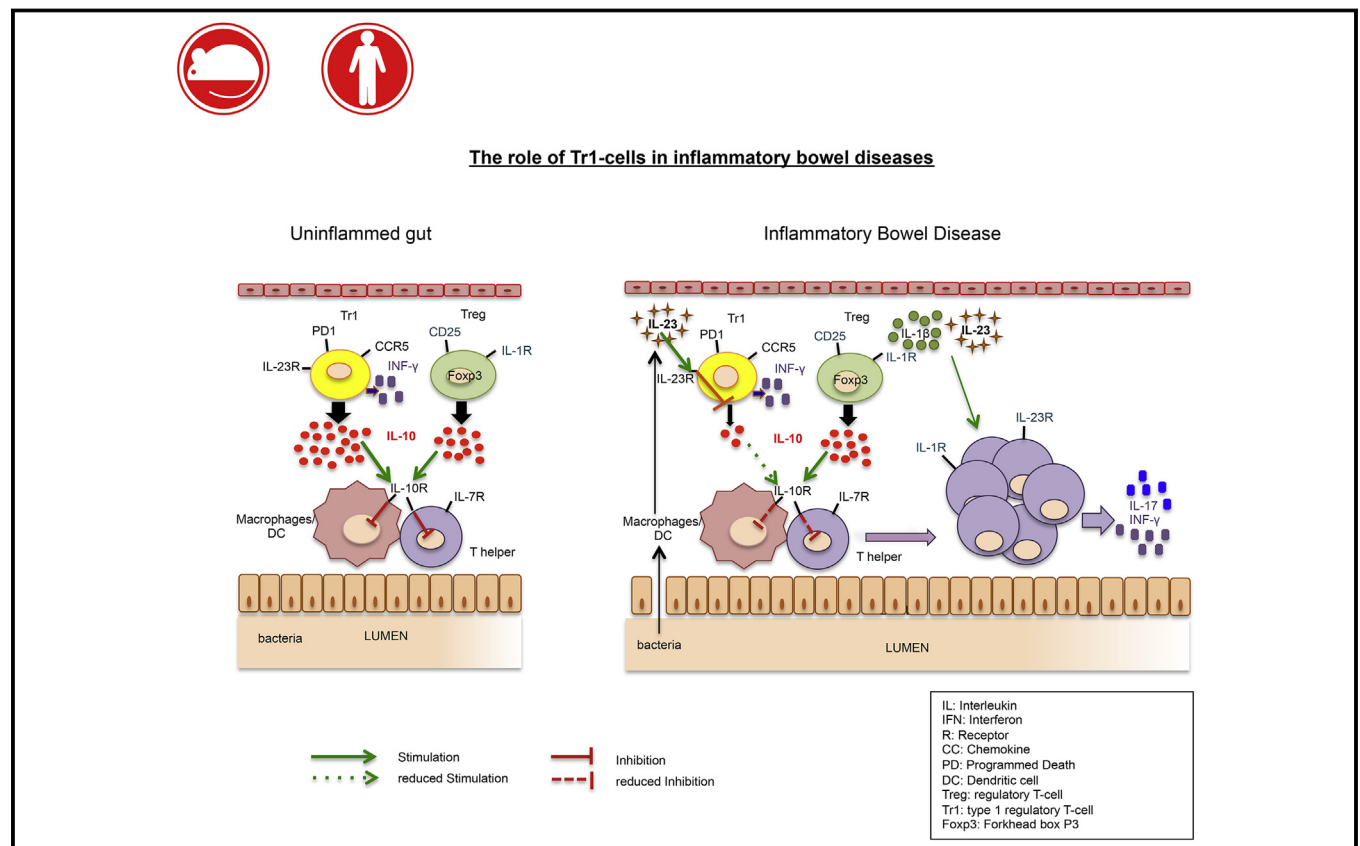


Intestinal IFN- γ -producing type 1 regulatory T cells coexpress CCR5 and programmed cell death protein 1 and downregulate IL-10 in the inflamed guts of patients with inflammatory bowel disease

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GRAPHICAL ABSTRACT



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Supported by a long-term EMBO fellowship (to N.G.), and a short-term EMBO fellowship (to F.F.) and the Cariplo foundation (grant 2013-0937 to J.G., F.C. and R.A.F.). J.G., M.P., and F.C. are supported by the Italian Ministry of Health (projects RF-2011-02348920 and GR-2011-02352001). J.G. is supported by the AIRC foundation (grant IG 2015 Id.17448). INGM is supported by the Romeo ed Enrica Invernizzi foundation. R.A.F. is supported by the Howard Hughes Medical Institute. J.S.A. has attended the PhD program of Molecular Medicine of the University of Milan (UNIMI).

Disclosure of potential conflict of interest: N. Gagliani’s institution received a grant from Yale University for this work. M. Paroni’s institution received a grant from the Ministry of Health (GR-2011-02352001) for this work. C. Frusteri is employed by IRCCS, Cà Granda Foundation, Ospedale Maggiore Policlinico, Gastroenterology and Endoscopy, Milan. S. Abrignani’s institution received a grant from Fondazione Invernizzi for this work. J. Geginat’s institution received grants from Cariplo Foundation and Ministry of Health for this work and from AISM, AIRC, Astellas, and Biotest AG for other works; he and his institution receive consultancy fees from Biotest AG. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication February 27, 2017; revised November 10, 2017; accepted for publication December 4, 2017.

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0091-6749/\$36.00

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<https://doi.org/10.1016/j.jaci.2017.12.984>

Background: IL-10 is an anti-inflammatory cytokine required for intestinal immune homeostasis. It mediates suppression of T-cell responses by type 1 regulatory T (T_R1) cells but is also produced by CD25⁺ regulatory T (Treg) cells.

Objective: We aimed to identify and characterize human intestinal T_R1 cells and to investigate whether they are a relevant cellular source of IL-10 in patients with inflammatory bowel diseases (IBDs).

Methods: CD4⁺ T cells isolated from the intestinal lamina propria of human subjects and mice were analyzed for phenotype, cytokine production, and suppressive capacities. Intracellular IL-10 expression by CD4⁺ T-cell subsets in the inflamed guts of patients with IBD (Crohn disease or ulcerative colitis) was compared with that in cells from noninflamed control subjects. Finally, the effects of proinflammatory cytokines on T-cell IL-10 expression were analyzed, and IL-1 β and IL-23 responsiveness was assessed.

Results: Intestinal T_R1 cells could be identified by coexpression of CCR5 and programmed cell death protein 1 (PD-1) in human subjects and mice. CCR5⁺PD-1⁺ T_R1 cells expressed IFN- γ and efficiently suppressed T-cell proliferation and transfer colitis. Intestinal IFN- γ ⁺ T_R1 cells, but not IL-7 receptor–positive T_H cells or CD25⁺ Treg cells, showed lower IL-10 expression in patients with IBDs. T_R1 cells were responsive to IL-23, and IFN- γ ⁺ T_R1 cells downregulated IL-10 with IL-1 β and IL-23. Conversely, CD25⁺ Treg cells expressed higher levels of IL-1 receptor but showed stable IL-10 expression.

Conclusions: We provide the first *ex vivo* characterization of human intestinal T_R1 cells. Selective downregulation of IL-10 by IFN- γ ⁺ T_R1 cells in response to proinflammatory cytokines is likely to drive excessive intestinal inflammation in patients with IBDs. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: Inflammatory bowel disease, regulatory T cells, IL-10, IL-23

IL-10 is an anti-inflammatory cytokine that plays a nonredundant role in maintaining intestinal immune homeostasis.^{1,2} IL-10–deficient mice and patients with genetic defects in the IL-10/IL-10 receptor pathway experience spontaneous intestinal inflammation and colitis in the presence of a normal gut microflora.^{3–5} Experiments with conditional knockout mice indicated that the protective IL-10 is produced by T cells,⁶ and studies with IL-10 receptor–deficient immune cells suggested that intestinal macrophages and T_H17 cells are directly controlled by IL-10.^{7–11} However, several T-cell subsets can produce IL-10, and the relevant cellular source of IL-10 in patients with inflammatory bowel diseases (IBDs) remained unclear.

Type 1 regulatory T (T_R1) 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.^{10,12–17} They produce variable levels of IFN- γ and are distinct from conventional regulatory T (Treg) cells, which are defined by expression of CD25 and forkhead box p3 (Foxp3). Nevertheless, CD25⁺Foxp3⁺ Treg cells can produce IL-10, which is important for their suppressive functions at barrier organs, including the gut.^{7,17} T_R1 cells are less defined than Foxp3⁺ Treg cells, but they can be induced under a variety of conditions *in vitro*.^{18–20}

Notably, *in vitro*–generated T_R1 cells are being tested currently for their therapeutic potential to treat different immune-mediated

Abbreviations used

CD:	Crohn disease
CRC:	Colorectal cancer
DC:	Dendritic cell
FACS:	Fluorescence-activated cell sorting
Foxp3:	Forkhead box p3
IBD:	Inflammatory bowel disease
IL-1R:	IL-1 receptor
IL-7R:	IL-7 receptor
IL-23R:	IL-23 receptor
LAG3:	Lymphocyte-activation gene 3
PD-1:	Programmed cell death protein 1
PMA:	Phorbol 12-myristate 13-acetate
STAT:	Signal transducer and activator of transcription
T_R1 :	Type 1 regulatory T
Treg:	Regulatory T
UC:	Ulcerative colitis

diseases, including Crohn disease (CD).^{21–23} However, *in vitro*–generated T_R1 cells are likely to differ from *in vivo*–occurring T_R1 cells. Previously, we identified *in vivo*–occurring Foxp3[–] IL-10 and IFN- γ –producing T_R1 -like cells with regulatory functions among CD4⁺CD25[–] IL-7 receptor (IL-7R)[–] T cells in human blood.²⁴ These T_R1 cells are also present in tonsils, where they regulate B-cell responses.²⁵ In mice *in vivo*–occurring T_R1 cells coexpress lymphocyte-activation gene 3 (LAG3) and CD49b and accumulate in the small intestine in an anti-CD3–induced, self-limiting colitis model.²⁶ Conversely, the characteristics of intestinal T_R1 cells in human subjects and their role in patients with IBDs remain to be defined.^{24,26} In particular, whether T_R1 cells have reduced anti-inflammatory functions in the intestines of patients with IBD is unknown.

