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INTESTINAL CD4^{+}CD25^{HIGH} REGULATORY T CELLS ARE EXPANDED IN ANKYLOSING SPONDYLITIS PATIENTS: A PUTATIVE ROLE FOR IL-10 IN PREVENTING INTESTINAL TH17 RESPONSE

Short title: regulatory T cells in spondyloarthritis

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Abbreviations: Ankylosing Spondylitis (AS), Crohn's Disease (CD)

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Abstract

Objective: subclinical gut inflammation has been demonstrated in ankylosing spondylitis (AS) patients. Aim of this study was to determine the frequency of regulatory $CD4^{+}CD25^{high}$ T cells (T_{reg}), and to evaluate T_{reg}-related cytokines (IL-2, TGF-β, IL-10), and transcription factors (FOXP3 and STAT5) in the ileum of AS patients.

Methods: Quantitative gene expression analysis, by rt-PCR, of T_{reg}-related cytokines (IL-2, TGF-β, IL-10) and transcription factor (STAT-5 and FOXP3) was performed on ileal biopsies of 18 AS and 15 active Crohn's disease (CD) patients, and 15 healthy subjects (HS). Tissue and circulating T_{reg} cells were also analyzed by flow cytometry.

Results: A significant up-regulation of IL-2, TGF-β, FOXP3, STAT-5 and IL-10 transcripts in the terminal ileum of AS patients displaying chronic ileal inflammation was observed. Flow cytometric analysis of T_{reg} cells showed significant peripheral expansion in both chronic inflamed AS and CD patients (1.08%±0.4% and 1.05±0.3% respectively), compared to HS (0.25±0.12; $p<0.05$). Interestingly a five-fold increase in the proportion of T_{reg} cells was observed in the gut of AS patients (5±3%; $p<0.001$) compared to HS (1.2%±0.4%), with 70 to 80% of these cells also producing IL-10. *In vitro* studies showed that block of IL-10 was sufficient to induce Th17 polarization on isolated lamina propria mononuclear cells (LPMC) from AS patients.

Conclusion: Here we provide the first evidence that an active T_{reg} cell response, mainly dominated by IL-10 production, occurs in the gut of AS patients, probably responsible for the absence of a clear TH17 polarization observed in the ileum of AS patients.

Introduction

Intestinal inflammation is a shared feature from Ankylosing spondylitis and Crohn's disease patients. Shared immunological and genetic similarities, in both clinical and preclinical stages, together with the observation that the 7% of patients with AS and initial chronic gut inflammation develops overt CD, strongly supports the hypothesis that of AS patients with more pronounced gut inflammation could be considered suffering from preclinical CD (1-3).

We have recently demonstrated that overexpression of IL-23, but not IL-17, is a pivotal feature of subclinical gut inflammation in AS patients (4). IL-23 has recently been shown to be a fundamental player in the intestinal immune responses balanced between tolerance and immunity. In particular IL-23, together with TGF-β, IL-1β and IL-6, has been demonstrated to be involved in the differentiation of the IL-17-producing $CD4^+$ T cells, namely Th17 lymphocytes, that are highly proinflammatory and induce severe autoimmunity (5). The participation of TGF-β in the differentiation of Th17 cells places them in close relationship with $CD4^+CD25^{high}$ T_{reg} cells, as TGF-β, among its numerous properties, is also able to induces expression of FOXP3 in naïve antigen-stimulated T cells in the peripheral immune compartment, leading to cells with regulatory or suppressor function (6). In consideration of the high levels of TGF-β transcripts we observed in the gut of AS patients without concomitant over-expression of IL-1β and IL-6, we aimed to investigate if the absence of a clear Th17 polarization, despite the high levels of IL-23 observed, was related to a T_{reg}-mediated suppression in the gut of AS patients. Here we provide the first evidence that $CD4^+CD25^{high}$ T cells are increased in the gut and in the peripheral blood of AS patients, with a concomitant significant intestinal increase of the T_{reg}-related transcripts (STAT-5, TGF-β and IL-10) suggesting that an active T_{reg} response,

mainly dominated by IL-10 production, occurs in AS. Thus we propose that T_{reg} response observed in the gut of AS patients, keeping auto reactive Th17 cells under check, is probably responsible for the absence of a clear TH17 polarization despite the high levels of IL-23.

