Intestinal IFN-γ-producing Tr1-cells co-express CCR5 and PD-1, and down-regulate IL-10 in the inflamed gut of IBD patients

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The role of Tr1-cells in inflammatory bowel diseases

Uninflammed gut

Inflammatory Bowel Disease

Macrophages/DC

T helper

bacteria

LUMEN

Stimulation
reduced Stimulation

Inhibition
reduced Inhibition

IL: Interleukin
IFN: Interferon
R: Receptor
CC: Chemokine
PD: Programmed Death
DC: Dendritic cell
Treg: regulatory T-cell
Tr1: type 1 regulatory T-cell
Foxp3: Forkhead box P3
Intestinal IFN-γ-producing Tr1-cells co-express CCR5 and PD-1, and
down-regulate IL-10 in the inflamed gut of IBD patients.

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Clinical implications:
IL-10 expression by intestinal Tr1-cells is a candidate prognostic parameter in
Crohn’s disease and Ulcerative Colitis. Cellular therapy of IBDs with IL-23
unresponsive Tr1-cells could be highly efficient.

Capsule summary:
Intestinal IFN-γ and IL-10 co-expressing-Tr1-cells that suppress T-cell activation
and colitis can be tracked in humans and mice by CCR5 and PD1 co-expression.
Tr1-cells are responsive to IL-23, and down-regulate IL-10 with IL-1β and IL-23
and in the inflamed gut of IBD patients, while IL-10 production by CD25⁺Tregs is
poorly affected.

Key words: IBD, regulatory T-cells, IL-10, IL-23

Abbreviations:
IBD: inflammatory bowel diseases, LPMC: lamina propria mononuclear cells, CD:
Crohn’s Disease, UC: Ulcerative Colitis, CRC: Colorectal cancer HD: healthy
donors, Tr1-cells: type 1 regulatory cells, CD25⁺Treg: CD25⁺ regulatory T-cell,
DC: dendritic cell
ABSTRACT

Background:

IL-10 is an anti-inflammatory cytokine that is required for intestinal immune homeostasis. It mediates suppression of T-cell responses by type-1 regulatory (Tr1-) cells, but is also produced by CD25+ Tregs.

Objective:

We aimed to identify and characterize human intestinal Tr1-cells, and to investigate if they are a relevant cellular source of IL-10 in inflammatory bowel diseases (IBDs).

Methods:

CD4+ T-cells isolated from the intestinal lamina propria of humans and mice were analyzed for phenotype, cytokine production and suppressive capacities. Intracellular IL-10 expression by CD4+ T-cell subsets in the inflamed gut of IBD patients with Crohn’s Disease or Ulcerative Colitis was compared to non-inflamed controls. Finally, the effects of pro-inflammatory cytokines on T-cell IL-10 expression were analyzed, and IL-10 expression in response to IL-23 responsiveness were assessed.

Results:

Intestinal Tr1-cells could be identified by the co-expression of CCR5 and PD-1 in humans and mice. CCR5+PD-1+ Tr1-cells expressed IFN-γ and efficiently suppressed T-cell proliferation and transfer colitis. Intestinal IFN-γ Tr1-cells, but neither IL-7R+ helper T-cells nor CD25+ Tregs, showed lower IL-10 expression in patients with IBDs. Tr1-cells were responsive to IL-23, and IFN-γ Tr1-cells down-regulated IL-10 with IL-1β and IL-23. Conversely, CD25+ Tregs expressed higher levels of IL-1R, but showed nevertheless stable IL-10 expression.

Conclusions:

We provide the first ex vivo characterization of human intestinal Tr1-cells. The selective down-regulation of IL-10 by IFN-γ Tr1-cells in response to pro-inflammatory cytokines is likely to drive excessive intestinal inflammation in IBDs.
INTRODUCTION

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that plays a non-redundant role to maintain intestinal immune homeostasis [1, 2]. IL-10-deficient mice and patients with genetic defects in the IL-10/IL-10R pathway spontaneously develop intestinal inflammation and colitis in the presence of a normal gut microflora [3, 4, 5]. Experiments with conditional k.o. mice indicated that the protective IL-10 is produced by T-cells [6], and studies with IL-10R-deficient immune cells suggested that intestinal macrophages and Th17-cells are directly controlled by IL-10 [7, 8, 9, 10, 11]. However, several T-cell subsets can produce IL-10 and the relevant cellular source of IL-10 in particular in patients with inflammatory bowel diseases remained unclear. Type 1 regulatory (Tr1) T-cells are characterized by high IL-10 producing capacities and the ability to inhibit T cell responses and colitis in an IL-10-dependent manner [10, 12, 13, 14, 15, 16, 17]. They produce variable levels of IFN-γ and are distinct from conventional regulatory T-cells, which are defined by the expression of CD25 and Foxp3. Nevertheless, also CD25+Foxp3−Tregs can produce IL-10 that is important for their suppressive functions at barrier organs, including the gut [7, 17]. Tr1-cells are less defined then Foxp3+Tregs, but they can be induced under a variety of conditions in vitro [18, 19, 20]. Notably, in vitro generated Tr1-cells are currently tested for their therapeutic potential to treat different immune-mediated diseases, including Crohn’s disease [21, 22, 23]. However, in vitro generated Tr1-cells are likely to differ from in vivo occurring Tr1-cells. We previously identified in vivo occurring Foxp3+ IL-10 and IFN-γ producing Tr1-like cells with regulatory functions among CD4+CD25−IL-7R− T cells in human blood [24]. These Tr1-cells are also present in tonsils where they regulate B-cell responses [25]. In mice, in vivo occurring Tr1-cells co-express LAG3 and CD49b, and accumulate in the small intestine in an anti-CD3-induced, self-limiting colitis model [26]. Conversely, the characteristics of intestinal Tr1-cells in humans, and their role in IBDs remain to be defined [24, 26]. In particular, if Tr1-cells have reduced anti-inflammatory functions in the intestines of IBD patients is unknown.

