Journal of Pediatric Gastroenterology and Nutrition Publish Ahead of Print

DOI: 10.1097/MPG.0000000000001559

Development and Function of Immune Cells in an Adolescent Patient with a Deficiency in the Interleukin-10 Receptor

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Running head: Impact of IL-10R deficiency on innate and adaptive immunity

Funding: This work was supported by the Dutch Sophia Research Foundation grant 557 and the Dutch Digestive Foundation grant (WO 10-37)

Word count: 3250, 5 Figures, 2 Supplementary Figures, 1 Supplementary Table

The authors have no conflicting financial interests.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.jpgn.org).

ABSTRACT (250)

Objective: Monogenic defects in the interleukin-10 (IL-10) pathway are extremely rare and cause infantile-onset inflammatory bowel disease (IBD)-like pathology. Understanding how immune responses are dysregulated in monogenic IBD-like diseases can provide valuable insight in "classical" IBD pathogenesis. Here, we studied long-term immune cell development and function in an adolescent IL-10 receptor *(IL10RA)*-deficient patient who presented in infancy with severe colitis and fistulizing perianal disease and is currently treated with immune suppressants.

Methods: Biomaterial was collected from the *IL10RA*-deficient patient, pediatric IBD patients and healthy controls. The frequency and phenotype of immune cells were determined in peripheral blood and intestinal biopsies by flow cytometry and immunohistochemistry. Functional changes in monocyte-derived dendritic cells and T cells were assessed by *in vitro* activation assays.

Results: The *IL10RA*-deficient immune system developed normally with respect to numbers and phenotype of circulating immune cells. Despite normal co-stimulatory molecule expression, bacterial lipopolysaccharide-stimulated monocyte-derived dendritic cells from the *IL10RA*-deficient patient released increased amounts of TNFα compared to healthy controls. Upon T-cell receptor ligation, *IL10RA*-deficient peripheral blood mononuclear cells released increased amounts of T cell cytokines IFNγ and IL-17 agreeing with high numbers of T-bet^{+} and IL-17^{+} cells in intestinal biopsies taken at disease onset. *In vitro*, the immunosuppressive drug thalidomide used to treat the patient decreased peripheral blood mononuclear cell-derived TNFα production.

Conclusions: With time and during immunosuppressive treatment the *IL10RA*- deficient immune system develops relatively normally. Upon activation, IL-10 is crucial for controlling

excessive inflammatory cytokine release by dendritic cells and preventing IFNγ and IL-17-mediated T-cell responses.

Keywords: Dendritic cell; T cell; Crohn's disease; ulcerative colitis; interleukin-10

What is known

- Monogenic defects in IL-10 and its receptor genes *IL10RA* and *IL10RB* cause infantile-onset IBD-like immunopathology with a severe phenotype that can only be treated with bone marrow transplantation.
- Sequence variants in the IL-10 gene predispose to the development of "classical" IBD, particularly ulcerative colitis.
- In mice, IL-10 orchestrates intestinal immune homeostasis by controlling innate and adaptive immune responses.
- IL-10 is secreted in the late phase of cellular activation and has a significant inhibitory effect on inflammatory responses.

What is new

- The immune system has developed relatively normally in an adolescent *IL10RA*-deficient patient receiving immunosuppressive therapy.
- Cytokine secretion by stimulated human dendritic cells (TNFα) and T cells (IFNγ and IL-17) was elevated in the absence of a functional IL-10 pathway.
- The immune patterns uncovered from patients with *IL10RA* deficiency can be useful for the identification of "classical" IBD patients with suboptimal IL-10 signaling.

INTRODUCTION

Chronic inflammation of the gastrointestinal tract, as seen in inflammatory bowel disease (IBD), is driven by uncontrolled immune responses to commensal microbiota. Over the years, over 160 susceptibility loci have been identified for classical IBD, some of which have led to the identification of gene polymorphisms that contribute to disease pathogenesis (1, 2). In addition, more than 50 monogenic defects have been identified in children with early-onset (before the age of 10 years) IBD-like gastrointestinal pathology classified as the A1a group in the Paris classification for pediatric-onset IBD (3-5). As many of these monogenic defects fall within inflammatory immune networks that are shared by genetic variants conferring susceptibility to classical IBD, detailed analysis of patients with monogenic disorders may yield immune patterns that are useful to categorize classical IBD.

One of the key genes that clusters within the IBD susceptibility loci and monogenic disorders encodes interleukin-10 (IL-10) (3), a potent immunoregulatory cytokine essential for mounting tolerance to harmless intestinal microbial antigens. In the intestine, IL-10 is mainly produced by myeloid cells including macrophages and dendritic cells, as well as regulatory T cells and B cells (6). Once secreted, IL-10 exerts its biological effects via a heterodimeric receptor complex composed of two IL-10 binding chains (IL-10RA) and two accessory chains (IL-10RB), the latter being shared with other receptors of IL-10 cytokine family members (7). The polymorphisms in the IL-10 pathway associated with IBD (8-11) and the discovery of *IL10* and *IL10R* loss-of-function mutations causing severe, infantile-onset IBD (12-15) reveal the importance of the IL-10 pathway in maintaining intestinal immune homeostasis. However, identifying the mechanisms by which IL-10 exerts suppression has been challenging.

With the aid of animal models, IL-10 has been recognized as a major regulator of intestinal innate and adaptive immune responses. Mice deficient in IL-10 or IL-10 receptor

develop intestinal inflammation, which is dependent on bacterial colonization (16, 17). Recent animal studies have demonstrated that IL-10 sensing by innate immune cells comprising macrophages and dendritic cells (DCs) is required for maintenance of intestinal homeostasis (18-20). Regarding adaptive immune responses, defective IL-10 receptor signaling in memory/effector T cells renders these cells more colitogenic than wild-type cells in a mouse model of transfer colitis (21). Although it is now widely appreciated that IL-10 orchestrates intestinal homeostasis, the precise immune cell dysfunctions resulting from a defective IL-10 pathway and their role in driving intestinal inflammation in human are only beginning to be uncovered. So far, only alterations in human macrophage differentiation and function have been described in patients with a defective IL-10 pathway (19, 22). Functional studies in human are limited because *IL10* and *IL10R*-deficient patients are extremely rare, with most having undergone a hematopoietic stem cell transplantation shortly after diagnosis. As genetic screening has become more accessible, it is expected that a substantial number of now adolescent IBD patients who suffered from early-onset IBD will be diagnosed with a monogenic disorder. A young age of presentation and progressive disease severity can be reasons for genetic screening to detect monogenic defects that are associated with IBD-like pathology in adolescent patients (23). Studying such adolescent patients may prove a valuable addition to identify the long-term consequences of a particular defect on immune development. Here, we studied an adolescent *IL10RA*-deficient patient currently treated with immune suppressants who presented with infantile IBD and was diagnosed with *IL10RA* deficiency at the age of 9 years. The aim of this study was to determine the effects of an inherited defect in the IL-10 pathway on both the development and function of innate and adaptive immune cells.