Patients and methods

Gut specimens and peripheral blood samples from patients with CD, AS or control subjects (HS) were consecutively obtained. The AS group consisted of 18 consecutive patients, 11 men and 7 women, ranging from 19 to 48 yrs of age without clinical symptoms of bowel inflammation. The diagnosis for each patient was made according to the modified New York criteria (7) and all the patients were HLA-B27 positive. Disease activity was evaluated by the use of the Bath Ankylosis Spondylitis Disease Activity Index (BASDAI) (8), with a BASDAI > 4 defining an active disease. The mean ± SD BASDAI score, at the time mucosal biopsies were obtained, was 6.8 (2.5). Mean disease duration since diagnosis was 4±2 months. At the time of samples collection, eleven out of 18 AS patients were taking non-steroidal anti-inflammatory drugs (NSAIDs). The CD group consisted of 15 patients (9 men and 6 women), ranging from 20 to 50 yrs of age. Patients with pure colonic involvement were excluded from the study. Disease activity in each patient with CD was analyzed according to the Crohn's Disease Activity Index (CDAI) (9) and endoscopic and histopathological data. Mean disease duration since diagnosis was 5±1.5 months. At the time of sample collection, patients of the Crohn's disease group were not receiving corticosteroids or immunosuppressant. The mean ± SD CD Activity index score was 320 (70) at the time of enrollment. The control group consisted of 15 normal subjects, (12 male and 3 female patients, age ranging from 41 to 68 yrs) undergoing to ileocolonscopy for routine evaluation. Ten AS patients, 7 CD patients and 7 controls in which sufficient m-RNA for new experiments was available, came from the previous published study (4). Paired specimens for histological analysis, QT-PCR and LPMCs isolation for flow cytometric analysis and functional assays were obtained from new consecutively enrolled patients and controls (8 patients with AS, 8 with CD and 8 HS). Collection of ileal biopsies

was approved by the ethical committee and the institutional review board of the University of Palermo.

Histomorphological grading

Tissue samples were processed as previously described (4) and divided in two main subgroups according to histology: normal gut histology or minor inflammatory changes (acute lesions) and chronic inflammation.

Quantitative TaqMan reverse transcriptase–polymerase chain reaction (RT-PCR) for Treg cytokines, FOXP3 and STAT5 molecules in ileal biopsies

Total RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen, Chatsworth, CA), with on-column DNase I digestion. A total of 1 μg of RNA was reverse-transcribed to complementary DNA (cDNA) using a ThermoScript First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA). For quantitative TaqMan real-time PCR, gene expression assays for β-actin or Eukaryotic 18S control and for IL-1β (Hs00174097_m1), IL-2 (Hs00914135_m1), IL6 (Hs00174131_m1), IL-10 (Hs00174086_m1), IL-17A (Hs00174383_m1), TGFβ1 (Hs00171257_m1), IL-23p19 (Hs00372324_m1), RoRc (Hs01076112_m1), FOXP3 (Hs01085835_m1), and STAT5A (Hs00559647_m1) were obtained from Applied Biosystems (Foster City, CA). Samples were run in triplicate at 20 ng ofcDNA per well and detected using an ABI Prism 7900HT instrument. Results were analyzed using ABI Prism 7900HT Sequence Detection System version 2.1 software. Relative quantification was assessed using the Ct method.