Here we show that intestinal IFN- γ – and IL-10–producing T_R1 cells can be identified by CCR5 and programmed cell death protein 1 (PD-1) coexpression in human subjects and mice. These T_R1 cells were IL-23 responsive, downregulated IL-10 with IL-1 β and IL-23, and had reduced IL-10–producing capacities in the intestines of patients with IBDs, suggesting that proinflammatory cytokines inhibit their anti-inflammatory functions in patients with 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 biopsy specimens or from patients with IBD undergoing therapeutic resection of highly inflamed parts of the intestine (Table E1). The intestinal mucosa was excised from the intestines of patients with CD and ulcerative colitis (UC) at the time of surgery. As a noninflamed control, intestinal samples were obtained from healthy tissue (at least 7 cm away from neoplastic tissue) of patients undergoing surgery for colorectal cancer (CRC; Table E1). As additional noninflamed controls, biopsy specimens from 3 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 ethics committee approved the use of specimens for research purposes (permission no. 2476), and biopsy specimens (protocol IISR-2014-100922) and informed consent were obtained from patients.

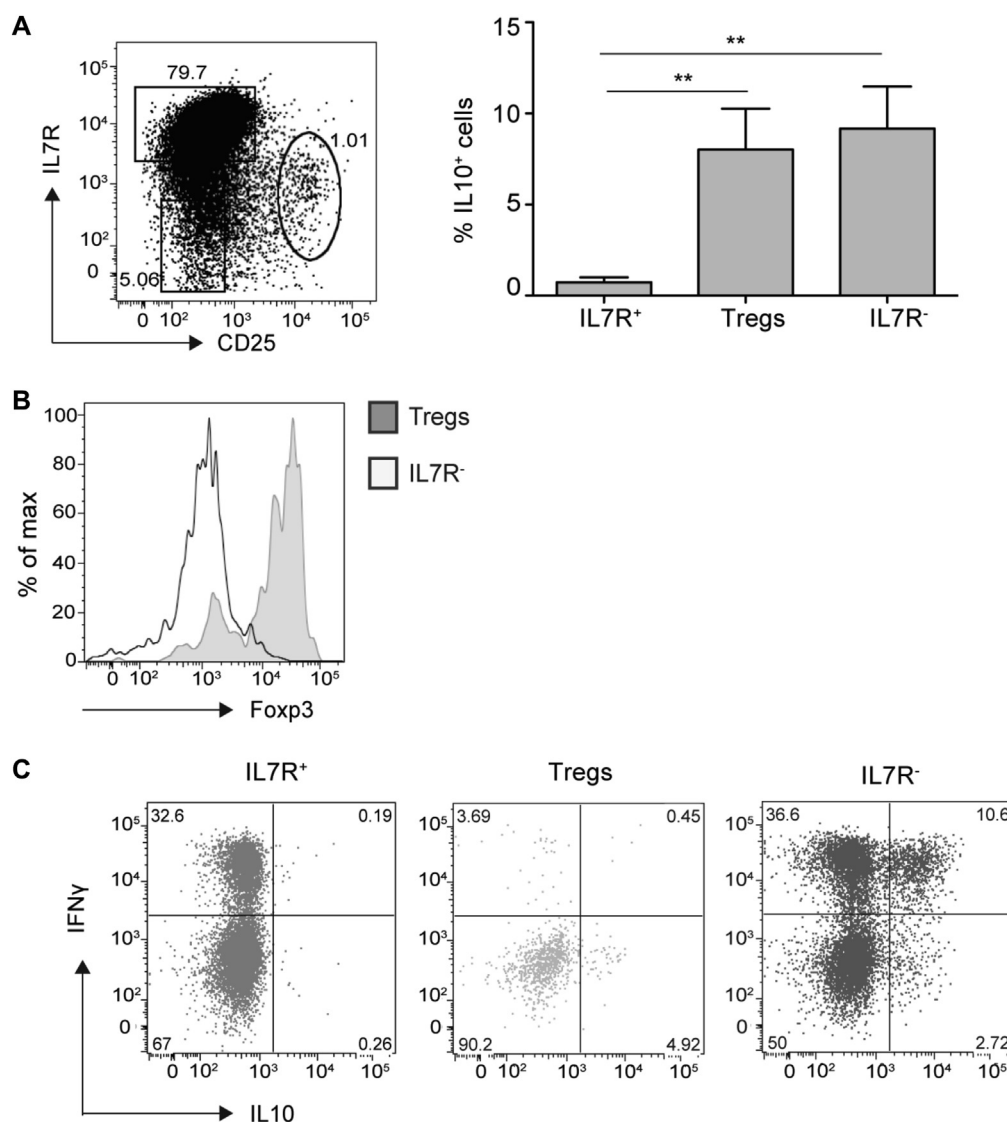


FIG 1. Intestinal human CD4⁺IL-7R⁻CD25⁻ T cells express IL-10 and IFN-γ. **A**, IL-7R versus CD25 expression among CD4⁺ T cells in the intestinal lamina propria (*left panel*). Percentages of IL-10-producing cells among intestinal IL-7R^{hi}CD25^{lo} (IL-7R⁺), IL-7R^{lo}CD25⁻ (IL-7R⁻), and IL-7R^{lo}CD25^{hi} (Treg) CD4⁺ T-cell subsets after brief polyclonal stimulation. Bars represent means ± SEMs (n = 9). **P < .005. **B**, Foxp3 expression of intestinal IL-7R⁻ and Treg T-cell subsets. One representative histogram overlay is shown. **C**, IL-10 and IFN-γ production by intestinal IL-7R⁺, IL-7R⁻, and Treg cells. Representative dot plots of the same donor are shown (n = 4).

Mice

C57BL/6, *Rag1*^{-/-}, C57BL/6 CD45.1⁺, and OTII mice were purchased from Jackson laboratories (Bar Harbor, Me). Foxp3^{RF} (Fir), IL-10^{eGFP} (Tiger), and IL-17^{eGFP} (Singer) reporter mice were generated at Yale University (New Haven, Conn). 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 Yale University.

Cell isolation

Human subjects. PBMCs and intestinal lamina propria mononuclear cells were isolated by means of Ficoll-Hypaque gradient, according to published protocols.²⁷ Lymphocyte subsets were purified by means of cell sorting on a FACSaria (BD, Franklin Lakes, NJ) based on surface marker expression (purity >95%). Thus T_H1 cells were sorted as CD4⁺IL-7R⁻CD25⁻CCR5⁺CCR6⁻PD-1⁺ cells, Treg cells as CD4⁺CD25⁺IL-7R^{lo} cells, and T_H cells as CD4⁺IL-7R⁺CD25^{lo} cells (see Fig E1 in this article's

Online Repository at www.jacionline.org). The cellular yield of lamina propria mononuclear cells was $12.5 \pm 8 \times 10^6$ cells, and we obtained $33 \pm 15 \times 10^3$ T_H1 cells and $65 \pm 40 \times 10^3$ Treg cells according to FACSaria counts.

Mice. For isolation of lamina propria cells from the mouse, small intestines were collected, Peyer patches were removed, and tissue pieces were incubated with 1.5 mmol/L EDTA and 1 mmol/L dithiothreitol at 37°C for 15 minutes, followed by mechanical disaggregation with the gentleMACS dissociator. Cells were then separated further with a Percoll gradient. Cells were isolated from the duodenums and jejunums of anti-CD3-treated mice.

Flow cytometry

Human subjects. Cells were stained with the following antibodies: CD4 (RPA-T4; BD), CCR5 (27D; BD), PD-1 (MIH4; BioLegend, San Diego, Calif), CD25 (M-A251; BioLegend), IL-10 (JES-19F1; BioLegend); IFN-γ (BioLegend), CD127 (eBioRDR5; eBioscience, San Diego, Calif), LAG3