METHODS

Patients

Blood was collected in EDTA tubes from an *IL10RA*-deficient patient, cohorts of pediatric IBD patients, age-matched healthy orthopedic patients without underlying inflammatory or intestinal disease (**Table 1**) and adult healthy laboratory volunteers (age between 22-62 years). All IBD patients had clinical characteristics and were diagnosed by endoscopy and histology of multiple biopsies according to the Porto criteria (24). All participants and/or their parents gave written informed consent and the study was approved by the local ethics committee.

At the age of 9 years, an IBD patient with infantile disease onset and from a consanguineous Turkish family was diagnosed with *IL10RA* deficiency through homozygosity mapping. Briefly, leukocyte DNA was hybridized to an Affymetrix GeneChip 260k Nsp1 SNP array (Santa Clara, CA, USA). Regions of homozygosity were identified in the patient and her unaffected sister using CNAG software (version 3)(25) The exons and flanking intron regions of *IL10RA* were sequenced after PCR amplification. A single nucleotide deletion resulting in a frame-shift and premature stop codon (c.585delT, p.Gly196Glufs*10) was identified (**Figure 1b, c**) and confirmed in an accredited diagnostic laboratory using an ABI 3730 DNA Analyzer and SeqPatient genetic analysis software (JSI Medical Systems). NM_001558.3 was used as a reference sequence. A similar mutation was found in her deceased brother (confirmation on DNA isolated from paraffin embedded intestinal biopsy). *IL10RA* deficiency was confirmed by immunohistochemistry in colonic biopsy specimens previously taken from the patient at time of diagnosis (**Figure 1d**), and functional assays on patients' peripheral blood mononuclear cells (**Figure 1e, f**).

The currently 13-year-old female with an *IL10RA* deficiency presented like other patients with mutations in the IL-10 pathway with abscesses and fissures in the perianal region in the first months of life (15, 26). A male sibling, who had also presented with perianal disease and severe gastrointestinal symptoms, died at the age of 18 months due to a bowel perforation after colonoscopy. Colonoscopy at the age of 2 months in the female patient showed mild left-sided colitis with normal crypt differentiation and mild focal inflammation with inflammatory cell infiltration in the crypt epithelium in mucosal biopsies. At the age of 10 months, colonic inflammation was more severe with tissue ulceration and abundant mononuclear and polymorphonuclear cell infiltration in the epithelium and lamina propria (**Supplemental Digital Content, Fig., *http://links.lww.com/MPG/A927***). The patient failed to achieve full clinical remission during treatment with exclusive elemental nutrition, antibiotics, sulfasalazine, prednisone, infliximab, and methotrexate. Colchicine was started because of suspicion of Behcet's disease at the age of 2 years. The patient's medical record timeline is shown in **Figure 1a**. Until now, the family has refused hematopoietic stem cell transplantation, because of the risk of the procedure in relation to the relatively mild clinical course. Currently, she has only occasionally had clinically mild gastrointestinal symptoms since the age of three while receiving thalidomide and intravenous immunoglobulin. Fecal calprotectin is only mildly elevated (151 microgram/g, normal values in IBD patients <100 microgram/g). Respiratory infections, that were severe from age 2 years, have decreased since the start of monthly intravenous immunoglobulins, as advised by the immunology/infectiology team. Between the ages 9 until 13 blood was repeatedly collected from the *IL10RA*-deficient patient before her monthly intravenous immunoglobulins infusion. At the time of the experiments the patient had no physical complaints.

Flow Cytometry

Whole blood samples were lysed and stained with antibodies directed against CD11c (B-ly6), HLA-DR (L243), CD3 (HIT3), CD4 (RPA-T4), CD8 (SK-1), CD38 (HIT2), CD62L (DREG-56), CD40 (5C3), CD80 (2D10), CD86 (2331), HLA-DR (L243), (all purchased from BD Biosciences (San Jose, CA, USA), eBioscience (San Diego, CA, USA) or Biolegend (San Diego, CA, USA)) and CCR9 (R&D Systems, Minneapolis, MN, USA). For intracellular cytokine staining, peripheral blood mononuclear cells were stimulated for 4h with phorbol 12-myristate 13-acetate (0.05 μg/ml, Sigma-Aldrich, St. Louis, MO USA) and ionomycin (0.5 μg/ml, Sigma-Aldrich) in the presence of Brefeldin A (3 μg/ml eBioscience) for the last 3h. Cells were stained with anti-CD3 (BD biosciences), fixed in 2% formaldehyde, permeabilized with saponin (Sigma-Aldrich), and labeled with anti-IL-21 (eBio3A3-N2; eBioscience), anti-IL-17A (eBio64DEC17; eBioscience), anti-IFNγ (4SB3, BD biosciences), or appropriate isotype controls (eBioscience). Foxp3 was stained using the Foxp3 Staining Buffer Set (eBioscience) according to manufacturer's protocol. Cells were analyzed using the FACSCanto II (BD Biosciences) and FlowJo software.

Cell isolation and Culture

Cells were cultured in Iscove's modified Dulbecco's medium (Lifetechnologies, Grand Island, NY, USA) supplemented with heat inactivated fetal calf serum, Glutamax (Lifetechnologies), 2-mercaptoethanol, penicillin and streptomycin. Peripheral blood mononuclear cells were isolated using a Ficoll-Hypaque gradient and stimulated with purified lipopolysaccharide from Escherichia coli Serotype 0111:B4 (100 ng/ml, Sigma-Aldrich) or anti-CD3 (500 ng/ml; Sanquin, the Netherlands) with or without IL-10 (25 ng/ml, R&D Systems) or thalidomide (1, 10 μg/ml, Celgene Corp.) for the indicated time-points. $CD14^+$ monocytes were isolated using CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach,

Germany) according to the manufacturer's instructions. Purified monocytes (>90%) were differentiated into monocyte-derived dendritic cells (moDCs) during a 6-day culture with 800 U/ml GM-CSF (Novartis, Basel, Switzerland) and 400 U/ml IL-4 (R&D). At day 6, LPS was added to the wells in the presence or absence of IL-10. Supernatants were collected at day 7.

Measurement of Cytokines in Cell Culture Supernatant

Cytokine production was measured in cell culture supernatants using enzyme-linked immunosorbent assays for TNFα (BD biosciences, Breda, the Netherlands), IFNγ (eBiosciences) or IL-17A (Duoset; R&D Systems) or by application of the standard protocol of the Human Inflammation Cytometric Bead Array specific for IL-8, IL-1β, IL-6, IL-10, TNF, IL-12p70 (BD Biosciences).

Immunohistochemistry on Paraffin Sections

Paraffin embedded biopsies were sectioned, deparaffinized, and endogenous peroxidase activity was quenched with 3% H_2O_2 in PBS for 20 min. Antigen retrieval was performed by microwave treatment in citrate buffer (10 mM, pH 6.0) or enzymatic treatment with 0.1% pepsin. The sections were blocked for 1h in 10% normal human serum plus 10% normal goat, rabbit or horse serum diluted in 10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% Tween-20, pH 8. Antibody incubation was performed overnight at 4°C using anti-IL-10RA (polyclonal rabbit, Millipore, Temecula, CA, USA), anti-CD3 (polyclonal rabbit, DakoCytomation, Glostrup, Denmark), anti-IL-17A (polyclonal goat IgG; R&D Systems, Abingdon, UK), anti-IL-21 (polyclonal rabbit IgG, Lifespan Biosciences, Seattle, WA, USA), anti-Foxp3 (mouse IgG_1 clone 236A/E7, eBiosciences, San Diego, CA, USA), anti-T-bet

(mouse IgG clone 4Bio, eBiosciences), or proper isotype control antibodies. Immunoreactive sites were detected with biotinylated secondary antibodies using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, The Netherlands). Tissues were counterstained with hematoxylin (Vector Laboratories). Images were acquired using a Leica DM5500B upright microscope and LAS image acquisition software (Leica Microsystems, Rijswijk, The Netherlands).