Immunohistochemistry for STAT5 phosphorylated

Immunohistochemistry for pSTAT5 was performed on 3-μm thick paraffin-embedded sections obtained from intestinal biopsies of patients and controls as previously described

(4). Briefly, following rehydration, antigen was unmasked using the DAKO Target retrieval solution (pH 6, DAKO) for 45 min at 95°C. Endogenous peroxidase was blocked with the DAKO Peroxidase blocking reagent for 10 min and non-specific bindings with the DAKO Protein Block for 20 min. The primary antibody, rabbit monoclonal anti-human STAT5 phosphorylated (Tyr 694) (IgG, Cell Signaling) was added (1:300 dilution in DAKO diluent) and incubated for 1h at room temperature. An isotype-matched irrelevant antibody was used as a negative control. Slides were then incubated for 30 min with peroxidase-conjugated DAKO Envision polymer and peroxidase activity was visualised using the DAB+ chromogen (DAKO) and slides lightly counterstained with hematoxylin before dehydration and mounting in DePex (VWR International). The number of p-STAT5 expressing cells was determined by counting immunoreactive cells on microphotographs taken from three random high-power microscopic fields (original magnification x400) under a Leica DM2000 optical microscope using a Leica DFC320 digital camera. Results were reported as mean±SD.

Culture media, reagents and antibodies

In all in vitro assays, cells were cultured in very low endotoxin medium RPMI 1640 (Sigma; St Louis, USA), 10 mM Hepes (Euroclone; Wetherby, Yorkshire, UK), 10% FBS (Euroclone), 100 U/ml penicillin/streptomycin, and 0.05 mM 2-ME (Sigma). Phosphate buffer saline (PBS) was obtained from Euroclone. Purified recombinant human IL-6 (R&D systems, Wiesbaden, Germany, and Minneapolis, MN), phorbol myristate acetate (PMA) and ionomycin (Sigma-Aldrich) were used for *in vitro* culture assays.

The following antibodies were used for flow cytometry analysis: FITC-conjugated anti-CD4; FITC-conjugated anti-CD68; FITC-conjugated anti-CD20; PE-conjugated anti-IL-10; PE-conjugated IgG isotype control MoAbs (Beckton Dikinson, Mountain, View, CA, USA);

APC-conjugated anti-IL-17 (Santa Cruz Biotechnology, Santa Cruz, CA); FITC-conjugated anti-Ki67 (Biocompare, San Francisco CA, USA).

Isolation of LPMCs and peripheral blood mononuclear cells (PBMC)

LPMCs were isolated from eight gut biopsy specimens of patients with AS, CD and of healthy controls as described by Van Damme (10). Briefly, the biopsy samples were washed with PBS, transferred to fresh PBS and stirred for 20 minutes at 37°C to remove blood and debris. Next, the samples were transferred to fresh PBS plus 1mM EDTA and stirred for another 60 minutes at 37°C to remove intraepithelial cells. Subsequently, LPMCs were obtained by cutting the biopsy into fragments of about 1-5 mm^3, which were then incubated at 37°C in collagenase type IV (25 U/ml; Sigma) for 3 h. No further purification of the cell populations was performed to retain the maximum number of cells. The isolated cells were counted and checked for viability using 0.1% trypan blue (viability ranged from 90 to 94%). Fresh cell suspension was either immediately analysed by fluorescence-activated cell sorter (FACS) analysis or cultured for 7 days in RPMI complete medium. On day 3, 50% of the culture medium was replaced by fresh medium supplemented with cytokines. On day 7, cells were harvested, and their phenotype was analysed by flow cytometry. In some experiments, cells were stimulated with cytokines in presence of anti-IL-10 antibody (10 µg/ml), or the respective rat IgG1 isotype control (10 µg/ml). All cultures were set up in triplicate.

PBMCs were isolated from heparinized blood samples of all patients and controls by density gradient centrifugation (Histopaque; Sigma). Cell viability (trypan blue dye exclusion) was always greater than 95 %. Fresh cell suspension was either immediately analysed by fluorescence-activated cell sorter (FACS) analysis or cultured for 7 days in RPMI complete medium.

Flow cytometry analysis of surface and intracellular antigens

Total cells were incubated with MoAbs for 30 min on ice and washed twice in PBS, cointaining 0.1% (w/v) NaN_3. After staining, the cells were fixed with 1% (w/v) paraformaldehyde (Sigma) in PBS for 30 min at room temperature before flow cytometric analysis. For intracellular staining, cells were stimulated with PMA (1µg/ml) plus ionomycin (0.5 µg/ml) for 4 hours. After 2 h Brefeldin A (10 µg/ml; Sigma) was added. After simulation, the cells were stained with cell surface antibodies, fixed in 4% paraformaldehyde, permeabilized with 0.1% saponin (Sigma), and then stained with antibodies to intracellular antigens. Three-colour flow cytometric analysis was performed using a FACSCalibur (Becton Dickinson). At least 50,000 cells (events) were acquired for each sample. LPMCs were expressed as percentage of cells within the lymphocytes gate. The acquired data were analyzed using the CellQuest software program (Becton Dickinson).