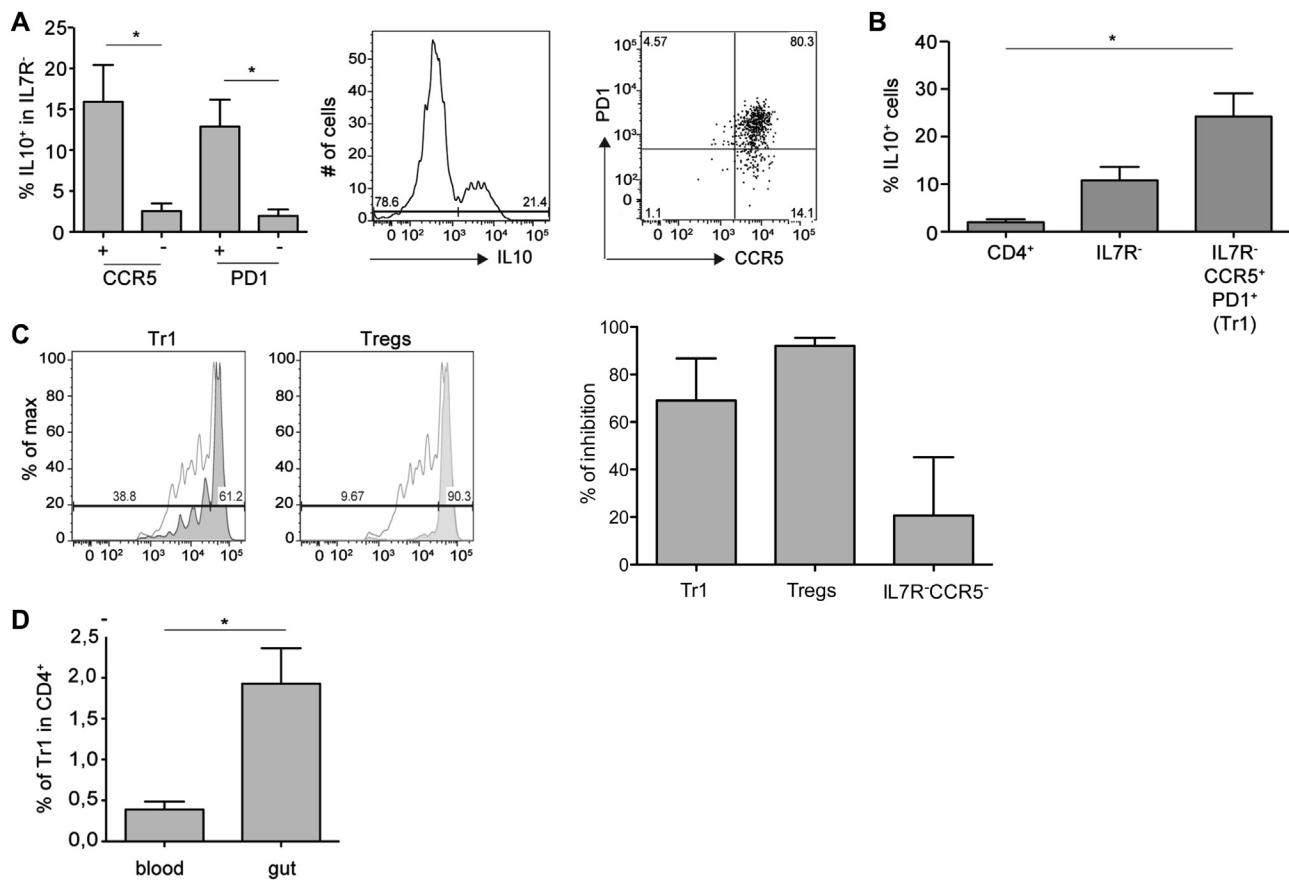


FIG 2. Intestinal human Tr1 cells coexpress CCR5 and PD-1. **A**, Left panel, Percentage of IL-10⁺ T cells among intestinal IL-7R⁻ T cells according to CCR5 or PD-1 expression. Right/central panels, Expression of CCR5 and PD-1 (right panel) in gated IL-10⁺CD4⁺IL-7R⁻ T cells (central panel). **B**, Percentage 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 and Treg cells. Left, Representative overlays of CellTrace profiles of intestinal CD4⁺IL-7R⁺ T cells in the absence (open) and presence (shaded) of intestinal Treg or Tr1 cells. Right, Suppression of CD4⁺IL-7R⁺ T-cell proliferation by autologous intestinal Tr1 cells, Treg cells, and CCR5⁻IL-7R⁻ control cells. Bars represent means $\pm$ SEM (n = 3). **D**, Frequencies of Tr1 cells among CD4⁺ T cells in peripheral blood and the noninflamed intestinal lamina propria of patients with CRC (gut; n = 8). *P < .05.

(REA351; Miltenyi Biotec, Bergisch Gladbach, Germany); phosphorylated signal transducer and activator of transcription (STAT) 3 (4/P-STAT3; BD), CD45RA (MEM-56; ImmunoTools, Friesoythe, Germany), and CD1c (AD5-8E7; Miltenyi Biotec). T-cell cytokine production was measured after stimulation with 0.1 μ Mol/L phorbol 12-myristate 13-acetate (PMA) and 1 μ g/mL ionomycin (Sigma-Aldrich, St Louis, Mo) in the presence of 10 μ g/mL Brefeldin A (Sigma); cells were fixed, permeabilized, stained, and analyzed by means of flow cytometry. For IL-10 and IFN- γ production of CD4⁺ T-cell subsets, lymphocytes were gated according to CD4, IL-7R, CD25, CCR5, and PD-1 expression (see Fig E1).

Mice. Murine cells were stained with anti-CD4 (GK1.5), anti-PD-1 (RMP1-30), anti-CCR5 (HM-CCR5), anti-CCR6 (29-2117), anti-CD45.2 (104), anti-LAG3 (C9B7W), and anti-CD49b (HM α 2), all from BioLegend. Samples were passed on a FACSCanto flow cytometer (BD) and analyzed with FlowJo (TreeStar, Ashland, Ore) or FACSDiva software.

Experimental colitis

Mice were injected with anti-CD3 (15 μ g, 145-2C11), isotype antibody, or PBS administered intraperitoneally 2 times every other day. For transfer colitis, T_H17 cells were differentiated *in vitro* from CD4⁺ T cells from Foxp3^{RFP}IL17^{eGFP} mice with TGF- β , IL-6, and IL-23 for 5 days with

anti-CD3 and irradiated antigen-presenting cells and a pure population of IL-17^{eGFP}Foxp3^{RFP} T_H17 cells purified by means of cell sorting. Tr1 and Treg cells were induced in Foxp3^{RFP}IL-10^{eGFP} mice with 2 anti-CD3 injections and purified from the small intestine. In some experiments IFN- γ and IL-17 production of purified Tr1 cells was measured after activation with coated anti-CD3 and anti-CD28 antibodies (5 μ g/mL each) for 96 hours by means of ELISA. IL-17⁺Foxp3⁻ cells (3×10^4) were injected into Rag1^{-/-} recipients in the presence or absence of 3×10^4 CCR6⁻CCR5⁺PD-1⁺IL-10⁺ Tr1 or CCR6⁻CCR5⁻PD-1⁻IL-10⁺ control cells.

Colonoscopies were performed in blind fashion by using the Coloview system. In brief, the colitis score was based on the granularity of mucosal surfaces, stool consistency, vascular pattern, translucency of the colon, and visible fibrin (0-3 points for each). Histologic examinations were performed according to standard methods on hematoxylin and eosin staining of paraffin-embedded small intestinal and colonic specimens.

Suppression assays

Human subjects. CD4⁺ intestinal T_H cells were sorted according to IL-7R and CD25 expression and labeled with CellTrace Violet (Life Technologies, Grand Island, NY). T_H cells (2.5×10^4) were cocultured at a 1:1 ratio

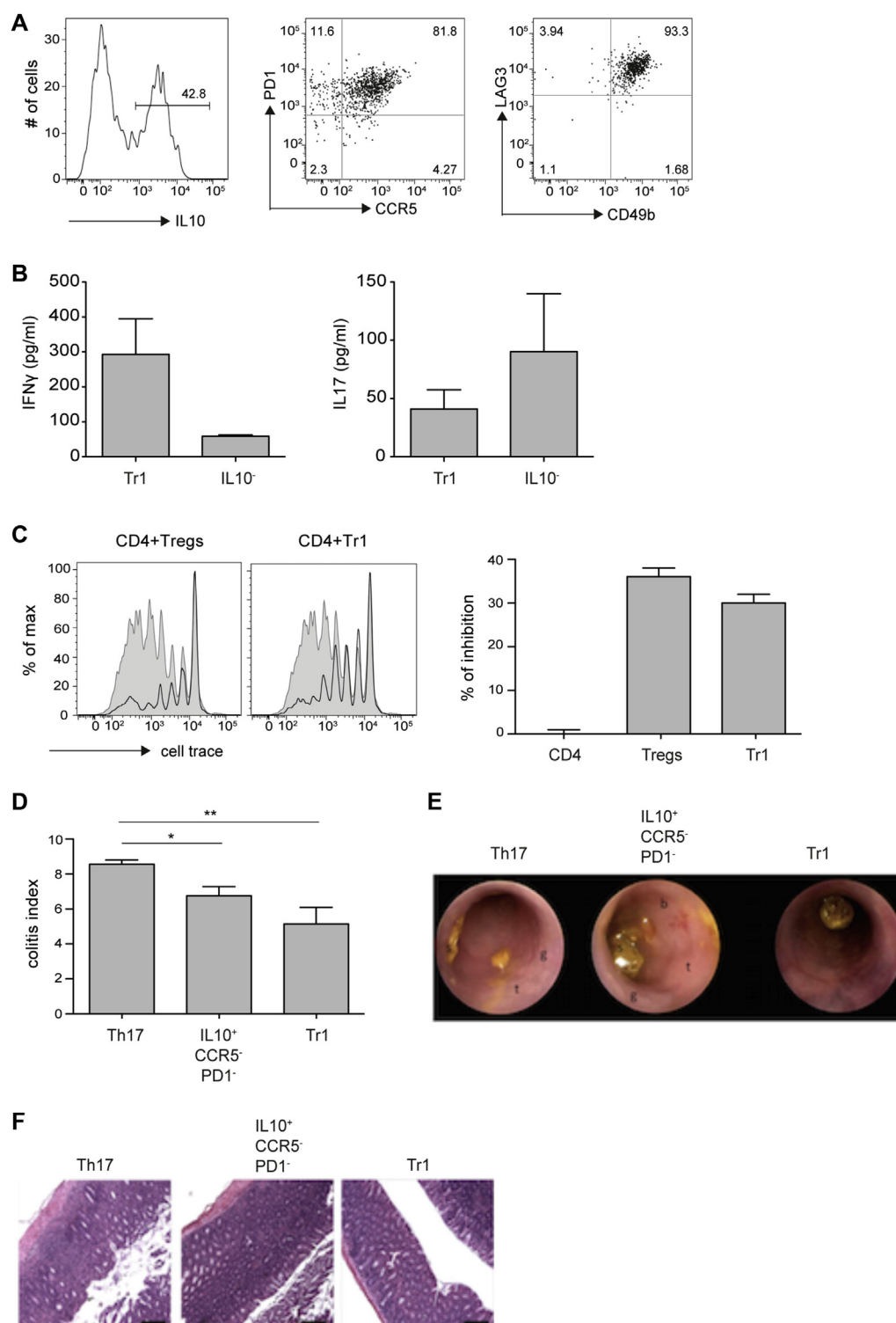


FIG 3. Intestinal murine CCR5 $^+$ PD-1 $^+$ Tr1 cells produce IFN- γ and inhibit transfer colitis. **A**, Intestinal CD4 $^+$ IL-10 $^{\text{GFP}}$ $^+$ Foxp3 $^{\text{RFP}}$ $^-$ cells (left histogram plot) were analyzed for coexpression of CCR5 and PD-1 (central dot plot), and CCR5 $^+$ PD-1 $^+$ cells were analyzed for coexpression of LAG3 and CD49b (right dot plot). **B**, IFN- γ and IL-17 production by CCR5 $^+$ PD-1 $^+$ Tr1 and IL-10 $^-$ control cells stimulated *ex vivo* with anti-CD3 and anti-CD28 antibodies was measured by means of ELISA. Bars represent means $\pm$ SEMs ($n = 3$). **C**, Inhibition of CD4 $^+$ T-cell proliferation by intestinal Tr1 and Treg cells. Left, Representative overlays of CellTrace profiles of total CD4 $^+$ T cells in the absence (open) and presence (shaded) of intestinal Treg and Tr1 cells. Right, Suppression of CD4 $^+$ T-cell proliferation by intestinal Tr1, Treg, and total CD4 $^+$ cells as a control. Bars represent means $\pm$ SEMs ($n = 3$). **D-F**, Inhibition of transfer colitis by intestinal Tr1 cells or CCR5 $^+$ PD-1 $^-$ IL-10 $^+$ T cells. **Fig 2, D**, Mean colitis index ($n > 4$). * $P < .05$ and ** $P < .005$. Representative endoscopies (**Fig 2, E**) and intestinal histology (**Fig 2, F**) of representative mice. Scale bars = 200 μm .