Here we show that intestinal IFN-γ and IL-10 producing Tr1-cells can be identified by CCR5 and PD-1 co-expression in humans and mice. These Tr1-cells
were IL-23 responsive, down-regulated IL-10 with IL-1β and IL-23, and had reduced IL-10 producing capacities in the intestines of IBD patients, suggesting that pro-inflammatory cytokines inhibit their anti-inflammatory functions in IBDs.

METHODS

Human samples.

Buffy-coat blood from healthy donors and surgically removed intestinal specimens from patients were obtained from the IRCCS Policlinico Ospedale Maggiore, Milan, Italy. Intestinal specimens were obtained from biopsies or from IBD patients undergoing therapeutic resection of highly inflamed parts of the intestine (Table 1). The intestinal mucosa was excised from the intestines of patients with Crohn’s disease (CD) and ulcerative colitis (UC) at the time of surgery. As non-inflamed control, intestinal samples were obtained from healthy tissue (at least 7 cm away from neoplastic tissue) of patients undergoing surgery for colon cancer (CRC, Table 1). As additional non-inflamed controls, biopsies from three patients presenting for gastroesophageal reflux-related symptoms were used. The mucosal layer was separated from the rest of the tissue by a pathologist and directly transferred to our laboratory. The ethical committee approved the use of specimens for research purposes (permission n. 2476) and biopsies (protocol IISR-2014-100922) and informed consent was obtained from patients.

Mice.

C57BL/6, Rag1<sup>-/-</sup>, C57BL/6 CD45.1<sup>+</sup>, and OTII mice were purchased from The Jackson laboratories. Foxp3<sup>RFP</sup> (Fir), IL-10<sup>eGFP</sup> (Tiger) and IL-17<sup>eGFP</sup> (Singer) reporter mice were generated at Yale University. Age- and sex-matched littermates between 8 and 12 weeks of age were used. Animal procedures were approved by the Institutional Animal Care and Use Committee of the Yale University.

Cell isolation.
Humans: Mononuclear cells from peripheral blood (PBMC) and the intestinal lamina propria (LPMC) were isolated by Ficoll-Hypaque gradient following published protocols [27]. Lymphocyte subsets were purified by cell sorting on a FACS Aria (BD) based on surface marker expression (purity of >95%). Thus, Tr1-cells were sorted as CD4+IL-7R-CD25+CCR6+PD1+, Tregs as CD4+CD25+IL-7Rlo and helper T-cells as CD4+IL-7R+CD25lo (supplementary Figure 1). The cellular yield of LPMC was 12.5±8×10⁶ cells, and we obtained 33±15×10³ Tr1-cells and 65±40×10³ Tregs according to the counts of the FACS Aria.

Mice: For the isolation of lamina propria cells from the mouse, small intestine was collected, Peyer’s Patches were removed, and tissue pieces were incubated with 1.5 mM EDTA and 1mM DTT at 37°C for 15 min, followed by mechanic disaggregation with gentleMACS™ dissociator. Cells were then further separated with a Percoll gradient. Cells were isolated from the duodenum and jejunum of anti-CD3-treated mice.

Flow cytometry.

Humans: Cells were stained with the following antibodies: CD4 (RPA-T4, BD), CCR5 (27D, BD), PD-1 (MIH4, Biolegend), CD25 (M-A251, Biolegend), IL-10 (JES-19F1, Biolegend), IFN-γ, CD127 (eBioRDR5, eBioscience), LAG-3 (REA351, Miltenyi), p-STAT3 (4/P-STAT3, BD), CD45RA (MEM-56, Immunotools), CD1c (AD5-BE7, Miltenyi). T cell cytokine production was measured after stimulation with 0.1μM phorbole ester (PMA) and 1μg/ml ionomycin (Sigma-Aldrich) in the presence of 10 μg/ml Brefeldin A (Sigma); cells were fixed, permeabilized, stained and analyzed by flow cytometry. For IL-10 and IFN-γ production of CD4+T-cells subsets, lymphocytes were gated according to CD4, IL-7R, CD25, CCR5 and PD-1 expression (supplementary Figure 1).

Mice: Murine cells were stained with anti-CD4 (GK1.5), anti-PD-1 (RMP1-30), anti-CCR5 (HM-CCR5), anti-CCR6 (29-2L17), anti-CD45.2 (104), anti-LAG-3 (C9B7W), anti CD49b (HMα2), all from Biolegend. Samples were passed on a FACSCanto flow cytometer (BD) and analyzed using FlowJo software (Tristar) or FACSDiva software.