Th17 and T_{reg} quantification

To determine the percentage of regulatory T cells, PBMCs and LPMCs were stained using a FITC-labeled anti-CD4 and a PerCP-labeled anti-CD25 and then analyzed with a FACSCalibur. Results were expressed as the percent of $CD4^+CD25^{high}$ lymphocytes. As suggested by Cao et al. (11), T_{reg} cells were defined as those $CD4^+$ lymphocytes showing higher expression of CD25 than autologous $CD8^+$ activated *in vitro* with phytohemagglutinin. To further characterize the phenotype and function of regulatory T cells, the intracellular production of IL-10 was investigated in $CD4^+CD25^{high}$ cells from PB and LP of patients and controls.

IL-17-producing T cells were determined in LPMCs by CD4 and IL-17 intracellular staining, and subsequent analysis on a FACSCalibur flow cytometry.

Cell cultures and stimulation assays

LPMCs ($1x10^5$) were cultured in RPMI 1640 complete medium. Where indicated, 100 or 500 ng/ml IL-6 (R&D systems), or neutralizing anti-IL10 (10 µg/ml; R&D systems) were added to the cultures. For culture with Dynabeads CD3/CD28 T cell Expander (Invitrogen Dynal), LPMCs were cultured in complete medium alone or with various cytokines at a bead/cell ratio of 1:1 in U-bottomed 96-well plates. After 7 days, cells were collected and analyzed by flow cytometry.

$CD4^+CD127^-CD25^+$ and $CD4^+CD127^-CD25^-$ T cell isolation and proliferation assay

Freshly isolated LPMCs from patients with AS were separated into $CD4^+CD127^-CD25^+$ and $CD4^+CD127^-CD25^-$ by double selection, using a regulatory T cell isolation kit for magnetic separation (Miltenyi Biotech), according to the manufacturer's instruction. The resulted purity of T_{reg} cells was 90-95% as determined by FACS analysis. The proliferation assay was performed by incubating $CD4^+CD127^-CD25^-$ T cells (1×10^5) with or without allogenic $CD4^+CD127^-CD25^+$ T_{reg} cells in the presence of Dynabeads CD3/CD28 T cell Expander in complete medium. The cells were seeded at different T responder: T_{reg} ratios (1:1 and 1:0.5) in triplicates. After 3 days of culture, cells were harvested and stained using anti-CD25-PerCP and anti-Ki67-FITC antibodies to evaluate the percentage of proliferating cells. The data were acquired using an FACSCalibur and analysed by the CellQuest software program.

Statistical analysis

Student t test or the nonparametric Mann–Whitney test was used to calculate statistical significance between groups. Spearman's rank correlation was performed to correlate TGF-β and FOXP3 expression levels in both AS and CD patients. P values less than 0.05 were considered significant.

Results

Prevalence and histomorphological evaluation of subclinical intestinal inflammation in AS patients

Of the 18 patients with active AS (mean BASDAI 6.8) who underwent colonscopy, evidence of subclinical intestinal inflammation was observed in 14 (77%). In all cases, microscopic inflammatory lesions were only observed in the distal ileum. On the basis of gut histology (12) patients with AS were divided into two main subgroups, patients with normal histology or minor inflammation (acute lesions, 10 patients) and patients with chronic inflammation (8 patients).

IL-23 mRNA is over-expressed in the gut of AS patients without a clear Th17 polarization

Over-expression of IL-23 and TGF-β without the concomitant increase of other pro-inflammatory cytokines (such as IL-6 or IL-1β) and a clear Th17 polarization previously described in AS patients (4), was confirmed in the present study (data not shown), suggesting a role for TGF-β independent by Th17 response. Sub-analysis of AS patients showed no differences in IL-23 expression or Th17 polarization between patients who were taking or not NSAIDs. Patients with CD displayed a marked increase in IL-17A, IL-6, IL-1β and ROR-c expression compared with controls (data not shown), confirming the coexistence of IL-23 up-regulation and Th17 responses in intestinal inflammation in CD.

