating immune responses in different T cell populations in humans, in order to determine whether functional T cell alterations are the result of primary effects of IL10R deficiency in T cells or are secondary to innate immune cell dysfunction. We show that IL10 suppresses $CD4^+$ T cell proliferation and limits generation of T_H17 cells: T cells obtained from IL10R-deficient patients exhibited enhanced proliferative capacity and T_H17 cell generation, which may contribute to the pathogenesis of the disease. Interestingly, IL10-dependent signals were dispensable for *in vitro* Treg generation and suppressive function of effector T cell proliferation. However, IL10R-deficient Tregs from a single patient that was studied were sensitive to IL1β stimulation leading to enhanced IL17 production.

T_H17 cells in mice are known to express the IL10R and are controlled by IL10 secreted from classic Tregs ($FOXP3^+$), TR1 cells ($FOXP3^-$)[17], B cells[30] and possibly other innate immune cells. Blocking IL10R signaling in mouse effector T cells leads to increased generation of T_H1 and T_H17 cells in the small intestine during an acute αCD3 model of intestinal inflammation[17]. Similarly, loss of IL10R expression in effector T cells ($CD45RB^{low}FOXP3^-$) results in T_H17-mediated colitis following transfer into *Rag1*$^{-/-}$ mice[18]. Our human data is consistent with these findings that T naïve cells acquire a T_H17 phenotype as a result of absence of IL10R signaling.

IL10 has been previously shown to limit the generation of $CD4^+$ IL17A-producing T cells in humans. Heo and colleagues demonstrated that under T_H17 polarizing conditions addition of IL10 decreases *RORC* expression and IL17 production in $CD4^+$ T cells in rheumatoid arthritis patients[31]. Similarly, blocking IL10 in intestinal immune cells from ankylosis spondylitis patients

significantly increased the frequency of T_H17 cells[32]. T_H17 cells are abundant in the intestinal lamina propria of Crohn's disease patients, and are thought to be a key immune population mediating tissue damage[33]. While IL10R expression in T_H17 cells from Crohn's disease patients has not been investigated, our data along with the studies above suggest that IL10R-dependent signals down-regulate the differentiation of $CD4^+$ T naïve cells into T_H17 cells.

While we have shown that IL10R signaling suppresses T_H17-cell differentiation in humans, we failed to identify a role for IL10R in the generation or in vitro suppressive function of human Tregs. Two studies in mice highlighted that loss of IL10R signaling in Tregs leads to aberrant function of Tregs and development of colitis[15, 16], and in one of these studies IL10R-deficienct Tregs lost their FOXP3 expression over time[15].

Hsu and colleagues recently showed that in humans IL10 potentiates in vitro Treg generation, increases their FOXP3 and enhances the suppressive function of these inducible Tregs[34]. They showed in a single IL10RA-deficeint patient that inducible Tregs suppressed normally effector T cell proliferation[34]. Normal peripheral blood Treg frequency has been described in several IL10R-deficient patients[6, 35]. We show similar frequencies of circulating Tregs in the blood and intestine, normal FOXP3 expression and intact generation of inducible Tregs among a large cohort of IL10- and IL10R-deficient patients. We also show intact *in vitro* suppressive function of IL10R-deficient Tregs. Thus, IL10R-dependent signals seem dispensable for Treg generation and suppressive function *in vitro*. However, since mice with a conditional deletion of *IL10Ra* in Tregs developed spontaneous colitis and splenic Tregs from these mice also suppressed normally *in vitro*, such assays likely do not adequately predict Treg function *in vivo*. Moreover, we show that IL1 downstream signaling is upregulated in IL10R-deficient Tregs, and that stimulation with IL1β results in increased conversion into IL17A-producing T cells in an IL10R-deficient patient. $IL17^+FOXP3^+$ T cells have been identified in the lamina propria of Crohn's disease patients, but not in patients with ulcerative colitis or healthy subjects[37]. These cells were reported to share phenotypic characteristics of both Tregs and T_H17 cells[37]. Whether the IL1β-

induced T_H17 cells in our studies retain suppressive capacity or acquire an effector phenotype is unclear. Valmori and colleagues reported that human T_H17 generated from Tregs contain several distinct populations that secrete IL17 but differ in their ability to suppress effector T cell proliferation[28]. Given the limited number of patients that were assessed, additional studies are required to characterize the functional effects of IL1α and IL1β on Tregs in IL10R-deficient patients and more broadly in IBD.

Our study has two main limitations: First, our functional studies were conducted in sorted populations under *in vitro* conditions, which may not reflect the true function of these cells *in vivo*. As an example, while mice with conditional deletion of IL10Ra in Tregs develop spontaneous colitis, these Tregs suppress normally *in vitro*[16]. A functional defect in these cells may be evident *in vivo*, as a result of a rich IL1 environment as we show. Second, some of the studies involving RNA analysis and lamina propria staining included a small number of patients. Therefore, additional studies are warranted to confirm our observations and specifically assess functional consequences of IL10R deficiency in different $CD4^+$ T cell populations in the gut.