Experimental colitis.
Mice were injected with anti-CD3 (15 μg, 145-2C11), isotype antibody, or PBS i.p. two times every other day. For transfer colitis, Th17 cells were differentiated in vitro from CD4+ T cells from Foxp3RFP IL17eGFP mice with TGF-β, IL-6 and IL-23 for 5 days with anti-CD3 and irradiated APC and a pure population of IL17eGFP+Foxp3RFP- Th17 cells purified by cell sorting. Tr1 and Treg cells were induced in Foxp3RFP IL-10eGFP mice with two anti-CD3 injections and purified from the small intestine. In some experiments IFN-γ and IL-17 production of purified Tr1-cells was measured following activation with coated anti-CD3 and anti-CD28 antibodies (5 μg/ml each) for 96 hours by ELISA. 3x10^4 IL17+Foxp3- cells were injected into Rag1-/- recipients in the presence or absence of 3x10^4 CCR6-CCR5- PD-1+IL-10+ Tr1 or CCR6-CCR5- PD-1+IL-10+ control cells. Colonoscopy was performed in a blinded fashion via the Coloview System. In brief, colitis score was based on granularity of mucosal surfaces, stool consistency, vascular pattern, translucency of the colon and fibrin visible (0-3 points for each). Histological examinations were performed as per standard methods upon H&E stainings of paraffin embedded small intestinal and colonic specimens.

**Suppression assays.**

Humans: CD4+ intestinal helper T cells were sorted according to IL-7R and CD25 expression and labeled with CellTrace™ Violet (Life Technologies). 2.5x10^4 helper T-cells were co-cultured at 1:1 ratio with sorted intestinal regulatory T subsets, i.e. CD25+IL-7R+ Tregs and CD25-IL-7R-CCR5+ Tr1-cells. 5x10^3 allogenic human myeloid CD1c+ DC from peripheral blood were added as stimulators. For dose-titration experiments, 5x10^3 of total CD4+ T-cells from peripheral blood were stimulated with anti-CD3/CD28 beads at a 1:5 ratio in the absence of presence of autologous intestinal Tregs or Tr1-cells at different ratios (1:1-1:16). Unlabeled responder cells were used as a negative control. After 4-5 days cells were stained and analyzed for CellTrace™ Violet dilution by FACS.

Mice: Murine responder CD45.2 CD4+ T cells were sorted and labeled with CellTrace™ Violet. They were co-cultured at 1:1 ratio with sorted regulatory T cell subsets in the presence of anti-CD3 and irradiated T cell depleted splenocytes. After 4 days cells were stained and analyzed for dye dilution by
FACS. To calculate the percent of suppression we set undivided cells as 100% of suppression and the percentage of proliferating T cells in the absence of regulatory T-cell subsets as 0% of suppression.

**T-cell culture**

Purified human Tr1-cells, Tregs and IL-7R$^+$ helper T-cells were stimulated in 96-well plates, which were pre-coated with ant-CD3 and -CD28 antibodies at 2 µg/ml, and 1000U/ml IL-2 in the absence or presence of recombinant IL-1β and IL-23 at 10 ng/ml. After 72 hours cells were detached from the plates and transferred to uncoated wells. After another 3 days of culture they were tested for IL-10 and IFN-γ production following brief stimulation with PMA and ionomycin as described above. The assay to measure IL-23 responsiveness was described previously [28]. Briefly, cells were extensively washed and incubated in the absence or presence of IL-23, and phospho(Y)-STAT3 measured by intracellular staining and flow cytometry. In the case of intestinal Tr1-cells, IL-23R was assessed by RT-PCR as described [28].

**Statistics**

Statistical significance was calculated using two-tailed Student’s t test in case of Gaussian distribution, otherwise Mann Whitney for unmatched or Wilcoxon for paired groups. In the case of multiple comparisons One-way ANOVA with Tukey post-test was used. p<0.05(*), P<0.005(**) and p<0.0005(***) were regarded as statistically significant.

**RESULTS**

**Human intestinal Tr1-cells that co-express IL-10 and IFN-γ can be identified by CCR5 and PD-1 expression.**

We first investigated whether human T-cells with Tr1-like characteristics as we previously identified in peripheral blood [24] and in tonsils [25] were also present in non-lymphoid tissues such as the intestinal lamina propria. The large majority of CD4$^+$ T-cells in the non-inflamed intestinal lamina propria obtained from colorectal cancer patients had an IL-7R$^{hi}$CD25$^{low}$ helper phenotype and only
A small fraction expressed IL-10 upon ex vivo stimulation with phorbole ester and ionomycin as detected by intracellular staining (Figure 1a). A minor population of CD4+CD25-IL-7R- T-cells could also be detected in the lamina propria, and a significantly higher fraction of these effector-like cells expressed IL-10 (Figure 1a and sFigure 1a and b). In addition, also CD25-IL-7Rlow Tregs were present at low frequencies in the intestinal lamina propria, and they also contained a significant fraction of cells that could express IL-10 upon stimulation. Consistent with these results, high amounts of IL-10 protein were detected in culture supernatants of FACS-purified, intestinal CD25+IL-7RlowTregs and of CD25+IL-7R- effector T-cell subsets following stimulation with PMA and ionomycin, while only low amounts were measured in supernatants of IL-7RhiCD25low helper T-cells (sFigure 1c). In contrast to Tr1-cells, the majority of CD25+IL-7RlowT-cells expressed Foxp3 (Figure 1b), indicating that they represented conventional CD25+Foxp3-Tregs. Moreover, the large majority of IL-10+CD4+CD25+IL-7R-T-cells also co-expressed IFN-γ (Figure 1c), whereas IL-10+CD25+Tregs did not.