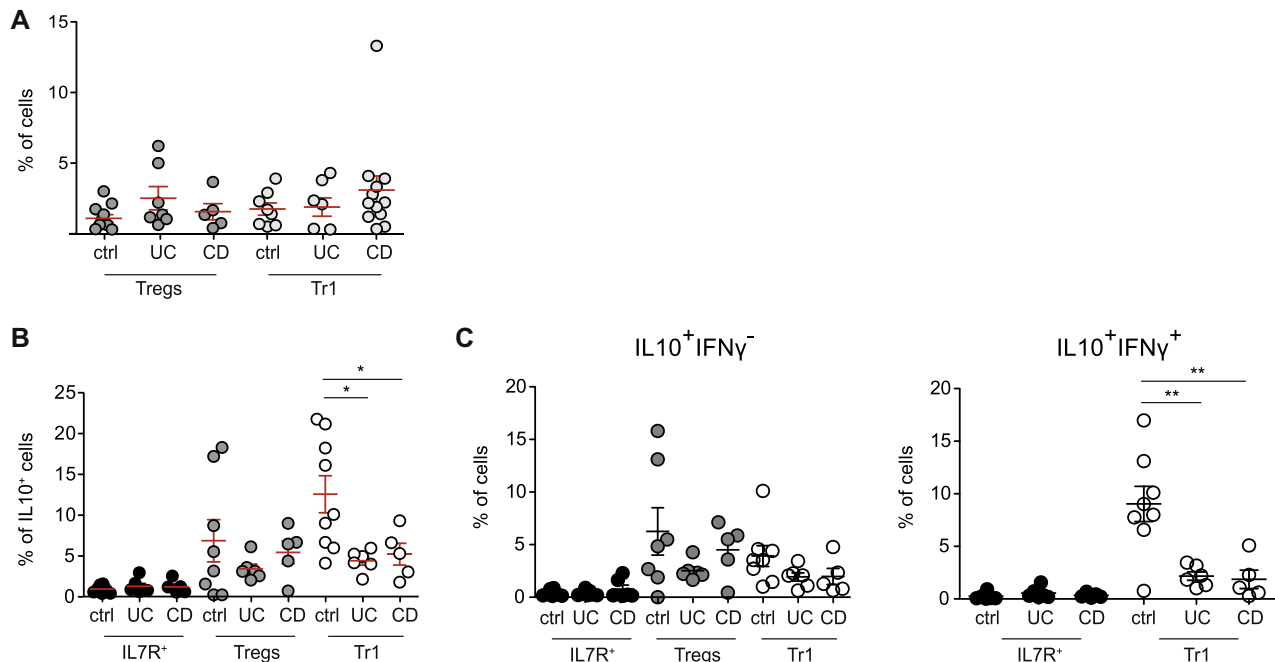


FIG 4. Selective downregulation of IL-10 by intestinal IFN- γ ⁺ Tr1 cells in patients with IBDs. **A**, Frequencies of Tr1 and Treg cells in the noninflamed lamina propria of control subjects with CRC (n = 8) or the inflamed lamina propria of patients with CD (n = 12) and patients with UC (n = 7). Individual patients and means $\pm$ SEMs are shown. **B**, Percentages of IL-10⁺ cells among intestinal IL-7R⁺ T, Treg, and Tr1 cells from patients with CD (n = 5), patients with UC (n = 6), and control subjects with CRC (ctrl; n = 12). **C**, Frequencies of IL-10⁺ cells among intestinal IL-7R⁺ T, Treg, and Tr1 cells that lacked IFN- γ production (left) or among IFN- γ ⁺ cells among Tr1 and IL-7R⁺ T cells (right) in patients with IBDs (UC and CD) and control subjects with CRC (ctrl). * $P < .05$ and ** $P < .005$.

with sorted intestinal Treg cell subsets (ie, CD25⁺IL-7R⁺ Treg and CD25⁺IL-7R⁺CCR5⁺ Tr1 cells). Allogeneic human myeloid CD1c⁺ dendritic cells (DCs) from peripheral blood (5×10^3) were added as stimulators. For dose-titration experiments, 5×10^3 total CD4⁺ T cells from peripheral blood were stimulated with anti-CD3/CD28 beads at a 1:5 ratio in the absence or presence of autologous intestinal Treg or Tr1 cells at different ratios (1:1–1:16). Unlabeled responder cells were used as a negative control. After 4 to 5 days, cells were stained and analyzed for CellTrace Violet dilution by using fluorescence-activated cell sorting (FACS).

Mice. Murine responder CD45.2 CD4⁺ T cells were sorted and labeled with CellTrace Violet. They were cocultured at a 1:1 ratio with sorted Treg 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 means of FACS. To calculate the percentage of suppression, we set undivided cells as 100% of suppression and the percentage of proliferating T cells in the absence of Treg cell subsets as 0% of suppression.

T-cell culture

Purified human Tr1, Treg, and IL-7R⁺ T_H cells were stimulated in 96-well plates precoated with anti-CD3 and anti-CD28 antibodies at 2 μ g/mL and 1000 U/mL IL-2 in the absence or presence of recombinant IL-1 β 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 after brief stimulation with PMA and ionomycin, as described above. The assay to measure IL-23 responsiveness was described previously.²⁸ Briefly, cells were washed extensively and incubated in the absence or presence of IL-23, and phospho(Y)-STAT3 measured by means of intracellular staining and flow cytometry. In the case of intestinal Tr1 cells, IL-23 receptor (IL-23R) was assessed by using RT-PCR, as previously described.²⁸

Statistics

Statistical significance was calculated by using the 2-tailed Student *t* test in case of Gaussian distribution; otherwise, the Mann-Whitney test for unmatched or Wilcoxon for paired groups. In the case of multiple comparisons, 1-way ANOVA with the Tukey posttest was used.

RESULTS

Human intestinal Tr1 cells that coexpress 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 blood²⁴ and tonsils,²⁵ were also present in nonlymphoid tissues, such as the intestinal lamina propria. The large majority of CD4⁺ T cells in the noninflamed intestinal lamina propria obtained from patients with CRC had an IL-7R^{hi}CD25^{low} helper phenotype, and only a small fraction expressed IL-10 on *ex vivo* stimulation with PMA and ionomycin, as detected by means of intracellular staining (Fig 1, A). 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 (Fig 1, A, and see Fig E1, A and B). In addition, CD25⁺IL-7R^{low} Treg cells were present at low frequencies in the intestinal lamina propria, and they also contained a significant fraction of cells that could express IL-10 on stimulation. Consistent with these results, high amounts of IL-10 protein were detected in culture supernatants of FACS-purified, intestinal CD25⁺IL-7R^{low} Treg cells and of CD25⁺IL-7R⁺ effector T-cell subsets after stimulation

with PMA and ionomycin, whereas only low amounts were measured in supernatants of IL-7R^{hi}CD25^{low} T_H cells (see Fig E1, C). In contrast to T_R1 cells, the majority of CD25⁺IL-7R^{low} T cells expressed Foxp3 (Fig 1, B), indicating that they represented conventional CD25⁺Foxp3⁺ Treg cells. Moreover, the large majority of IL-10⁺CD4⁺CD25⁺IL-7R⁺ T cells coexpressed IFN- γ (Fig 1, C), whereas IL-10⁺CD25⁺ Treg cells did not.

The large majority of IL-10⁺CD4⁺CD25⁺IL-7R⁺ T cells expressed CCR5 and PD-1 (Fig 2, A), whereas CCR6 was largely absent (data not shown). These surface markers allowed strong enrichment for IL-10⁺ T cells in the intestine (Fig 2, B). Moreover, purified intestinal CCR5⁺IL-7R⁺ T_R1 cells secreted significantly higher amounts of IL-10 protein compared with CCR5⁺IL-7R⁺ control T cells (see Fig E1, C). *Ex vivo*-purified intestinal CD4⁺CD25⁺IL-7R⁺CCR5⁺ T_R1 cells (see Fig E2 in this article's Online Repository at www.jacionline.org) possessed regulatory functions and inhibited the proliferation of autologous intestinal T_H cells induced by CD1c⁺ DCs nearly as efficiently as conventional CD25⁺ Treg cells (Fig 2, C). Conversely, intestinal CD4⁺CD25⁺IL-7R⁺ control cells that lacked CCR5 expression did not suppress T-cell proliferation (Fig 2, C), which is consistent with their low IL-10 expression (Fig 2, A, and see Fig E1, C). Finally, when we compared frequencies of CD4⁺CD25⁺IL-7R⁺CCR5⁺ T_R1 cells among total CD4⁺ T cells in peripheral blood and the noninflamed intestinal lamina propria of patients with CRC, we found that T_R1 cells were strongly enriched in the gut (Fig 2, D).

LAG3 is expressed by T_R1 cells in human subjects and mice.^{26,29} Only a small fraction of human CD4⁺CD25⁺IL-7R⁺CCR5⁺ T_R1 cells (1.6% $\pm$ 1.4%, n = 10) showed LAG3 surface expression (see Fig E3, A, in this article's Online Repository at www.jacionline.org). However, we found that human CD4⁺CD25⁺IL-7R⁺CCR5⁺ T_R1 cells, which did not express LAG3 at the cell surface, nevertheless expressed LAG3 mRNA (see Fig E3, B). Finally, in line with what was previously reported,²⁶ CD4⁺CD25⁺IL-7R⁺CCR5⁺ T_R1 cells efficiently upregulated LAG3 protein expression at the cell surface after T-cell receptor stimulation *in vitro* (see Fig E3, C).