Statistical Analysis

Significance was determined using Student's t-test or Mann–Whitney *U*-test performed on GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). P-values of <0.05 were regarded as significant.

RESULTS

Normal distribution of circulating dendritic cells and T cell subsets in the *IL10RA*-deficient patient during treatment

Primary immunodeficiency caused by monogenic defects often leads to abnormal immune cell development, cellular dysfunction, or both. To determine whether an inherited defect in the IL-10 pathway causes abnormal immune cell development, we determined the frequency of the major circulating immune cell populations in the *IL10RA*-deficient patient during adolescence. While in remission, the percentages of circulating $CD11c^+HLA\text{-}DR^+$ dendritic cells (**Figure 2a**), $CD4^+$ and $CD8^+$ T cells (**Figure 2b**) of the patient were within the normal range for pediatric IBD patients (in remission or with active disease) and age-matched orthopedic patients. Similarly, the frequency of $Foxp3^+$ cells within the $CD4^+$ T cell population was comparable between the *IL10RA*-deficient patient and pediatric IBD patients and pediatric controls (**Figure 2c**). To investigate changes in circulating mucosal $CD4^+$ T cells in particular, we analyzed the combined cell-surface expression of $CD62L^{neg}$ and CD38 as well as the gut-homing marker CCR9 within the $CD4^+$ T cell population (27, 28). Both the percentages of circulating $CD4^+CD62L^{neg}CD38^+$ cells and gut-homing $CD4^+CCR9^+$ cells were not different to pediatric controls or pediatric IBD patients (**Figure 2d**). Moreover, no differences were observed for $CD4^+CD62L^{neg}CD38^{neg}$ cells, $CD4^+CD62L^+CD38^{neg}$ cells, and $CD4^+CD62L^+CD38^+$ cells (data not shown). Consistent with the unchanged T-cell frequencies, the percentages of $CD3^+$ T-cell subpopulations producing the effector cytokines IL-17, IFNγ and IL-21 were comparable to pediatric IBD patients in remission (**Figure 2e**). Thus, in the absence of a functional IL-10 pathway, during treatment with thalidomide, colchicine, and intravenous immunoglobulins the immune system has developed with normal

frequencies of $CD11c^{+}HLA\text{-}DR^{+}$ DCs and circulating effector memory T cell subsets.

Functional consequences of defective IL-10 signaling on monocyte-derived dendritic cells

The functional impact of primary immunodeficiency on innate and adaptive immunity helps to elucidate underlying mechanisms of disease. Recent evidence from animal studies demonstrates that loss of IL-10R expression by innate immune cells causes severe colitis (18-20). Therefore, we investigated whether alterations in the *in vitro* generation and function of DCs, as part of the innate immune system, occur in the absence of a functional IL-10 pathway. Monocytes from the *IL10RA*-deficient patient differentiated normally into DCs in response to GM-CSF and IL-4. As expected (29), LPS-induced upregulation of co-stimulatory molecules was inhibited by exogenous IL-10 in healthy control moDCs, but not in *IL10RA*-deficient moDCs. Post stimulation, expression levels of HLA-DR and the co-stimulatory molecules CD40, CD80, and CD86 were comparable between the *IL10RA*-deficient moDCs and healthy control moDCs (**Figure 3a**). In contrast, significantly higher amounts of TNFα were released by LPS-stimulated *IL10RA*-deficient moDCs, while IL-10 production was similar to healthy control moDCs (**Figure 3b**). Comparable to that observed for TNFα, *IL10RA*-deficient moDCs released higher amounts of the chemotactic cytokine CXCL-8 in response to LPS (data not shown). These data demonstrate that *IL10RA*-deficient moDCs develop normally and do not hyper-express co-stimulatory molecules, but when stimulated with LPS, these cells secrete significantly more pro-inflammatory cytokines.

IL-10 fails to suppress effector T-cell responses in the *IL10RA*-deficient patient

Considering that DCs are potent inducers of primary T-cell immune responses and that IBD is mediated by abnormally activated effector T cells predominantly secreting IFNγ and IL-17

(30), we next investigated whether the *IL10RA*-deficient patient exhibits alterations in effector T-cell responses. IL-10 inhibited secretion of the effector T cell cytokines IFNγ and IL-17 by anti-CD3-stimulated peripheral blood mononuclear cells from healthy controls (**Figure 3c**). Interestingly, upon anti-CD3 stimulation, peripheral blood mononuclear cells from the *IL10RA*-deficient patient secreted significantly enhanced amounts of IFNγ, and to a lesser extent IL-17, which could not be inhibited by exogenous IL-10 (**Figure 3c**). To ascertain whether these effector T cell cytokines or the transcription factor critical for their production had possibly played a role in patients' colitis, we performed immunohistochemical analysis of paraffin-embedded biopsies obtained from the *IL10RA*-deficient patient at disease onset. At the age of 2 and 10 months, descending colon tissue showed an influx of lamina propria and intra-epithelial $CD3^+$ cells (**Figure 4a-c**). Co-staining of serial sections revealed that the majority of the $CD3^+$ cells co-localized with IL-21^+, IL-$17A^+$, and/or T-bet^+ cells in the inflamed mucosa (**Figure 4c**). For comparison, descending colon tissue of an age-matched (5 months old) non-IBD patient suffering from cow's milk protein intolerance is shown (**Figure 4d**). Foci of $Foxp3^+$ cells were detected in the intestinal mucosa lesions, suggesting that the intestinal inflammation is not simply a consequence of defective $Foxp3^+$ T cell recruitment. In conclusion, these data demonstrate that IL-10 is essential for suppressing IFNγ and IL-17-secreting effector T cells, and that in the absence of a functional IL-10 pathway, inflammatory lesions in the colon contain both effector T cell subsets.

Thalidomide inhibits LPS-mediated TNFα production but not anti-CD3-driven IFNγ and IL-17 production by peripheral blood mononuclear cells

Given that IL-10R signaling is crucial for controlling the function of moDCs and cytokine secretion by effector T cells, we next addressed the effect of the patient's current therapy on

both innate and adaptive immune responses *in vitro*. Since treatment with thalidomide primarily promoted clinical remission of the intestinal symptoms in the *IL10RA*-deficient patient, we developed an *in vitro* assay system in which we studied the effects of thalidomide on innate and adaptive immune responses to bacterial LPS and T-cell receptor ligation, respectively. Thalidomide treatment had no significant effect on anti-CD3-mediated IFNγ and IL-17 cytokine secretion by peripheral blood mononuclear cells from healthy controls (**Figure 5a, c**) and the *IL10RA*-deficient patient (**Figure 5b, d**). However, thalidomide significantly decreased TNFα production by LPS-stimulated peripheral blood mononuclear cells from both healthy controls (**Figure 5e**) and the *IL10RA*-deficient patient (**Figure 5f**). PBMC from the *IL10RA*-deficient patient seemed less sensitive to the effects of thalidomide, possibly because the cells have already been exposed to this drug in the patient. Altogether, these results suggest that inhibition of innate responses may contribute to disease suppression.