The large majority of IL-10+CD4+CD25+IL-7R- T-cells expressed CCR5 and PD-1 (Figure 2a), while CCR6 was largely absent (data not shown). These surface markers allowed to strongly enrich for IL-10+ T-cells in the intestine (Figure 2b). Moreover, purified intestinal CCR5+IL-7R-Tr1-cells secreted significantly higher amounts of IL-10 protein as compared to CCR5-IL-7R- control T-cells (sFigure 1c). Ex vivo purified intestinal CD4+CD25+IL-7R-CCR5+Tr1-cells (sFigure 2) possessed regulatory functions, and inhibited the proliferation of autologous intestinal helper T-cells induced by CD1c+DC nearly as efficiently as conventional CD25+Tregs (Figure 2c). Conversely, intestinal CD4+CD25+IL-7R- control cells that lacked CCR5 expression failed to suppress T-cell proliferation (Figure 2c), consistent with their low IL-10 expression (Figure 2a and sFigure 1c). Finally, when we compared the frequencies of CD4+CD25+IL-7R+CCR5+Tr1-cells among total CD4+ T-cells in peripheral blood and the non-inflamed intestinal lamina propria of colorectal cancer (CRC) patients, we found that Tr1-cells were strongly enriched in the gut (Figure 2d).

LAG-3 is expressed by Tr1-cells in humans and mice [26, 29]. Only a small fraction of human CD4+CD25+IL-7R-CCR5+ Tr1-cells (1.6 ± 1.4%, n=10) showed
LAG-3 surface expression (sFigure 3a). However, we found that human CD4+CD25-IL-7R-CCR5+Tr1-cells, that did not express LAG-3 at the cell surface, nevertheless expressed LAG-3 m-RNA (sFigure 3b). Finally, in line with what previously reported [26], CD4+CD25-IL-7R-CCR5+ Tr1-cells efficiently up-regulated LAG-3 protein expression at the cell surface following TCR stimulation in vitro (sFigure 3c).

Intestinal murine CCR5+PD-1+Tr1-cells suppress transfer colitis.

We next investigated CCR5 and PD-1 expression on intestinal Tr1-cells in mice, and the in vivo suppressive capacities of intestinal Tr1-cells that co-expressed CCR5 and PD-1. Intestinal Tr1-cells can be tracked in IL-10 and Foxp3 double reporter (FirxTiger) mice [26, 30] following repetitive anti-CD3 injections, a treatment that induces a self-limiting colitis. As observed in humans, the large majority of intestinal IL-10eGFP+Foxp3RFP- Tr1-cells co-expressed CCR5 and PD-1 (Figure 3a), and they also co-expressed LAG-3 and CD49b on the cell surface, as expected [26]. Consistent with the high expression of the IL-10eGFP reporter, purified murine CCR5+PD-1+Tr1-cells in from the small intestine secreted high amounts of IL-10 protein upon stimulation with anti-CD3 and anti-CD28 antibodies (sFigure 4a and b). CCR5+PD-1+Tr1-cells also secreted substantial amounts of IFN-γ, but little IL-17 (Figure 3b). Consistently, they did not express CCR6 (data not shown), which is characteristic for Th17-cells [31, 32], but not for human IL-7R-Tr1-cells [25]. Finally, IL-10 producing CCR5+PD-1+ T-cells suppressed the in vitro proliferation of CD4+T-cells with a similar efficiency as Foxp3RFP+Tregs (Figure 3c), confirming that they represent Tr1-cells. Intestinal Tr1 cells suppress Th17 cells in an IL-10-dependent manner in vivo and inhibit colitis [10]. To assess the in vivo suppressive capacities of CCR5+PD-1+Tr1-cells, we transferred in vitro generated Th17-cells (CD4+Foxp3RFP-IL-17eGFP+) into Rag1−/− mice in the presence or absence of purified intestinal Tr1-cells. Transfer of Th17-cells caused disease in the intestine as assessed by the colitis score and by histological evaluation (Figure 3d/e). Co-transfer of IL-10eGFP+CCR5+PD-1+Tr1-cells strongly inhibited colitis induction, while IL-10eGFP+ control T-cells that lacked CCR5 and PD-1 were also suppressive, but were less potent (Figure 3d/e).
IL-10 and IFN-γ co-expressing Tr1-cells down-regulate IL-10 production in the inflamed gut of IBD patients, and with IL-1β plus IL-23 in vitro.

Since intestinal CCR5^+ Tr1-cells inhibited transfer colitis in mice, we asked if they could also play a protective role in human IBDs. We first analyzed if CCR5^+IL-7R^- Tr1-cells or CD25^+ Tregs were reduced in the intestinal lamina propria of patients (Table 1) with Crohn's Disease (CD) or Ulcerative colitis (UC), the two principal forms of IBDs, as compared to the non-inflamed, normal lamina propria obtained from colon cancer control patients (CRC). We observed a moderate increase of CD25^+Tregs in the lamina propria of UC patients and of CCR5^+IL-7R^- Tr1-cells in CD patients, which did however not reach statistical significance (Figure 4a). Notably, there was also no significant difference between the frequencies of these regulatory T-cell subsets in inflamed and non-inflamed biopsies from the same IBD patients (sFigure 5). Intestinal IL-7R^-Tr1-cells had markedly reduced capacities to express IL-10 in both types of IBDs, while IL-10 expression by CD25^+Tregs and IL-7R^hi helper T-cells was not significantly altered (Figure 4b). Interestingly, Tr1-cells that co-expressed IL-10 and IFN-γ were much more strongly affected than those that expressed IL-10 alone, and consequently only IFN-γ and IL-10 co-expressing Tr1-cells were significantly reduced (Figure 4c).