Intestinal murine CCR5⁺PD-1⁺ T_R1 cells suppress transfer colitis

We next investigated CCR5 and PD-1 expression on intestinal T_R1 cells in mice and the *in vivo*-suppressive capacities of intestinal T_R1 cells that coexpressed CCR5 and PD-1. Intestinal T_R1 cells can be tracked in IL-10 and Foxp3 double-reporter (Firx-Tiger) mice^{26,30} after repetitive anti-CD3 injections, a treatment that induces self-limiting colitis. As observed in human subjects, the large majority of intestinal IL-10⁺eGFP⁺Foxp3^{RFP} T_R1 cells coexpressed CCR5 and PD-1 (Fig 3, A), and they also coexpressed LAG3 and CD49b on the cell surface, as expected.²⁶ Consistent with high expression of the IL-10⁺eGFP reporter, purified murine CCR5⁺PD-1⁺ T_R1 cells from the small intestine secreted high amounts of IL-10 protein on stimulation with anti-CD3 and anti-CD28 antibodies (see Fig E4 in this article's Online Repository at www.jacionline.org). CCR5⁺PD-1⁺ T_R1 cells also secreted substantial amounts of IFN- γ but little IL-17 (Fig 3, B). Consistently, they did not express CCR6 (data not shown), which is characteristic for T_H17 cells^{31,32} but not for human IL-7R⁺ T_R1 cells.²⁵ Finally, IL-10-producing CCR5⁺PD-1⁺ T cells

suppressed *in vitro* proliferation of CD4⁺ T cells with a similar efficiency as Foxp3^{RFP} Treg cells (Fig 3, C), confirming that they represent T_R1 cells.

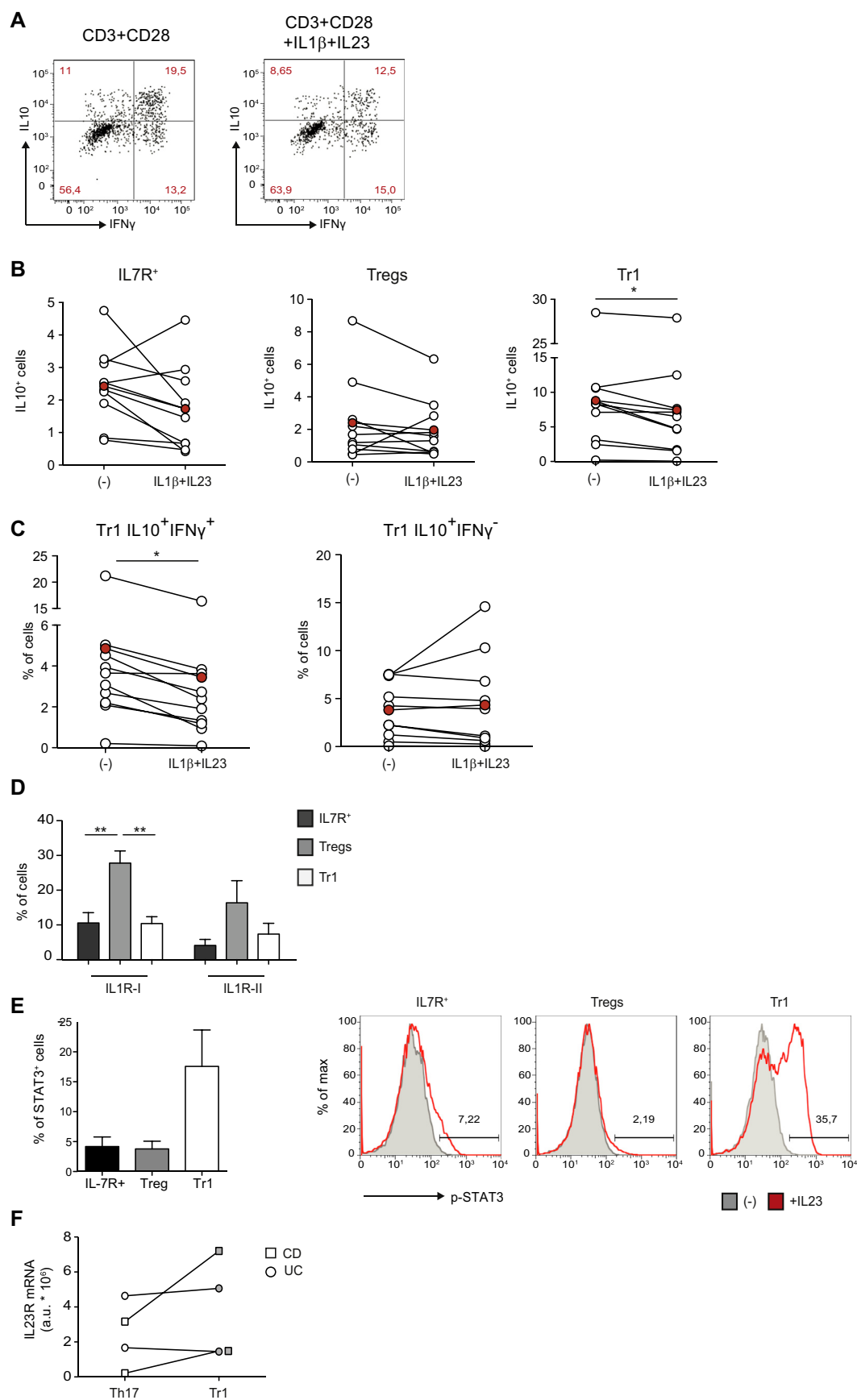
Intestinal T_R1 cells suppress T_H17 cells in an IL-10-dependent manner *in vivo* and inhibit colitis.¹⁰ To assess the *in vivo*-suppressive capacities of CCR5⁺PD-1⁺ T_R1 cells, we transferred *in vitro*-generated T_H17 cells (CD4⁺Foxp3^{RFP}IL-17^{eGFP}) into Rag1^{-/-} mice in the presence or absence of purified intestinal T_R1 cells. Transfer of T_H17 cells caused disease in the intestine, as assessed based on the colitis score and by means of histologic evaluation (Fig 3, D and E). Cotransfer of IL-10^{eGFP}CCR5⁺PD-1⁺ T_R1 cells strongly inhibited colitis induction, whereas IL-10^{eGFP} control T cells that lacked CCR5 and PD-1 were also suppressive but less potent (Fig 3, D and E).

IL-10 and IFN- γ -coexpressing T_R1 cells downregulate IL-10 production in the inflamed guts of patients with IBD and with IL-1 β plus IL-23 *in vitro*

Because intestinal CCR5⁺ T_R1 cells inhibited transfer colitis in mice, we asked whether they could also play a protective role in patients with IBDs. We first analyzed whether numbers of CCR5⁺IL-7R⁺ T_R1 cells or CD25⁺ Treg cells were reduced in the intestinal lamina propria of patients (Table E1) with CD or UC, the 2 principal forms of IBD, compared with the noninflamed lamina propria obtained from control subjects with CRC. We observed a moderate increase in numbers of CD25⁺ Treg cells in the lamina propria of patients with UC and of CCR5⁺IL-7R⁺ T_R1 cells in patients with CD, which did not reach statistical significance (Fig 4, A). Notably, there was also no significant difference between the frequencies of these Treg cell subsets in inflamed and noninflamed biopsy specimens from the same patients with IBD (see Fig E5 in this article's Online Repository at www.jacionline.org). Intestinal IL-7R⁺ T_R1 cells had markedly reduced capacities to express IL-10 in patients with both types of IBD, whereas IL-10 expression by CD25⁺ Treg cells and IL-7R^{hi} T_H cells was not significantly altered (Fig 4, B). Interestingly, T_R1 cells that coexpressed IL-10 and IFN- γ were much more strongly affected than those that expressed IL-10 alone, and consequently, only numbers of IFN- γ and IL-10-coexpressing T_R1 cells were significantly reduced (Fig 4, C).

To understand why T_R1 cells downregulated IL-10 production in the intestines of patients with IBDs, we activated circulating IL-7R⁺CCR5⁺ T_R1, CD25⁺ Treg, and IL-7R^{hi} T_H cells in the presence of the proinflammatory cytokines IL-1 β and IL-23, which play critical pathogenic roles in colitis.³³⁻⁴⁰ Only T_R1 cells had reduced capacities to express IL-10 after culture with IL-1 β and IL-23 (Fig 5, A and B). Furthermore, as observed in the intestines of patients with IBDs, the effect was selective for IL-10 and IFN- γ -coexpressing T_R1 cells (Fig 5, C). Conversely, no significant effect on IL-10 was detected in CD25⁺ Treg or IL-7R^{hi} T_H cells, either in total IL-10⁺ cells (Fig 5, B) or in IFN- γ -expressing subsets (see Fig E6, A, in this article's Online Repository at www.jacionline.org). This was also true when the fold increase was calculated to eliminate the variability of IL-10 production between different subjects (see Fig E6, B). Interestingly, neither addition nor neutralization of TNF- α , an efficient therapy for IBD, had a clear effect on IL-10 production (data not shown).

To understand whether the selective effect of IL-1 β and IL-23 on IL-10 production by T_R1 cells might be caused by a different



responsiveness to these proinflammatory cytokines, we assessed IL-1 receptor (IL-1R) surface expression and IL-23–induced STAT3 activation. Activated CD25⁺ Treg cells expressed high levels of IL-1R1 and IL-1R2, whereas T_R1 cells and T_H cells expressed these receptors only at low levels (Fig 5, D), arguing against a critical role for IL-1 β in reduced IL-10 production by T_R1 cells. However, IL-23 efficiently induced STAT3 phosphorylation in activated T_R1 cells (Fig 5, E, and see Fig E7 in this article's Online Repository at www.jacionline.org), whereas IL-23 responsiveness was lower in IL-7R⁺ T_H and CD25⁺ Treg cells, although the latter tendency did not reach statistical significance ($P = .088$).

To control whether intestinal T_R1 cells could respond to IL-23, we purified T_R1 cells from the lamina propria of patients with IBDs and quantified IL-23R mRNA expression (Fig 5, F). Notably, IL-23R expression is efficiently induced on naive T cells by T_H17-promoting cytokines²⁸ and is expressed together with CCR6 on T_H17 cells,^{31,41,42} particularly in the gut.⁴³ Therefore we compared IL-23R mRNA expression of intestinal T_R1 cells with that of intestinal CCR6⁺IL-7R⁺CD25⁺ T_H17 cells as a positive control. IL-23R mRNA was detectable in all cases in both intestinal T-cell subsets. Interestingly, T_R1 and T_H17 cells expressed similar levels of IL-23R mRNA in patients with UC, whereas T_R1 cells expressed even higher levels in patients with CD (Fig 5, E).