DISCUSSION

We investigated the long-term impact of a defective IL-10 pathway on immune system development and function in an adolescent patient with an *IL10RA* loss-of-function mutation who presented with severe early-onset IBD within the first year of life. In the absence of a functional IL-10 pathway, we observed no alterations in the composition of major immune cell populations in the blood. Yet, we demonstrate that upon stimulation, IL-10 is crucial for controlling excessive inflammatory cytokine release by moDCs and for optimal suppression of T-cell responses. These findings identify DCs and T cells as essential targets of IL-10 action in controlling cell-mediated immune responses in human.

During treatment, the number of circulating $CD11c^+$ DCs was normal in the *IL10RA*-deficient patient, indicating sufficient bone marrow output and differentiation of the myeloid cell lineage. DCs generated *in vitro* from peripheral blood monocytes showed normal upregulation of MHCII and co-stimulatory molecules but when stimulated with a bacterial stimulus, *IL10RA*-deficient moDCs secreted significantly higher amounts of TNFα. The *in vitro* generation of functional DCs occurred independently from any confounding effects of ongoing intestinal inflammation or drug therapy, suggesting a cell-intrinsic effect of *IL10R* deficiency on pro-inflammatory cytokine production by these cells. The differential regulation of co-stimulatory molecule expression and cytokine production is notable, as previous neutralization studies proposed a role for IL-10 in both autocrine co-stimulation and cytokine release (31, 32). However, our data are in line with observations in $CD11c^+$-specific *Il10r*-deficient mice, in which only cytokine release and not co-stimulatory molecule expression by $CD11c^+$ cells was affected (33). In our study, cytokine production by cells of the *IL10R*-deficient patient were compared to those from adult healthy volunteers. Cytokine production may differ between children and adults. However, it has been shown that in healthy children, cytokine production by PBMC is lower compared to adults upon mitogen and antigen

stimulation (34). Therefore, the degree with which the cytokine production by cells from the *IL10RA*-deficient patient is enhanced may be an underestimation.

Similar as observed for DCs, the composition of T-cell subpopulations in peripheral blood did not differ between the *IL10RA*-deficient patient and pediatric controls and pediatric IBD patients. This is of interest as IL-10 has been proposed to act as a cofactor for thymocyte proliferation (35). Consistent with prior findings (36), exogenous IL-10 inhibited IFN□□and IL-17 secretion by *in vitro*-activated peripheral blood mononuclear cells from control subjects. Reciprocally, in the *IL10RA*-deficient patient an enhancement in IFN□□T-bet and IL-17 expression was seen in mucosal tissue and peripheral blood mononuclear cells. In mice, T helper 17 (Th17) cells, but not IFN□-producing T helper type 1 (Th1) cells, express a functional IL-10 receptor and are sensitive to the IL-10 inhibitory effects (37). Besides a direct effect, IL-10 signaling in regulatory T cells has also been implicated in limiting pathogenic Th17 cell responses (38). Moreover, several groups have demonstrated that especially IL-10 sensing by innate immune cells is required for suppression of T cell-driven intestinal inflammation (18-20). Whether IL-10 regulates human IFN□□and IL-17-secreting T cells directly or indirectly via innate immune cells or by favoring Treg cell-mediated suppression awaits further studies.

The observed inhibitory effect of thalidomide on TNFα production by LPS-stimulated *IL10RA*-deficient peripheral blood mononuclear cells suggests that the inhibition of TNFα secretion contributes to suppression of innate responses and may be involved in the clinical remission of the intestinal symptoms. However, as the patient failed to respond to infliximab, mechanisms other than TNFα inhibition may have contributed to disease control. Thalidomide is known to inhibit NF-κB activity (39) as well as activation of caspase-1 and thereby secretion of biologically active IL-1β protein (40). As thalidomide suppresses IL-12 production by human monocytes (41), it is tempting to speculate that thalidomide may be

effective in inhibiting Th1 cytokine responses. Moreover, thalidomide binds cereblon, an upstream regulator of interferon regulatory factor (IRF)4 (42, 43). IRF4 is an important regulator of T_H17 cells, and *Irf4*-deficient mice display reduced *Il17* gene expression during chronic intestinal inflammation (44). Although thalidomide had no effect on IFN□ and IL-17 production *in vitro*, we cannot exclude an effect of thalidomide on T cells *in vivo*.

The abnormalities caused by monogenic defects in IBD-like disease hold valuable knowledge on the mechanisms that may predispose to "classical" IBD. Our findings show that *IL10R* deficiency has a major impact on the cytokine secretion by human moDCs (TNFα) and T cell cytokine production (IFNγ and IL-17), suggesting that these defects may contribute to the clinical manifestation of the IBD-like gastrointestinal pathology. Although the primary limitation of this study is that it involves only one *IL10RA*-deficient patient, we expect similar results in other patients with defects in the IL-10 pathway. Besides moDCs and T cells, it has been recently reported that monocyte-derived macrophages from *IL10R*-deficient patients secrete significantly more pro-inflammatory cytokines (19). Interestingly, the biological process term 'regulation of cytokine production' is significantly enriched among the 163 IBD susceptibility loci, and further detailed analysis showed an enrichment for genes expressed especially by DCs and to a lesser degree $CD4^+$ T cells (2). These and our findings underscore the importance of aberrant activated innate immune cells and $CD4^+$ T cells in inflammatory bowel disease pathology. The identified immune abnormalities in monogenic disease may be useful for the functional understanding and classification of polygenic classical IBD phenotypes. It can be envisaged that a subgroup of IBD patients may exhibit milder forms of IL-10 deregulation. Future studies will focus on the immunological alterations in *IL10R*-deficient patients and uncover biomarkers and immune assays to identify individuals with suboptimal IL-10 signaling among polygenic classical IBD patients.

Collectively, our data provide fundamental insights into the immune system development and functions in an *IL10R*-deficient patient. Our findings promote our knowledge of intestinal immune regulation, which is required to advance our ability to classify the varying clinical pathologies of IBD.

ACKNOWLEDGMENTS

The authors thank Dr T. Cupedo and Dr. B. Löwenberg for critical reading of the manuscript; and Dr. C. Klein and Dr. D. Kotlarz for genetic analysis.

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FIGURE LEGENDS

Figure 1. Functional analysis of the *IL10RA* mutation.