IL-2, IL-10, TGF-β, FOXP3 and STAT5 molecules mRNA expression is increased in ileal biopsies of AS patients

Given the strong up-regulation of TGF-β in AS patients without a concomitant Th17 polarization, we next investigated if an up-regulation of cytokines and transcription factors involved in T_{reg} immune responses, could occur in the inflamed ileum of AS patients. As

previously demonstrated (4), significant up-regulation of TGF-β was observed in AS patients with chronic ileal inflammation (4-fold increase compared to controls, $p<0.01$) and in CD patients (3-fold increase compared to controls, $p<0.05$) (data not shown). Over-expression of Foxp-3 (Fig.1A) was also observed in chronic inflamed AS and CD patients, but the correlation between TGF-β and Foxp3 was statistical significant only in AS patients ($R=0.9$, $p<0.001$) (Fig.2A-B), strongly suggesting that the high levels of TGF-β observed could act as a T_{reg} cytokine in the chronic inflamed ileum of AS.

We next evaluated IL-2 and STAT5 mRNA expression levels. As shown in Fig. 1B and C, significantly higher levels of IL-2 and STAT5 mRNA transcripts were detected in both chronic inflamed AS and CD patients, when compared to normal/acutely inflamed AS patients or normal controls, suggesting that the IL-2/STAT5 axis is functioning in AS and CD patients.

Finally we investigated IL-10 mRNA expression levels. To date, in fact, the best characterized regulatory mechanism in the intestine seems to be IL-10-dependent. When compared to HS and AS patients with acute inflammation or normal histology, IL-10 mRNA was significantly over-expressed in chronic inflamed AS patients and CD patients, (Fig. 1D) ($p<0.001$ and $p<0.01$ respectively), suggesting that an active T_{reg} response, sufficient to overcome intestinal inflammatory responses occurs in gut inflammation, apparently in AS but not in CD.

p-STAT5 expressing cells are increased in the chronic inflamed ileal specimens from AS patients

STAT5 signaling is important for FOXP3 expression and Treg cell function. Since that function of STAT molecules, is not reflected by transcript levels but by their phosphorylation status, we next investigated phospho-STAT5 protein expression in ileal

specimens from patients with AS, CD and controls. As shown in Figure 3, phospho-STAT5 expressing cells were rarely detected in the immune cell infiltrates in controls and AS patients with normal histology/acute inflammation (Figure 3 A, B, E), but were abundant in chronic inflamed AS and CD patients (Figure 3 C-E).

IL-10 producing $CD4^{+}CD25^{high}$ Treg cells are expanded in the gut of AS patients

Firstly we evaluated the percentage of IL-10+ cells in lymphoid B and T and myeloid cells. AS shown in Figure (4A) $CD4^{+}$ T cells were the main cellular sources of IL-10 in AS patients with chronic ileal inflammation. We next evaluated, by flow cytometry, the percentage of $CD4^{+}CD25^{high}$ T_{reg} cells occurring in LPMC of AS and CD patients and normal controls. Isolated cells were gated for $CD4^{+}$ T cells, and the percentage of $CD4^{+}CD25^{high}$ cells among $CD4^{+}$ cells was determined. Interestingly, LPMCs isolated from gut biopsy specimens of AS patients with chronic ileal inflammation showed a significantly higher frequency of $CD4^{+}CD25^{high}T_{reg}$ cells compared with non inflamed AS or CD patients and healthy controls (5±3%; 1.12±0.4%; 1.8±0.2%; 1.2%±0.4%; $p<0.001$) (Fig.4B). Considering the mRNA IL-10 expression levels increase in ileal biopsy from AS patients, we also evaluate IL-10 production by LP-derived T_{reg} cells. LPMCs were stained for CD4, CD25 and intracellular IL-10 after PMA stimulation. The percentage of IL-10-producing T_{reg} cells was significantly higher in LPMCs of AS patients with chronic intestinal inflammation and CD patients compared with non inflamed AS and healthy subjects (70±12%, 50±10%; $p<0.001$ and $p<0.05$ respectively; Fig 4C).