To understand why Tr1-cells down-regulated IL-10 production in the intestines of IBD patients, we activated circulating IL-7R^-CCR5^+Tr1-cells, CD25^+Tregs and IL-7R^hi helper T-cells in the presence of the pro-inflammatory cytokines IL-1β and IL-23, which play critical pathogenic roles in colitis [33, 34, 35, 36, 37, 38, 39, 40]. Only Tr1-cells had reduced capacities to express IL-10 following culture with IL-1β and IL-23 (Figure 5a/b). Furthermore, as observed in the intestines of IBD patients, the effect was selective for IL-10 and IFN-γ co-expressing Tr1-cells (Figure 5c). Conversely, no significant effect on IL-10 was detected in CD25^+Tregs or IL-7R^hi helper T-cells, neither in total IL-10^+ cells (Figure 5b) nor in IFN-γ expressing subsets (sFigure 6a). This was also true when the fold-increase was calculated to eliminate the variability of IL-10 production between different individuals (sFigure 6b). Interestingly, neither
addition nor neutralization of TNF-α to IBD, had a clear effect on IL-10 production (data not shown).

To understand if the selective effect of IL-1β and IL-23 on IL-10 production by Tr1-cells might be caused by a different responsiveness to these pro-inflammatory cytokines, we assessed IL-1R surface expression and IL-23-induced STAT3 activation. Activated CD25⁺Tregs expressed high levels of IL-1R1 and also of IL-1R2, while Tr1-cells and helper T-cells expressed these receptors only at low levels (Figure 5d), arguing against a critical role for IL-1β in the reduced IL-10 production by Tr1-cells. However, IL-23 efficiently induced STAT3 phosphorylation in activated Tr1-cells (Figure 5e and sFigure 7), while IL-23 responsiveness was lower in IL-7R⁺ helper T-cells and in CD25⁺Tregs, although the latter tendency did not reach statistical significance (p=0.088). In order to control if also intestinal Tr1-cells could respond to IL-23, we purified Tr1-cells from the lamina propria of IBD patients and quantified IL-23R m-RNA expression (Figure 5f). Notably, IL-23R expression is efficiently induced on naïve T-cells by Th17-promoting cytokines [28], and is expressed together with CCR6 on Th17-cells [31, 41, 42], in particular in the gut [43]. We therefore compared IL-23R m-RNA expression of intestinal Tr1-cells to that of intestinal CCR6⁺IL-7R⁺CD25⁻Th17-cells as a positive control. IL-23R m-RNA was detectable in all cases in both intestinal T-cell subsets. Interestingly, Tr1-cells and Th17-cells expressed similar levels of IL-23R m-RNA in UC patients, while Tr1-cells expressed even higher levels in CD patients (Figure 5e).

DISCUSSION

Tr1-cells are characterized by IL-10 production, low Foxp3 expression and their capacities to inhibit T-cell responses. Here we identified CCR5 and PD1 co-expression as a characteristic phenotype of IFN-γ and IL-10 producing Tr1-cells in the intestine, allowing for the first time to track and to isolate Tr1-cells directly in the human intestinal lamina propria.

Intestinal Tr1-cells were negative for IL-7R, CD25 and CCR6, distinguishing them from memory T cells, Foxp3⁺ Tregs [44] and Th17/22 cells [31], respectively. They expressed, however, the Th1-associated chemokine receptor CCR5 that allows immune cells to enter non-lymphoid tissues. Indeed, Tr1-cells produced
high amounts of IFN-γ and were enriched in the gut. In addition, they also express PD-1, an inhibitory receptor that directly induces IL-10 in T-cells [45] and that inhibits DC maturation [46]. PD-1 expression on Tr1-cells is consistent with the notion that they are chronically activated cells [24, 47, 48]. In vivo occurring Tr1-cells also express LAG-3 [26, 29], an inhibitory receptor that binds MHC class II, and indeed we found that both human and mouse CCR5+Tr1-cells expressed LAG-3. However, the large majority of human CCR5+IL-7R- Tr1-cells expressed LAG-3 on the cell surface only following TCR stimulation. This data suggest a possible heterogeneity among Tr1-cells, which could result from different stages of activation, maturation and/or function. This aspect is currently under investigation. In any case, the here reported phenotype allows, for the first time, the identification and isolation of Tr1-cells that produce high levels of IL-10 and IFN-γ directly in the human gut.

IL-10 produced by T-cells has a non-redundant role to prevent excessive inflammation in response to the gut microbiota [49]. Both Foxp3+Tregs and Foxp3+Tr1-cells were identified as possible relevant sources of IL-10 in experimental colitis in mice [9, 10, 12], but the contributions of these two regulatory T-cell subsets in human IBD is very difficult to address and therefore largely unknown. Our data points to a critical role of IL-10 and IFN-γ co-producing Tr1-cells in IBD, because only this T-cell population showed reduced IL-10 expression in the intestinal lamina propria of IBD patients. Reduced IL-10 expression by Tr1-cells was detected both in UC and CD patients, indicating that it is a common feature of IBDs. Of note, murine Tr1-cells control colitogenic Th17-cells and macrophages in an IL-10-dependent manner in vivo [7, 8, 9, 10, 11], and also human IL-10 and IFN-γ co-expressing IL-7R-Tr1-cells suppress T-cell responses via IL-10 [24]. Preliminary dose-response experiments with anti-CD3 and -CD28 coated beads and autologous peripheral CD4+ T-cells as responder cells (data not shown) suggested that intestinal Tr1-cells and CD25+Tregs had a similar suppressive potential, and that both subsets could suppress T-cell activation directly.