(**a**) The *IL10RA*-deficient patient's medical record timeline **(b)** Sequencing of the *IL10RA* region revealed a homozygous single nucleotide deletion (c.585delT, p.Gly196Glufs*10) in exon 5 resulting in a frame-shift and premature stop codon. (**c**) Intron-exon organization of the full-length and mutated *IL10RA* gene. The premature stop codon predicts a truncated receptor lacking the transmembrane and intracellular regions. Exons are shown in boxes, introns as lines, and mRNA as thick lines. (**d**) Immunohistochemical detection of IL-10R in paraffin-embedded colon descendens sections from the *IL10RA*-deficient patient and a pediatric IBD patient using an antibody raised against the cytoplasmic domain of human IL-10RA (residues 257-278). (**e**) Control or patient whole blood was stimulated with IL-10 (25 ng/ml) for 15 min or IL-6 (100 ng/ml) for 30 min, and STAT3 phosphorylation (Tyr^{705}) was quantified by flow cytometry. Gated on monocytes for IL-10 stimulation or the whole cell population for IL-6 stimulation. (**f**) Control and patient peripheral blood mononuclear cells were stimulated with LPS (100 ng/ml) in the absence or presence of IL-10 (25 ng/ml). After 6h, supernatants were collected and TNFα secretion was measured by ELISA. *P<0.05.

Figure 2. Similar numbers of circulating CD11c^{+}HLA-DR^{+} dendritic cells and T cell populations in the *IL10RA*-deficient patient and pediatric controls

Flow cytometric analysis for CD11c, HLA-DR, CD4, CD8, CD62L, CD38, CCR9, and/or Foxp3 was performed on peripheral blood from pediatric IBD patients, pediatric controls and the *IL10RA*-deficient patient (**a**) Frequency of the CD11c^{+}HLA-DR^{+} dendritic cell population (**b**) Frequencies of CD4^{+} and CD8^{+} T lymphocytes. (**c**) Frequency of Foxp3^{+} cells gated on CD4^{+} lymphocytes. **(d)** Frequencies of CD62L^{neg}CD38^{+} and CCR9^{+} cells gated on CD4^{+} lymphocytes. **(e)** Peripheral blood mononuclear cells were stimulated for 4h *in vitro* and stained for intracellular IFNγ, IL-21, IL-17A, and appropriate isotype controls followed by flow cytometry analysis. Representative cytokine dot plots for the *IL10RA*-deficient patient are shown; the numbers below the dot plots indicate the ranges of cytokine expression in five pediatric IBD patients in remission.

Figure 3. IL-10 controls excessive inflammatory cytokine release by dendritic cells and T cells

(**a**) Adult healthy control and patient monocyte-derived dendritic cells were matured with LPS (100 ng/ml) in the presence or absence of IL-10 (25 ng/ml). After 20h, cells were analyzed by flow cytometry for expression of CD40, CD80, CD86, and HLA-DR. Mean fluorescence intensity values (MFI) for all conditions are shown below histograms. Data shown are from one of three representative experiments. (**b**) Amount of TNFα secreted by monocyte-derived dendritic cells as determined by cytometric bead array. IL-10 was measured in supernatants using cells from five adult healthy controls and supernatants from five replicate cultures with cells from the *IL10RA*-deficient patient (c) Adult healthy control and patient peripheral blood mononuclear cells were stimulated with anti-CD3 (500 ng/ml) in the absence or presence of IL-10 (25 ng/ml). After 48h, supernatants were assayed for IFNγ and IL-17A using an ELISA. Data are mean ± SD of one of four representative experiments; **P*<0.05.

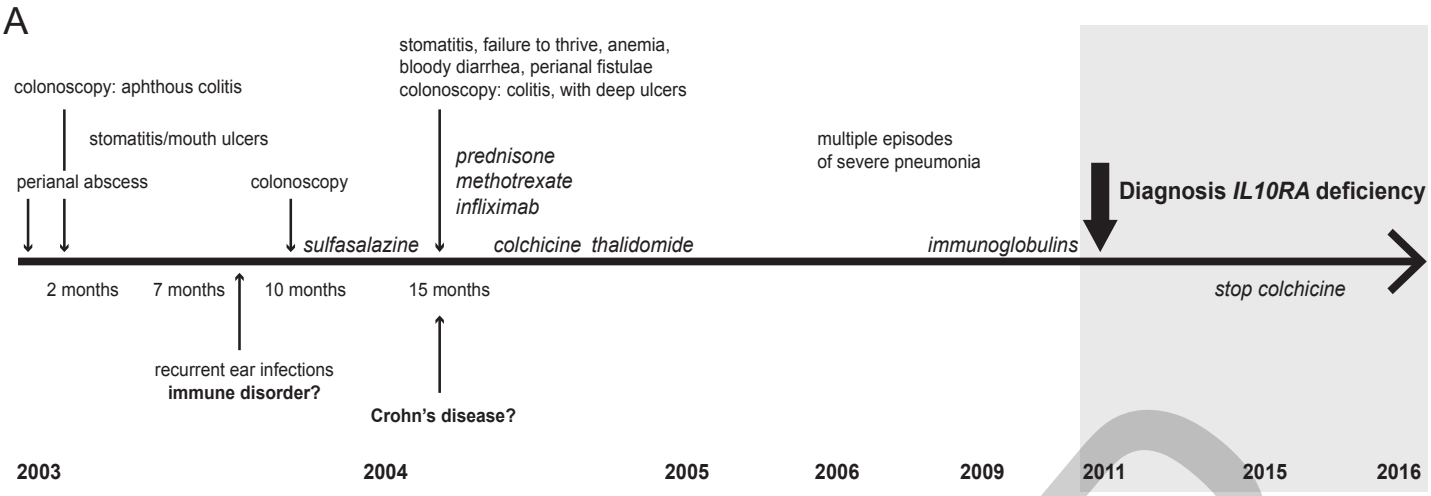
Figure 4. Presence of $CD3^+$, $IL\text{-}21^+$, $IL\text{-}17^+$, $T\text{-}bet^+$ and $Foxp3^+$ cells in the inflamed intestinal mucosa of the *IL10RA*-deficient patient

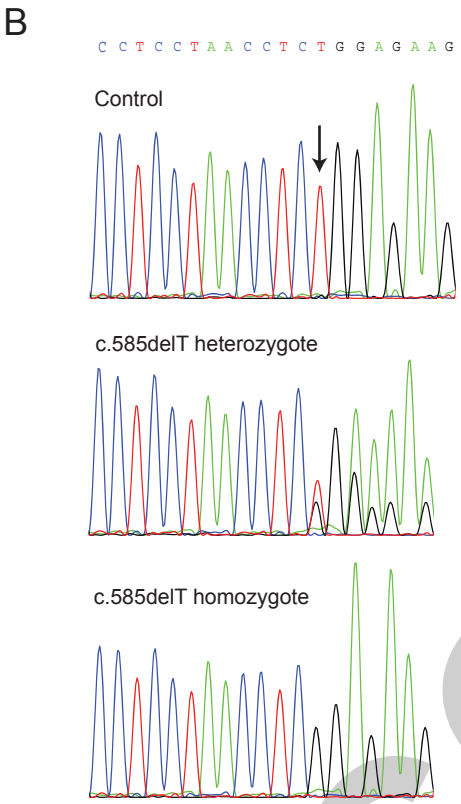
Representative immunohistochemical staining for CD3, IL-21, IL-17A, T-bet, and Foxp3 in serial sections of paraffin-embedded biopsies of the colon descendens from the *IL10RA*-deficient patient taken at (**a**) 2 months and (**b,c**) 10 months (**d**) age-matched (5 months old) non-IBD patient suffering from cow's milk protein intolerance. Polyclonal rabbit anti-goat Ig isotype control staining for IL-21 and CD3, goat IgG1 isotype control staining for IL-17A, mouse IgG_1 isotype control staining for Foxp3, and mouse IgG_1 isotype control staining for T-bet are shown in the small inserts in the top panel. Original magnification x20.