DISCUSSION

T_R1 cells are characterized by IL-10 production, low Foxp3 expression, and their capacity to inhibit T-cell responses. Here we identified CCR5 and PD-1 coexpression as a characteristic phenotype of IFN- γ and IL-10–producing T_R1 cells in the intestine, allowing tracking and isolation of T_R1 cells directly in the human intestinal lamina propria.

Intestinal T_R1 cells were negative for IL-7R, CD25, and CCR6, distinguishing them from helper T cells, Foxp3⁺ Treg cells,⁴⁴ and T_H17/T_H22 cells,³¹ respectively. However, they expressed the T_H1-associated chemokine receptor CCR5, which allows immune cells to enter nonlymphoid tissues. Indeed, T_R1 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 cells⁴⁵ and inhibits DC maturation.⁴⁶ PD-1 expression on T_R1 cells is consistent with the notion that they are chronically activated cells.^{24,47,48} *In vivo*–occurring T_R1 cells also express LAG3,^{26,29} an inhibitory receptor that binds MHC class II, and we found that both human and mouse CCR5⁺ T_R1 cells expressed LAG3. However, the large majority

of human CCR5⁺IL-7R⁺ T_R1 cells expressed LAG3 on the cell surface only after T-cell receptor stimulation. These data suggest a possible heterogeneity among T_R1 cells, which could result from different stages of activation, maturation, and/or function. This aspect is currently under investigation. In any case, for the first time, the phenotype reported here has allowed identification and isolation of T_R1 cells that produce high levels of IL-10 and IFN- γ directly in the human gut.

IL-10 produced by T cells has a nonredundant role in preventing excessive inflammation in response to the gut microbiota.⁴⁹ Both Foxp3⁺ Treg and Foxp3⁺ T_R1 cells were identified as possible relevant sources of IL-10 in experimental colitis in mice,^{9,10,12} but the contributions of these 2 Treg cell subsets in human IBDs are very difficult to address and therefore largely unknown. Our data point to a critical role of IL-10 and IFN- γ –coproducing T_R1 cells in patients with IBDs because only this T-cell population showed reduced IL-10 expression in the intestinal lamina propria of patients with IBDs. Reduced IL-10 expression by T_R1 cells was detected in both patients with UC and those with CD, indicating that it is a common feature of IBDs. Of note, murine T_R1 cells control colitogenic T_H17 cells and macrophages in an IL-10–dependent manner *in vivo*,⁷⁻¹¹ and human IL-10 and IFN- γ –coexpressing IL-7R⁺ T_R1 cells suppress T-cell responses through IL-10.²⁴ Preliminary dose-response experiments with anti-CD3– and anti-CD28–coated beads and autologous peripheral CD4⁺ T cells as responder cells (data not shown) suggested that intestinal T_R1 and CD25⁺ Treg cells had 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 patients with CRC as a noninflamed control, as do many studies on human intestinal immune cells given the difficulty to analyze the gut of healthy subjects. It is possible that the presence of intestinal tumors or the increased age of patients with CRC influences the properties of intestinal Treg cells. However, the similar frequencies of T-cell subsets at inflamed and noninflamed sites of the same patients with IBD and the similar IL-10 expression among CD4⁺ T cells from noninflamed biopsy specimens of a few patients with gastrointestinal reflux (data not shown) compared with intestinal CD4⁺ T cells from patients with CRC suggest that these factors did not have a major effect on these analyzed parameters.

Interestingly, in non-immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome autoimmune enteropathy caused by dysfunctional Foxp3⁺ Treg cells, we observed enhanced IL-10 production of CD4⁺ T cells, suggesting that T_R1 cells were not defective. In addition, we observed uncon-

FIG 5. T_R1 cells are responsive to IL-23 and downregulate IL-10 in response to IL-23 and IL-1 β . **A**, IL-10 and IFN- γ expression of T_R1 cells on *in vitro* culture with anti-CD3 and anti-CD28 antibodies in the absence or presence of IL-1 β and IL-23. Data show 1 representative donor. **B**, Frequencies of IL-10⁺ cells among circulating Treg, T_R1, and IL-7R⁺ cells. Individual donors (open dots) and mean (solid dots; $n = 10$) IL-10⁺ cell percentages after culture with anti-CD3 plus anti-CD28 antibodies in the absence or presence of IL-1 β and IL-23 for each cell type are shown. **C**, Frequencies of IL-10⁺ cells among T_R1 cells that coproduce IL-10 and IFN- γ (left) or that produce only IL-10 (right) in the absence or presence of IL-1 β and IL-23. Individual donors (open dots) and mean (solid dots) are shown. **D**, IL-1R1 and IL-1R2 expression by activated Treg, IL-7R⁺, and T_R1 cells. Bars represent means $\pm$ SEMs ($n = 4$). **E**, IL-23 responsiveness of activated Treg, IL-7R⁺, and T_R1 cells. Left, Mean of pphospho-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 specimens of patients with CD (squares) and patients with UC (circles). Each dot represents 1 patient ($n = 4$). * $P < .05$ and ** $P < .005$.

trolled IL-17 production by CD8⁺ intraepithelial lymphocytes, which was not observed in patients with CD.⁵⁰ These findings are consistent with the view that Foxp3⁺ Treg and T_R1 cells have unique functions in the control of intestinal immune homeostasis. The reduced IL-10 production by T_R1 cells could be explained by their responsiveness to IL-23, a highly pathogenic cytokine in patients with colitis.³³⁻³⁸

IL-1β is another pathogenic cytokine in patients with colitis^{39,40} and inhibits IL-10 production by human T_H17 cells.⁵¹ However, CD25⁺ Treg cells 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 Treg cells. IL-23 is thought to render T_H17 cells pathogenic^{52,53} and to inhibit the generation of Foxp3⁺ Treg cells from naive precursor cells.³³

Inhibition of IL-10 production by established T_R1 cells is a third mechanism that is likely to be highly relevant in patients with IBDs and is consistent with increased T-cell IL-10 production in IL-23R knockout mice.^{38,54} IL-23R expression is characteristic for T_H17 cells that express CCR6 and secrete IL-17.⁴² Therefore it was surprising that IL-7R⁺ CCR5⁺ T_R1 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 T_R1 cells responded to IL-23 and downregulated IL-10 in the gut. The physiologic function of IL-10 downregulation is probably to allow for a more efficient response against invading intestinal bacteria, but on chronic exposure to these bacteria because of a leaky intestinal barrier, the same mechanism could promote chronic inflammation and colitis. Thus IL-10 production of intestinal T_R1 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 patients with CD²² because T_R1 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, patients with IBDs with low intestinal IL-23 expression might respond better to T_R1 cell therapy.

Clinical implications: IL-10 expression by intestinal T_R1 cells is a candidate prognostic parameter in patients with CD and UC. Cellular therapy of IBDs with IL-23-unresponsive T_R1 cells could be highly efficient.

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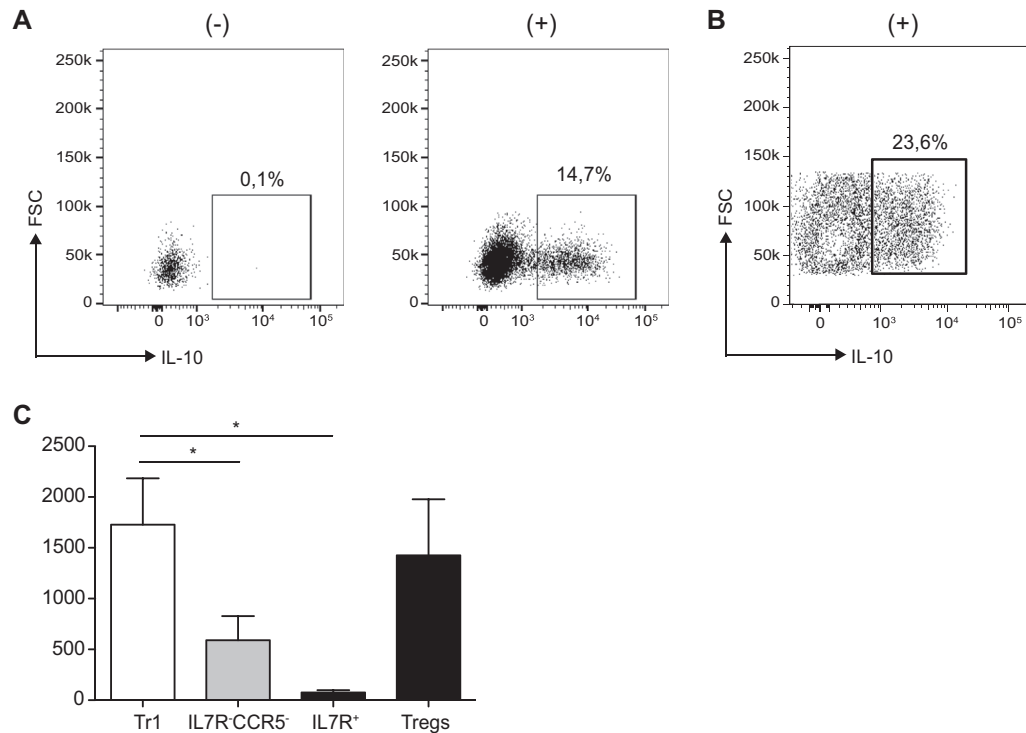


FIG E1. IL-10 production by human intestinal T_R1 cells. **A**, Intestinal T_R1 cells gated as $CD4^+IL-7R^-CCR5^+$ cells were either left untreated (*left panel*) or were stimulated with PMA and ionomycin for 5 hours in the presence of Brefeldin A in the last 3 hours. IL-10 expression was detected based on intracellular staining. Numbers indicate the fraction of IL-10⁺ cells. **B**, IL-10 expression of clones derived from peripheral blood $CD4^+IL-7R^-CCR5^+$ T_R1 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 and stimulated with PMA and ionomycin for 64 hours, and IL-10 protein production was measured in culture supernatants by means of ELISA. The mean IL-10 production of T_R1 , Treg, and $IL-7R^+$ T_H cells and $CCR5^-IL-7R^-$ control cells was calculated (in picograms per milliliter of IL-10 of 10^6 cells per milliliter, $n = 4$). * $P < .05$. FSC, Forward scatter.