A caveat of the present study is that we used the healthy lamina propria of colorectal cancer patients as non-inflamed controls, as do many studies on human intestinal immune cells given the difficulty to analyze the gut of healthy individuals.
individuals. It is possible that the presence of intestinal tumors or the increased age of CRC patients influence the properties of intestinal T-cells. However, the similar frequencies of regulatory T-cell subsets at inflamed and non-inflamed sites of the same IBD patients, and the similar IL-10 expression among CD4+ T-cells from non-inflamed biopsies of a few gastrointestinal reflux patients (data not shown) as compared to intestinal CD4+ T-cells from CRC patients suggested that these factors did not have a major impact on the here analyzed parameters.

Interestingly, in non-IPEX autoimmune enteropathy caused by dys-functional Foxp3+Tregs, we observed enhanced IL-10 production of CD4+ T-cells, suggesting that Tr1-cells were not defective. In addition, we observed uncontrolled IL-17 production by CD8+ intraepithelial lymphocytes, which was not observed in CD patients [50]. These findings are consistent with the view that Foxp3+Tregs and Tr1-cells have unique functions in the control of intestinal immune homeostasis. The reduced IL-10 production by Tr1-cells could be explained by their responsiveness to IL-23, a highly pathogenic cytokine in colitis [33, 34, 35, 36, 37, 38]. IL-1β is another pathogenic cytokine in colitis [39, 40], and inhibits IL-10 production by human Th17-cells [51]. However, CD25+Tregs that expressed the highest levels of IL-1Rs maintained normal IL-10 production in cultures with IL-1β, suggesting that IL-23 might be the more relevant cytokine to inhibit IL-10 production by regulatory T-cells. IL-23 is thought to render Th17-cells pathogenic [52, 53], and to inhibit the generation of Foxp3+Tregs from naive precursor cells [33]. The inhibition of IL-10 production by established Tr1-cells is a third mechanism that is likely to be highly relevant in IBDs, and is consistent with increased T-cell IL-10 production in IL-23R k.o. mice [38, 54]. IL-23R expression is characteristic for Th17-cells that express CCR6 and secrete IL-17 [42]. It was therefore surprising that IL-7R:CCR5+Tr1-cells, which lack CCR6 and IL-17 expression [24, 25], were IL-23 responsive. However, IL-23R expression is directly regulated by the transcription factor T-bet [55], which also induces IFN-γ expression, providing a plausible explanation why selectively IFN-γ producing Tr1-cells responded to IL-23 and down-regulated IL-10 in the gut. The physiological function of IL-10 down-regulation is probably to allow for a more efficient response against invading intestinal bacteria, but upon chronic exposure to these bacteria due to a leaky intestinal
barrier the same mechanism could promote chronic inflammation and colitis. Thus, IL-10 production of intestinal Tr1-cells might be a prognostic marker of IBDs. In addition, IL-23 responsiveness is also potentially a novel relevant parameter for T-cell therapy of CD patients [22], since Tr1-cells that do not respond to IL-23 are expected to maintain IL-10 production and to be thus more efficient to cure colitis. Furthermore, IBD patients with low intestinal IL-23 expression might respond better to Tr1 cell therapy.

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CONFLICT OF INTEREST:
The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1: Intestinal human CD4^+IL-7R^-CD25^-T-cells express IL-10 and IFN-γ.

a) IL-7R versus CD25 expression among CD4^+ T-cells in the intestinal lamina propria (left panel). Percentages of IL-10-producing cells among intestinal IL-7R^hiCD25^-/lo (IL-7R^+), IL-7R^-CD25^- (IL-7R^-) and IL-7R^loCD25^hi (Tregs) CD4^+ T-cell subsets following brief polyclonal stimulation. Bars represent Mean ± SEM (n=9). b) Foxp3 expression of intestinal IL-7R^- and Tregs T-cell subsets. One representative Histogram Overlay is shown. c) IL-10 and IFN-γ production by intestinal IL-7R^+ , IL-7R^- and Tregs. Representative dot plots of the same donor are shown (n=4).

Figure 2: Intestinal human Tr1-cells co-express CCR5 and PD-1.
a) Left panel: percentage of IL-10^+ T-cells among intestinal IL-7R^- T-cells according to CCR5 or PD-1 expression. Right/central panels: Expression of CCR5 and PD-1 (right panel) in gated IL-10^+CD4^+IL-7R^- T-cells (central panel). b) Percentage of IL-10^+ T-cells among total intestinal CD4^+, CD4^+IL-7R^- (IL-7R^-) and
CD4⁺IL-7R⁻CCR5⁻PD-1⁺ (Tr1) T-cells (n=5). c) Inhibition of CD4⁺ T-cell proliferation by autologous intestinal Tr1 cells and Tregs. Left: Representative Overlays of CellTrace profiles of intestinal CD4⁺IL-7R⁺ T-cells in the absence (open) and presence (filled) of intestinal Tregs or Tr1 cells. Right: suppression of CD4⁺IL-7R⁺ T-cell proliferation by autologous, intestinal Tr1 cells, Tregs and CCR5⁻IL-7R⁻ control cells. Bars represent Mean ± SEM (n=3). d) Frequencies of Tr1-cells among CD4⁺ T-cells in peripheral blood and in the non-inflamed intestinal lamina propria of CRC patients (“gut”, n=8).