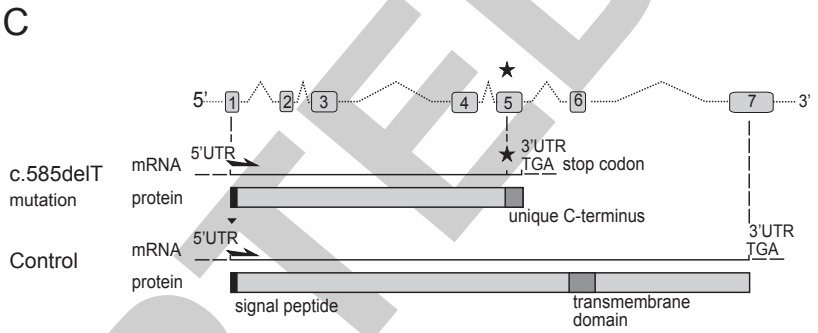
Figure 5. Thalidomide inhibits LPS-mediated TNFα release, but not the anti-CD3-driven IFNγ and IL-17 release

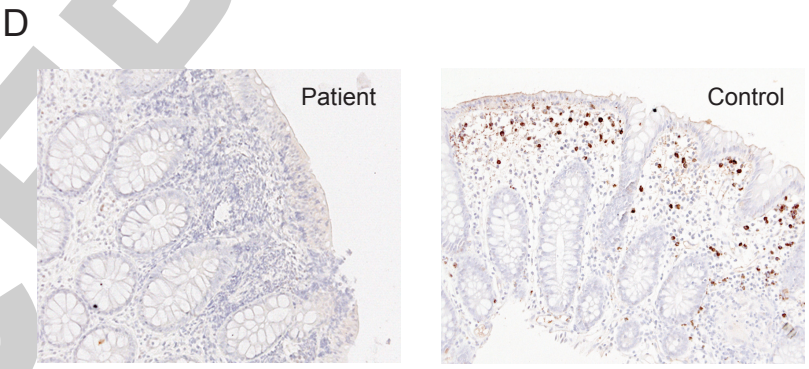
(**a-d**) Peripheral blood mononuclear cells were stimulated with anti-CD3 (500 ng/ml) in the presence of thalidomide (1 or 10 μg/ml) or DMSO control (1 or 10 μg/ml). After 48h, cytokine levels in culture supernatants were measured by ELISA. IL-17 and IFNγ response for (**a, c**) healthy adult donors and (**b, d**) the *IL10RA*-deficient patient are shown. The relative difference was calculated considering the percent of cytokine secretion upon anti-CD3 stimulation as 100%. (**e,f**) Peripheral blood mononuclear cells were stimulated with LPS (100 ng/ml) in the presence of thalidomide (1 or 10 μg/ml) or DMSO control (1 or 10 μg/ml). After 24h, cytokine levels were measured in the supernatant. TNFα response for (**e**) healthy adult donors and (**f**) the *IL10RA*-deficient patient are shown. The relative difference was calculated considering the percent of cytokine secretion upon LPS stimulation as 100%. Data are mean ± SD of one of three representative experiments; **P*<0.05