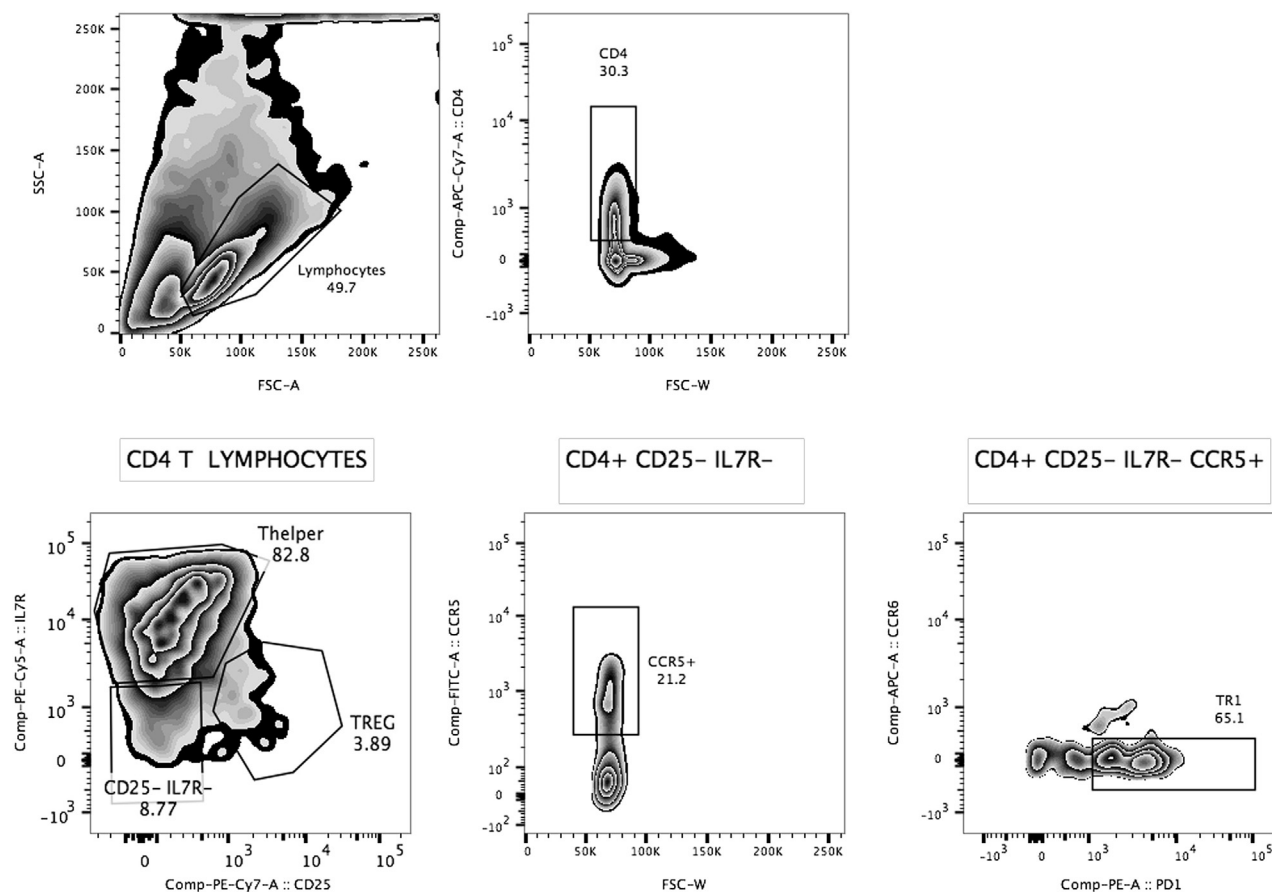


FIG E2. Gating strategy for human intestinal T_R1 cells. Total lamina propria mononuclear cells were gated on small cells as lymphocytes (upper left), CD4⁺ T cells were gated according to CD4 staining and forward scatter (FSC)-W to exclude doublets (upper right), and CD4⁺ T cells were gated according to IL-7R and CD25 expression to subdivide them into IL-7R⁺CD25^{-/lo} helper cells, CD25⁺IL-7R^{lo} Treg cells, and IL-7R⁻CD25⁻ effector-like cells (lower left). Among IL-7R⁻CD25⁻ effector-like cells, CCR5 was used to enrich for IL-10⁺ T_R1 cells (lower center). For functional experiments, PD-1 or CCR6 expression was used (lower right). SSC, Side scatter.

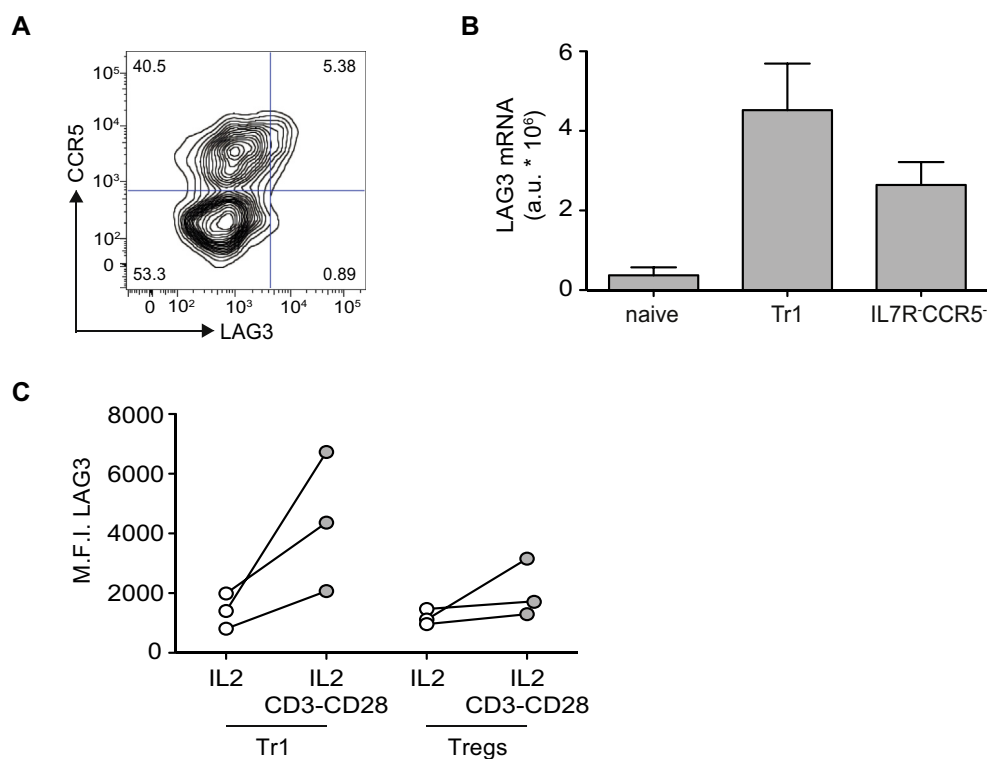


FIG E3. LAG3 surface and mRNA expression in human CCR5⁺IL-7R⁻ T_R1 cells. **A**, LAG3 versus CCR5 surface expression on circulating human CD4⁺IL-7R⁻CD25⁻ T cells in 1 donor. **B**, LAG3 mRNA expression in the indicated T-cell subsets that were purified as surface LAG3-negative cells. Bars represent means $\pm$ SEMs (n = 4). **C**, Induction of LAG3 surface expression by T_R1 and Treg cells on *in vitro* culture with IL-2 in the absence or presence of anti-CD3 plus anti-CD28, as indicated. M.F.I., Mean fluorescence intensity.

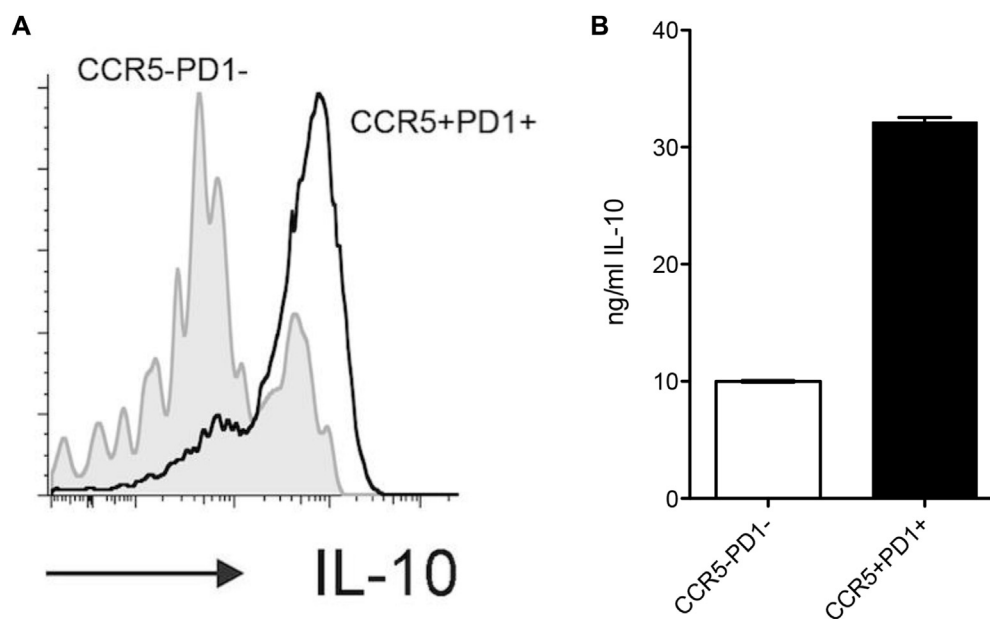


FIG E4. IL-10 production by murine intestinal T_R1 cells. **A**, Intestinal $CD4^+Foxp3^-$ T cells from FirxTiger mice that had been injected twice with anti-CD3 antibodies were analyzed for $IL-10^{eGFP}$ expression in gated $CCR5^+PD-1^+$ T_R1 cells and $CCR5^-PD-1^-$ control cells. Shown is a representative histogram overlay ($n = 4$). **B**, FACS-purified $CCR5^+PD-1^+$ T_R1 cells and $CCR5^-PD-1^-$ control cells were stimulated for 96 hours with anti-CD3 and anti-CD28 antibodies, and IL-10 levels were measured in culture supernatants by means of ELISA. Shown is the mean of duplicates in 1 representative experiment ($n = 2$).