**Figure 3: Intestinal murine CCR5⁺PD-1⁺Tr1-cells produce IFN-γ and inhibit transfer colitis.**

a) Intestinal CD4⁺IL-10⁺Foxp3⁻ cells (left Histogram Plot) were analyzed for co-expression of CCR5 and PD-1 (central dot plot) and CCR5⁺PD-1⁺ cells for LAG-3 and CD49b co-expression (right dot plot). b) IFN-γ and IL-17 production by CCR5⁺PD1⁺Tr1 and IL10⁻ control cells, stimulated ex-vivo with anti-CD3 and anti-CD28 antibodies, was measured by ELISA. Bars represent Mean ± SEM (n=3). c) Inhibition of CD4⁺T-cell proliferation by intestinal Tr1 cells and Tregs: Left: representative overlays of CellTrace profiles of total CD4⁺ T-cells in the absence (open) and presence (filled) of intestinal Tregs and Tr1 cells. Right: suppression of CD4⁺ T-cell proliferation by intestinal Tr1 cells, Tregs and total CD4⁺ cells as control. Bars represent Mean ± SEM (n=3). d-f) Inhibition of transfer colitis by intestinal Tr1 cells or CCR5⁻PD-1⁻IL-10⁺T-cells. d) Mean colitis index (n>4). e) Representative endoscopies and f) intestinal histology of representative mice. Scale bars: 200μm.

**Figure 4: Selective down-regulation of IL-10 by intestinal IFN-γ⁺Tr1-cells in IBDs.**

a) Frequencies of Tr1 cells and Tregs in the non-inflamed lamina propria of CRC control patients (n=8) or the inflamed lamina propria of CD (n=12) and UC (n=7) patients. Individual patients and mean ± SEM are shown b) Percentages of IL-10⁺ cells among intestinal IL-7R⁺T-cells, Tr1 cells and Tregs from CD (n=5), UC (n=6) and CRC control patients (ctrl, n=12) patients. c) Frequencies of IL-10⁺ cells among intestinal IL-7R⁺T-cells, Tr1 cells and Tregs that lacked IFN-γ production
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Figure 5: Tr1-cells are responsive to IL-23, and down-regulate IL-10 in response to IL-23 and IL-1β.

a) IL-10 and IFN-γ expression of Tr1-cells upon in vitro culture with anti-CD3 and CD28 antibodies in the absence or presence of IL-1β and IL-23. Data show one representative donor. b) Frequencies of IL-10+ cells among circulating Tregs, Tr1 cells and IL-7R+ cells. Individual donors (empty dots) and mean (filled dots) of the percentages of IL-10+ cells following culture with anti-CD3+CD28 antibodies in the absence or presence of IL-1β and IL-23 for each cell type are shown. c) Frequencies of IL-10+ cells among Tr1 cells that co-produce IL-10 and IFN-γ (left) or that produce only IL-10 (right) in the absence or presence of IL-1β and IL-23. Individual donors (empty dots) and mean (filled dots) are shown. d) IL-1R1 and R2 expression by activated Tregs, IL-7R+ and Tr1 cells. Bars represent Mean ± SEM (n=4). e) IL-23 responsiveness of activated Tregs, IL-7R+ and Tr1 cells. Left: Mean of p-STAT3+ cells in IL-23-stimulated T-cell subsets (n=5). Right: representative histogram overlays of IL-23-induced STAT3 phosphorylation in T cell subsets. f) IL-23 mRNA expression in CD4+CCR6+IL-7R+CD25- T-cells (“Th17”) versus CD4+CCR5+IL-7R-CD25- T-cells (“Tr1”) purified ex vivo from intestinal specimen of CD (squares) and UC (rounds) patients. Each dot represents one patient (n=4).

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44 Banham AH. Cell-surface IL-7 receptor expression facilitates the purification of FOXP3(+) regulatory T cells. Trends Immunol 2006;27:541-4.


Table 1. Baseline clinical characteristics of the enrolled IBD and CRC patients

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*according to Montreal classification [1]. SD: standard deviation
Figure 1

(a) Flow cytometry analysis of IL7R expression on CD25+ cells.

(b) Flow cytometry analysis of Foxp3 expression in Tregs and IL7R- cells.

(c) Flow cytometry analysis of IFNγ and IL10 production in IL7R+, Tregs, and IL7R- cells.
Figure 2
**Figure 3**

**a**

Graph showing the number of cells over a range of IL10 values.

**b**

Graph showing IFN levels (pg/mL) and IL-17 levels (pg/mL) for Tr1 and IL10 groups.

**c**

Graphs showing CD4+ Tregs and CD4+Tr1 levels and percentage of max cell trace.

**d**

Bar graph showing the collitis index for Th17, IL10-CCR5-PD1, and Tr1 groups.