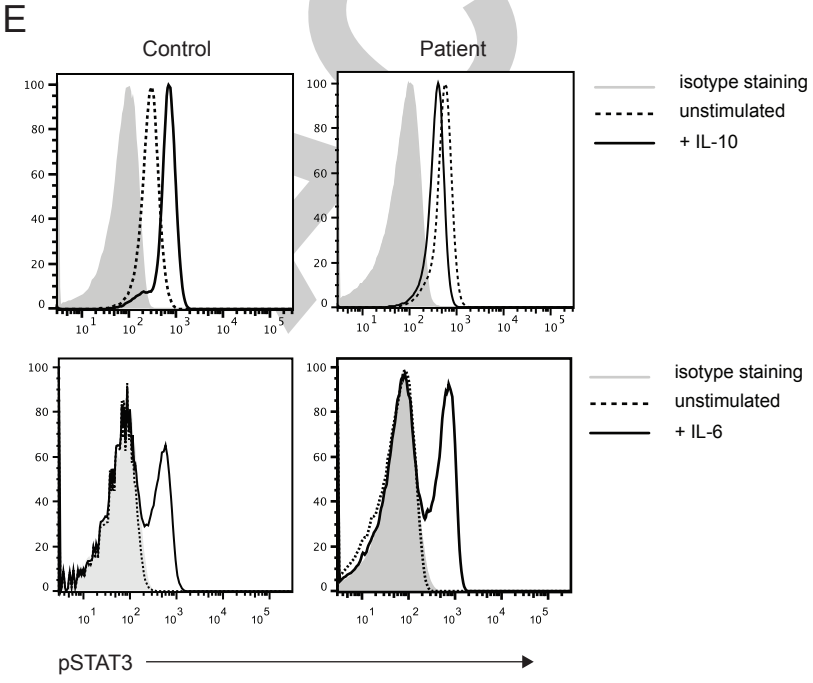
Figure 1











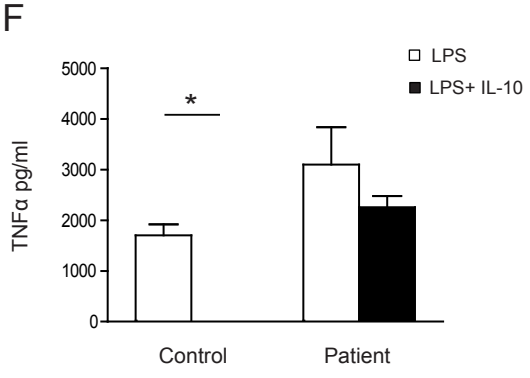
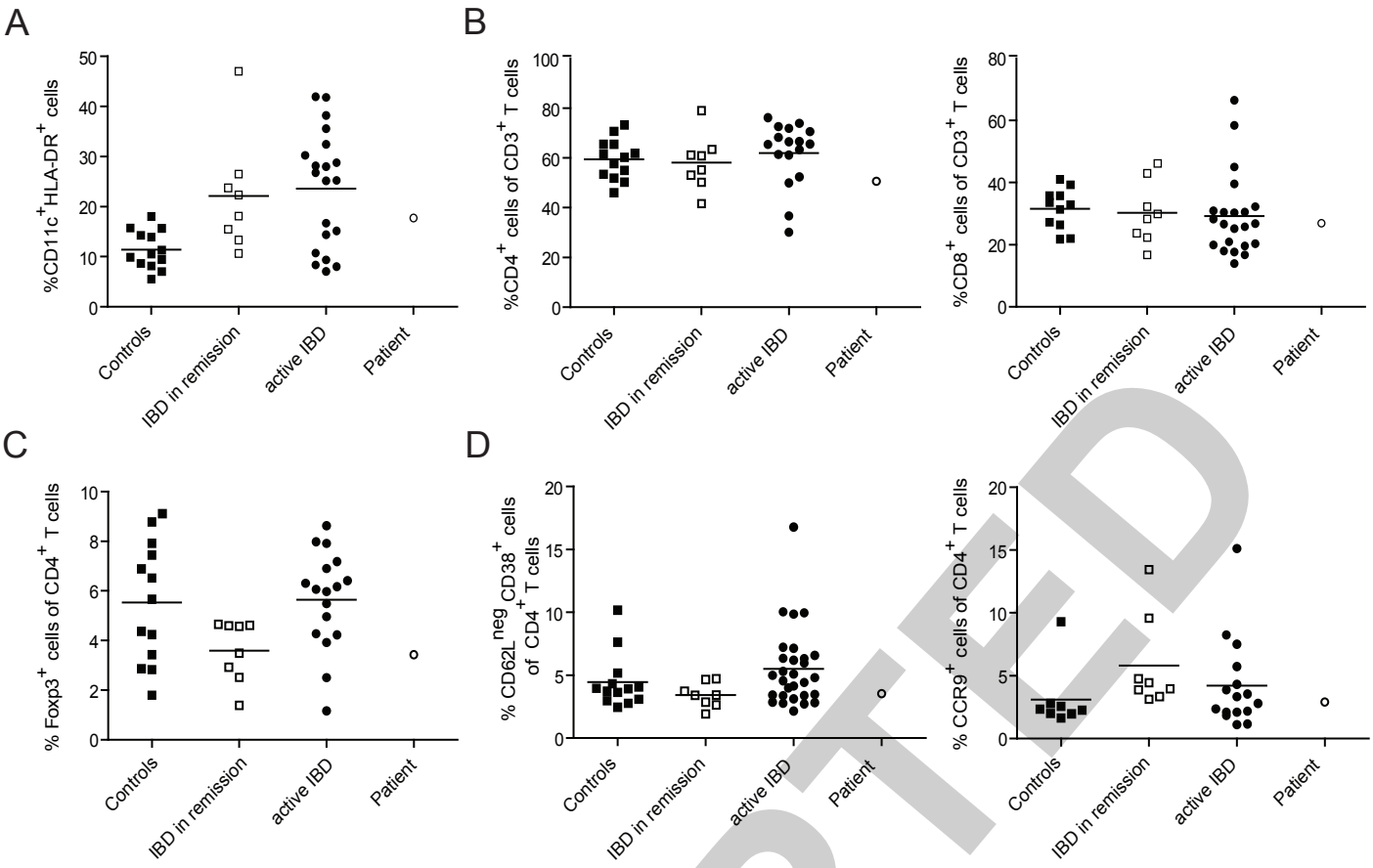


Figure 2



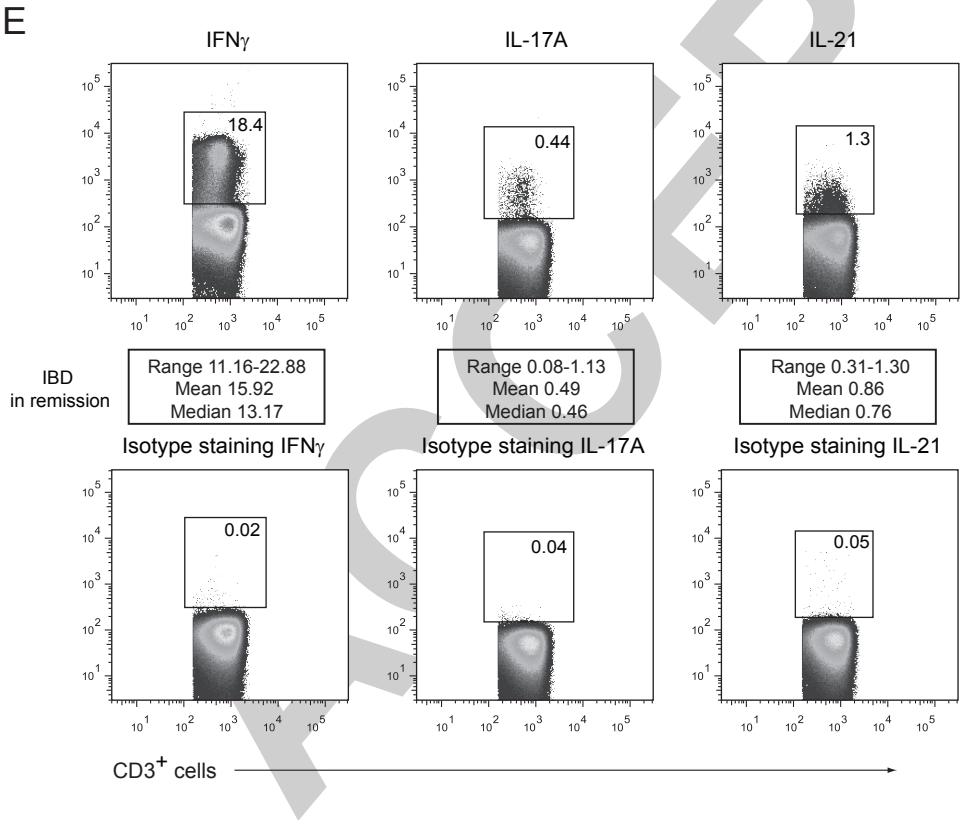
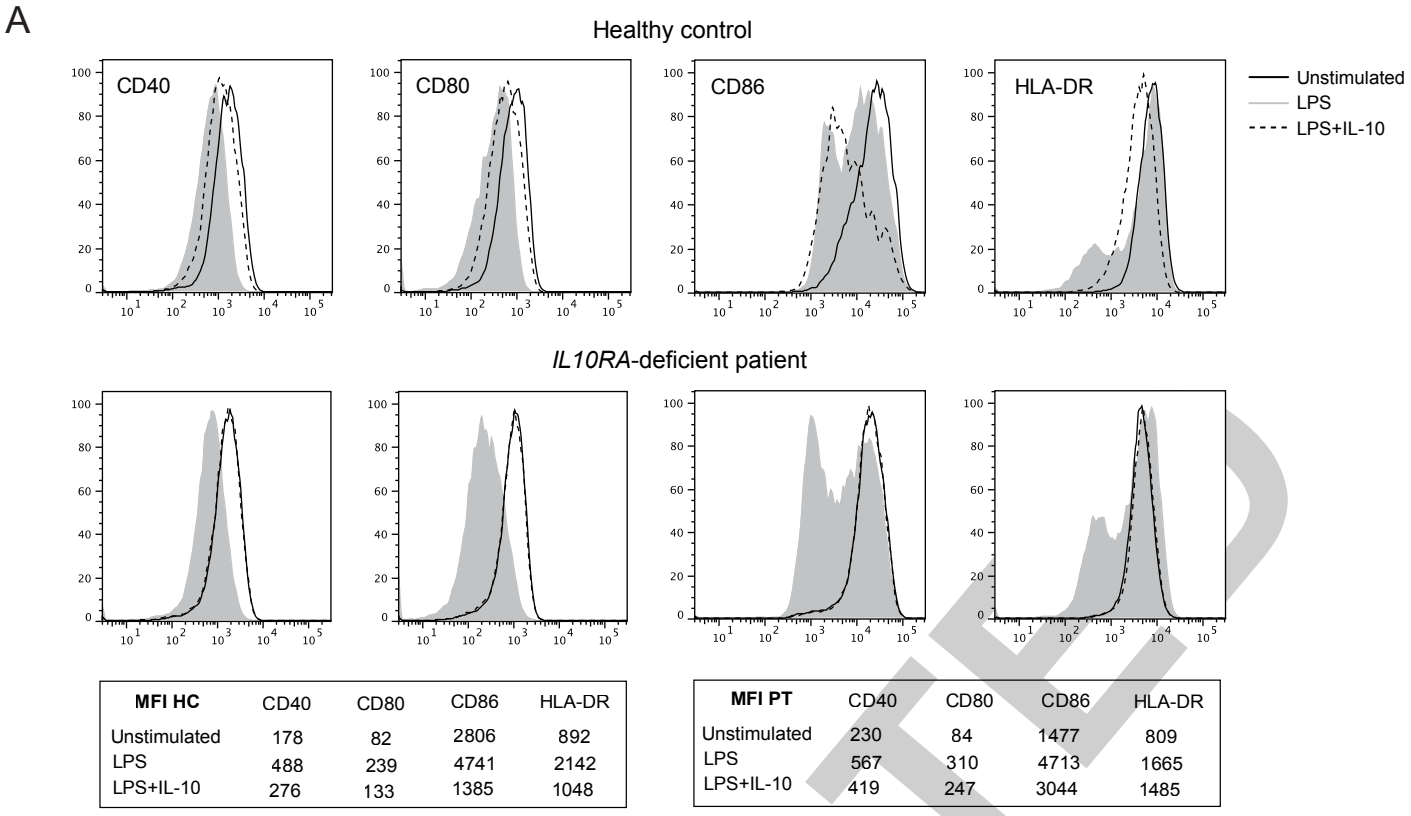