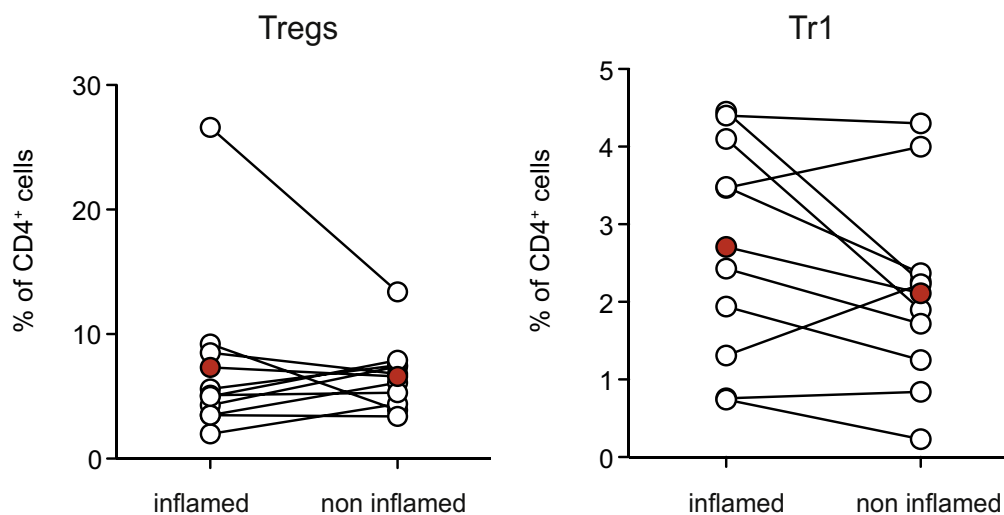


FIG E5. Frequencies of T_{R1} and Treg cells in inflamed and noninflamed guts of patients with IBDs. Biopsy specimens of 10 patients with IBDs that were taken at inflamed and distal noninflamed sites for diagnostic purposes were analyzed for frequencies of $CD4^{+}CD25^{+}IL-7R^{-}$ Treg and $CD4^{+}IL-7R^{-}CD25^{-}CCR5^{+}CCR6^{-}$ T_{R1} cells. Biopsy specimens of the same patients are indicated by a *line* and *open circles*; *solid circles* indicate the mean.

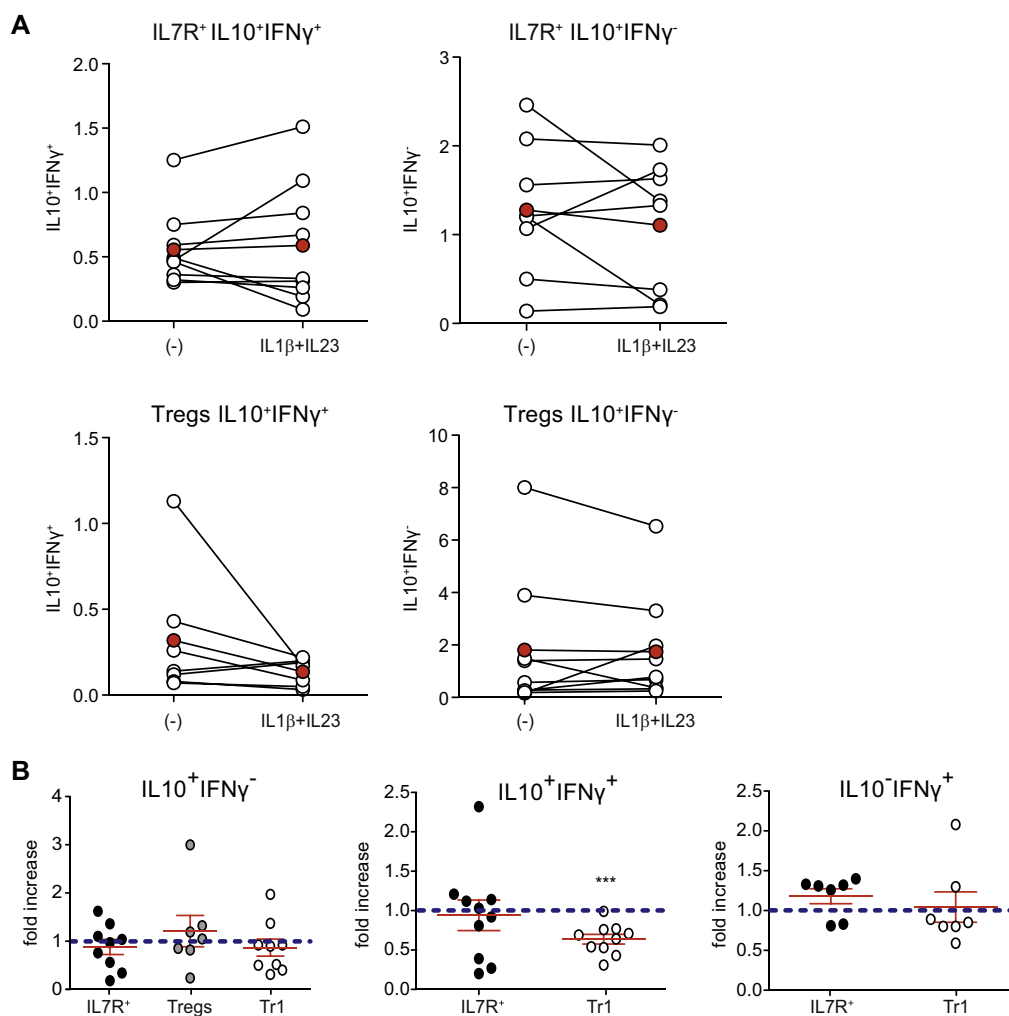


FIG E6. Selective inhibitory effect of IL-1 β and IL-23 on IL-10 expression by IFN- γ ⁺ T_H1 cells. **A**, Frequencies of IL-10⁺IL-7R⁺ T_H cells (*upper panels*) and IL-10⁺CD25⁺ Treg cells (*lower panels*) that coexpressed IFN- γ (*left panels*) or expressed only IL-10 (*right panels*) are shown after *in vitro* activation with anti-CD3 and anti-CD28 antibodies in the absence and presence of IL-1 β and IL-23. The same donors are indicated by a line and open circles; solid circles indicate the mean. Statistical significance was calculated by using a paired Student *t* test and was in all cases greater than .05. **B**, Effect of IL-1 β and IL-23 on different T-cell subsets that expressed IFN- γ , IL-10, or both, as indicated, was also calculated as fold increase. Dotted line, No change. ****P* < .0005.

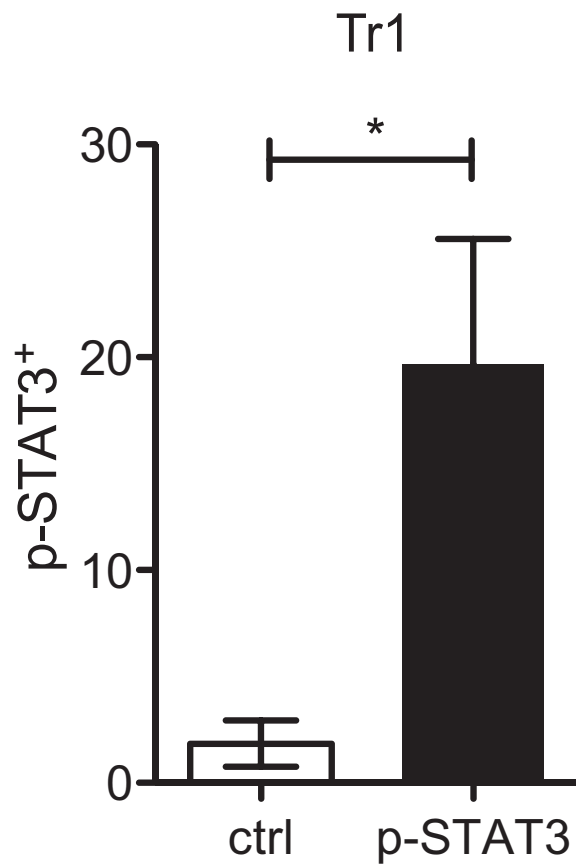


FIG E7. IL-23 responsiveness of T_R1 cells. T_R1 cells were activated *in vitro* with anti-CD3 and anti-CD28 antibodies and IL-2 for 6 days. After extensive washing, cells were incubated in the absence or presence of IL-23, and STAT3 phosphorylation was analyzed ($n = 5$). * $P < .05$.

TABLE E1. Baseline clinical characteristics of enrolled patients with IBDs and CRC

Clinical variables	Patients with CD (n = 18)	Patients with UC (n = 16)	Patients with colon cancer (n = 23)
Male/female sex (no.)	8/10	9/7	15/8
Age at enrolment (y), mean $\pm$ SD	34.8 $\pm$ 12.5	40.1 $\pm$ 11.2	66.2 $\pm$ 11.2
Disease duration (y), mean $\pm$ SD	6.3 $\pm$ 2.4	9.3 $\pm$ 4.5	
Smoking status, yes/no/ex-smoker	8/6/4	3/9/4	8/11/4
CD location (no.)*			
L1 (ileal)	10	—	—
L2 (colonic)	4	—	—
L3 (ileocolonic)	4	—	—
L4 (upper disease)	0	—	—
CD behavior (no.)*			
B1 (nonstricturing, nonpenetrating)	5	—	—
B2 (stricturing)	9	—	—
B3 (penetrating)	4	—	—
UC location			
E1 (proctitis)	—	0	—
E2 (left-sided)	—	7	—
E3 (extensive)	—	9	—
Concomitant therapy at enrolment			
Antibiotics (no.)	4	0	4
Mesalamine (no.)	5	11	—
Thiopurines (no.)	3	4	—
Corticosteroids (no.)	1	6	—
Anti-TNF (no.)	1	0	—

*According to Montreal classification.