Our findings may have important clinical implications concerning therapy of IL10R-deficient patients. Since the T_H17 pathway is highly upregulated in human IL10R deficiency, therapies targeting important cytokines involved in this axis, such as IL23, IL6 and IL1 might be beneficial in suppressing the hyper-inflammatory response. We have recently reported that treatment with anakinra, an IL1 receptor antagonist, led to significant improvement in two IL10R-deficient patients with severe medical-refractory IBD, and was associated with a decrease in intestinal IL17 production[14]. This therapy was used as a bridge to allogeneic HSCT. We are interested in assessing whether treatment with ustekinumab (an anti IL12/IL23 monoclonal antibody), which has shown promising results in Crohn's disease studies[38, 39] is effective in IL10R deficient patients given the role of IL23 in driving T_H17 responses.

In conclusion, our data highlights a unique T_H17 cell signature in patients with IL10R deficiency and severe infantile-onset IBD. We show that these lymphocyte alterations result from inherent defects in adaptive immune cells due to loss of IL10R signaling, which is likely independent of innate immune dysfunction. Targeting key cytokines required for the generation of T_H17 cells may have a role in suppressing intestinal inflammation in patients with IL10 and IL10R deficiency.

Legends to Figures

Figure 1: Enhanced Proliferation of IL10R-deficient naïve CD4$^+$ T cells. Sorted T naïve cells ($CD4^+CD127^+CD25^-$) were labeled with CFSE and plated with αCD3/αCD28-coated beads. Proliferation was assessed following 3-4 days. Figure displays (**A**) a representative flow cytometry plot of proliferation in an IL10R-deficient patient vs. control followed by (**B**) analyses from five separate experiments with different patients. Each circle or square represents a different value of proliferation within the same experiment. P value=0.067.

Figure 2: IL10R-deficient CD4$^+$ T naïve cells express a T_H17 signature. (**A**) Differentially expressed transcripts in sorted T naïve cells from two IL10R-deficient patients vs. three healthy controls. Red, green and black colors represent high, low and mean expression levels, respectively (**B**) Ingenuity pathway analysis predicts upregulation of T_H17 cells in IL10R-deficient T naïve cells.

Figure 3: Increased generation of T_H17 cells in IL10R-deficient patients. Sorted $CD4^+$ T naïve cells were cultured in polarizing conditions skewing them into IL17A production. Figure depicts (**A**) representative example of IL17A production in an IL10R-deficient patient vs. control and (**B**) cumulative data of different subjects. (**C**) Percentages of IL17A and interferon-γ (IFNγ) producing cells in colonic lamina propria cells obtained from inflamed rectal biopsies of patient 1 vs. control. Cells gated on $CD3^+CD4^+$. (**D**) Representative RNA *in situ* hybridization images (40X magnification) of *RORC* staining in colonic tissue from patient 1, Crohn's disease patient and healthy control, and (**E**) ileal tissue from patient 11 vs. control, followed by counts of transcripts per high power field (HPF) on the right. At least 5 images were taken per biopsy and *RORC* positive cells were counted per each HPF. * P value < 0.05; ** P value < 0.01; *** P value <0.001.

Figure 4: IL10R-dependent signals are not required for generation of Tregs. (**A**) Representative FACS plots demonstrating Treg frequency in the blood of an IL10R-deficient

patient vs. control, followed by (**B**) cumulative data. $CD4^+$ Tregs were identified based on FOXP3 or $CD25^+CD127^-$ expression. (**C**) FOXP3 relative expression in sorted Tregs from 2 IL10R-deficient patients vs. 3 healthy controls. Values indicate ratio of expression in Tregs vs. sorted T naïve cells. Sorted $CD4^+$ T naïve cells were plated in polarizing conditions skewing them into Tregs. Figure depicts (**D**) a representative example of Treg generation in an IL10R-deficient patient vs. control, followed by (**E**) results from different experiments. Each circle or square represents a different value of Treg generation within the same experiment. P value=non-significant.

Figure 5: Comparable intestinal Treg frequency between IL10R-deficient patients and controls. (**A**) Flow cytometry image showing percentage of Tregs in colonic lamina propria cells obtained from inflamed rectal biopsies from patient 1 vs. a control subject. Cells gated on $CD3^+CD4^+$. Representative RNA *in situ* hybridization images for *FOXP3* and *IL10* expression (40X magnification) in (**B**) colonic tissue of patient 1, Crohn's disease patient and a healthy control, and from (**C**) ileal tissue of patient 11 vs. a healthy subject, followed by counts of cells per HPF on the right. Star sign marks FOXP3 expression and + sign marks IL10 expression. At least 5 images were taken per biopsy and *IL10/FOXP3* positive cells were counted per each HPF. * P value < 0.05; ** P value < 0.01; *** P value <0.001.

Figure 6: The *in vitro* suppressive capacity of Tregs is not dependent on IL10R signaling. CFSE-labeled sorted (**A**) control T naïve cells or (**B**) IL10R-deficient T naïve cells were cultured with either control Tregs (blue) or patient-derived Tregs (red). Degree of proliferation was compared to proliferation without any Tregs (grey). Results representative of two separate experiments from different patients and controls.

Figures 7: IL10R-deficient Tregs are predicted to upregulate IL1 signaling. (**A**) Differentially expressed transcripts in sorted Tregs from two IL10R-deficient patients vs. three healthy controls. Red, green and black colors represent high, low and mean expression levels, respectively (**B**) Ingenuity pathway analysis predicts upregulation of *IL1A* and *IL1B* signaling in IL10R-deficient Tregs. (**C**) Sorted Tregs from an IL10R-deficient patient and a healthy control were stimulated for 4 days with αCD3/αCD28 beads and IL2, with our without IL1β. Figure depicts IL17A production determined by ELISA.

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