Figure 3



MFI HC	CD40	CD80	CD86	HLA-DR
Unstimulated	178	82	2806	892
LPS	488	239	4741	2142
LPS+IL-10	276	133	1385	1048

MFI PT	CD40	CD80	CD86	HLA-DR
Unstimulated	230	84	1477	809
LPS	567	310	4713	1665
LPS+IL-10	419	247	3044	1485

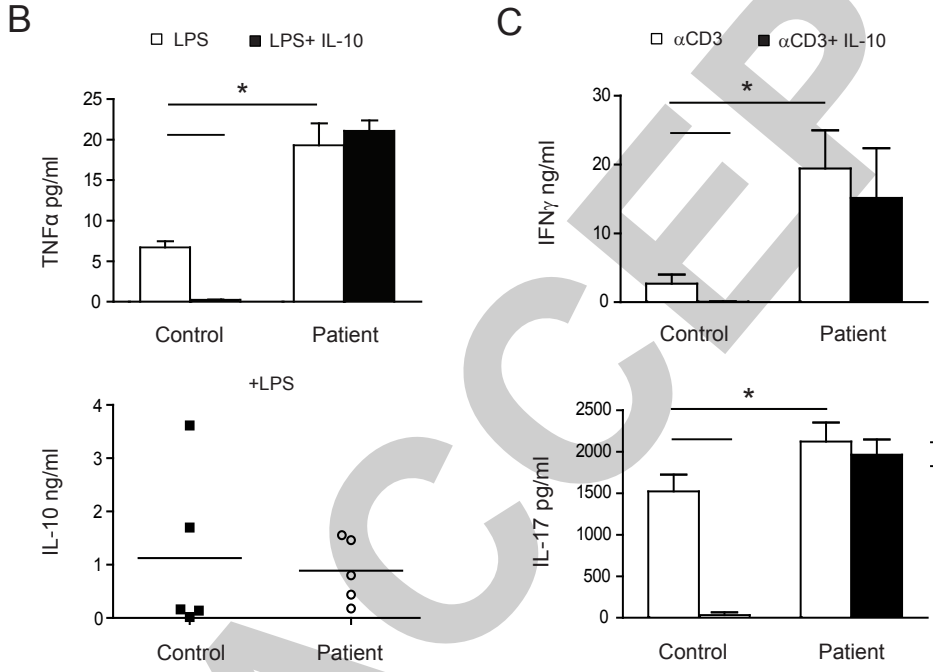


Figure 4

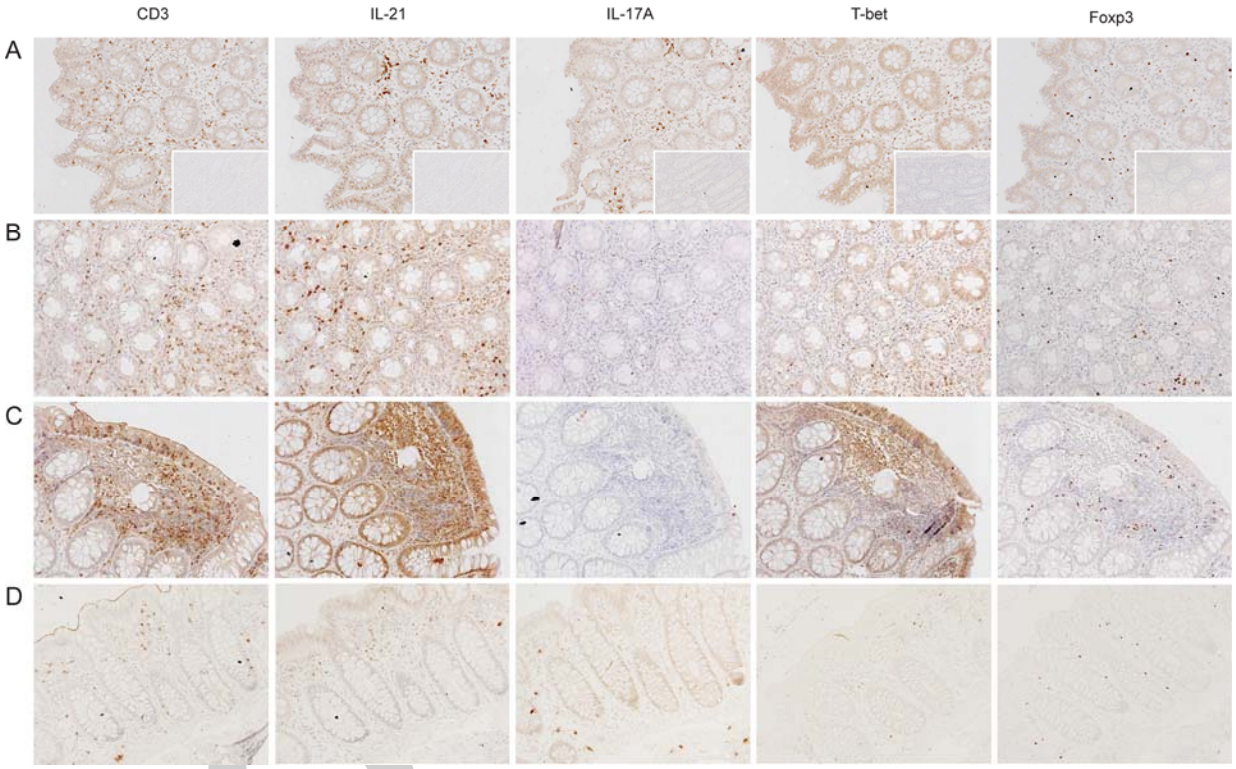


Figure 5