**e**

Histological images of Th17, IL10-CCR5-PD1, and Tr1 groups.
Figure 4
Figure 5
Supplementary Material

Table 1: Characteristics of IBD and CRC patients

supplementary Figure 1: IL-10 production by human intestinal Tr1-cells

Intestinal Tr1-cells, gated as CD4+IL-7R-CCR5+, were left either untreated (left panel) or stimulated with PMA and Ionomycin for 5 hours in the presence of Brefeldin A in the last 3 hours. IL-10 expression was detected by intracellular staining. Numbers indicate the fraction of IL-10+ cells. b) IL-10 expression of clones derived from peripheral blood CD4+IL-7R-CCR5+ Tr1-cells was used in some experiments as a positive control. c) Intestinal CD4+ T-cells were sorted according to IL-7R, CCR5 and CD25 expression, stimulated with PMA and Ionomycin for 64h, and IL-10 protein production was measured in culture supernatants by ELISA. The mean IL-10 production of Tr1-cells, Tregs, IL-7R+ helper T-cells and CCR5-IL-7R- control cells was calculated (pg/ml IL-10 of 10^6 cells/ml, n=4).

supplementary Figure 2: Gating strategy for human intestinal Tr1-cells

Total LPMC were gated on small cells as lymphocytes (upper left), CD4+ T-cells were gated according to CD4 staining and FSC-W to exclude doublets (upper right), CD4+ T-cells were gated according to IL-7R and CD25 expression to sub-divide them into IL-7R+CD25-/-lo helper cells, CD25+IL-7R+bTregs and IL-7R-CD25- effector-like cells (lower left). Among IL-7R-CD25- effector-like cells CCR5 was used to enrich for IL-10+ Tr1-cells (lower central). For functional experiments PD-1 or CCR6 expression were used in addition (lower right).

supplementary Figure 3: LAG3 surface and m-RNA expression in human CCR5+IL-7R- Tr1-cells.

a) LAG-3 versus CCR5 surface expression on circulating human CD4+IL-7R-CD25- T-cells in one donor b) LAG-3 m-RNA expression in the indicated T-cell subsets that were purified as surface LAG-3-negative. Bars represent Mean ± SEM (n=4). c) Induction of LAG-3 surface expression by Tr1 cells and Tregs upon in vitro cultures with IL-2 in the absence or presence of anti-CD3+CD28 as indicated.
supplementary Figure 4: IL-10 production by murine intestinal Tr1-cells
a) Intestinal CD4+Foxp3−T-cells from FIRxTiger mice that had been injected twice with anti-CD3 antibodies were analyzed for IL-10 GFP expression in gated CCR5+PD-1+ Tr1-cells and CCR5+PD-1− control cells. Shown is a representative histogram overlay (n=4). b) FACS-purified CCR5+PD-1+ Tr1-cells and CCR5+PD-1− control cells were stimulated for 96 hours with anti-CD3 and anti-CD28 antibodies and IL-10 was measured in culture supernatants by ELISA. Shown is the mean of duplicates in one representative experiment (n=2).

supplementary Figure 5: Frequencies of Tr1-cells and Tregs in the inflamed and non-inflamed gut of IBD patients. Biopsies of 10 IBD patients that were taken at inflamed and distal, not-inflamed sites for diagnostic purposes were analyzed for the frequencies of CD4+CD25+IL-7R−Tregs and CD4+IL-7R−CD25−CCR5+CCR6− Tr1-cells. Biopsies of the same patients are indicated by a line and open circles; filled circles indicate the mean.

supplementary Figure 6: Selective inhibitory effect of IL-1β and IL-23 on IL-10 expression by IFN-γ Tr1-cells. a) The frequencies of IL-10+IL-7R+helper T-cells (upper panels) and IL-10+CD25+Tregs (lower panels) that co-expressed IFN-γ (left panels) or expressed only IL-10 (right panels) are shown after in vitro activation with anti-CD3 and anti-CD28 antibodies in the absence and presence of IL-1β and IL-23. The same donors are indicated by a line and open circles; filled circles indicate the mean. Statistical significance was calculated by a paired student's t-test and was in all cases >0.05. b) The effect of IL-1β and IL-23 on different T-cell subsets that expressed IFN-γ and/or IL-10 as indicated was also calculated as fold-increase. (no change: dotted line, *** p<0.0005)

supplementary Figure 7: IL-23 responsiveness of Tr1-cells. Tr1-cells were activated in vitro with anti-CD3 and anti-CD28 antibodies and IL-2 for 6 days. After extensive washing, cells were incubated in the absence or presence of IL-23 and STAT3 phosphorylation analyzed (n=5).
Graphical Abstract: In the intestine CD25⁺Foxp³⁺Tregs and CCR5⁺PD1⁺Tr1-cells are two relevant sources of the anti-inflammatory cytokine IL-10. In IBDs pro-inflammatory cytokines IL-1β and IL-23 are produced in response to intestinal bacteria. IL-1β is sensed preferentially by Tregs, but is insufficient to reduce Treg IL-10 production. Conversely, IL-23 could act selectively on Tr1-cells to inhibit IL-10 production. IL-7R⁺ helper T-cells are consequently more efficiently activated by macrophages or DC, expand and induce excessive inflammation.
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3
Supplementary figure 4
Supplementary Figure 5
Supplementary Figure 6
Supplementary Figure 7

Tr1

 ctrl p-STAT3

 p-STAT3^+

 0 10 20 30

 *