Frequency of circulating $CD4^{+}CD25^{+}$ T_{reg} in the peripheral blood of patients with AS

In order to determine whether an increased number of $CD4^{+}$,$CD25^{+}$ T_{reg} in the gut of AS patients reflects a systemic modulation of these cells in the peripheral circulation, we analyzed PBMC from patients with AS, CD and HS. As shown in Fig. 4D flow cytometric

analysis of T_{reg} cells showed significant peripheral expansion in AS (1.08%±0.4%) at levels similar to those observed in CD patients (1.05±0.3%), compared to HS (0.25±0.12; $p<0.05$), indicating that increase gut frequency of T_{reg} in AS might be not related to a local compartmentalization.

LP derived $CD4^+CD127^-CD25^{bright}$ Treg of AS patients have a direct suppressive effect on T cells

We next investigated the functional properties of $CD4^+$CD25high T_{reg} cells isolated from the ileum of 8 normal subjects and 8 AS patients by testing their ability to suppress the proliferative responses of allogenic PB $CD4^+CD25^-$ T responder cells (T_{resp}) of healthy subjects. Lamina propria $CD4^+CD127^-CD25^+$ cells were selected by Ab-coated magnetic microbeads, and their ability to suppress the proliferative responses of allogenic peripheral blood T_{resp} cells to polyclonal CD3/CD28 TCR stimulation was tested *in vitro*. Isolated $CD4^+CD127^-CD25^-$ T_{resp} cells were stimulated with anti-CD3/CD28 microbeads alone or in the presence of LP $CD4^+CD127^-CD25^+$ cells at different ratio. After 7 days $CD4^+$ T-cell proliferation was evaluated by flow cytometry. T_{reg} cells from AS inhibited T_{resp} proliferation by an average of 81±2.64% at a 1:1 ratio (Fig. 5A). At 0.5:1 ratio the percentage of inhibition was 56±4% (Fig. 5A). These data indicate that T_{reg} cells from LP of AS patients have a normal direct suppressive effect on the activation of T_{resp} cells isolated from normal individuals. T_{reg} cells from CD inhibited T_{resp} proliferation by an average of 77±4% at a 1:1 ratio (Fig. 5B). At 0.5:1 ratio the percentage of inhibition was 51±6% (Fig. 5B). T_{reg} cells from controls inhibited T_{resp} proliferation by an average of 78±5% at a 1:1 ratio (Fig. 5C). At 0.5:1 ratio the percentage of inhibition was 60±5.6% (Fig. 5C).

Block of IL-10 induces expansion of intestinal Th17 population in AS patients

In consideration of the high transcript levels of IL-10 observed in AS patients, we evaluated the effect of the block of this cytokine on intestinal Th17 population. Since the higher levels of TGF-β and IL-23 observed in the gut of AS patients we considered LPMC from AS patients pre-activated by these cytokines.

The frequency of IL-17-producing CD4+T cells among $CD4^+$ T lymphocytes derived from LPMC of AS patients was 1.2±0.35%. LPMC from AS patients were cultured in the presence of different concentrations of IL-6 alone (100 or 500 ng/ml), in the absence of exogenous IL-23 and TGF-β with or without a neutralizing mouse anti-human IL-10 antibody. As shown in Fig.6, addition of IL-6 at 100 ng/ml did not modify the frequency of Th17 cells, whereas at 500 ng/ml IL-6 was sufficient to overcome IL-10 block, inducing significant TH17 expansion. Inhibition of IL-10 induces a significant expansion of Th17 cells (5±0.25%; $p<0.05$) at level similar to those observed with highest concentration of IL-6. Additional effect on Th17 expansion was observed when highest concentration of IL-6 were used together with IL-10 block.

Discussion

Regulation of the immune intestinal homeostasis is now thought to be dependent upon the functions of $CD4^+CD25^+$ T_{reg} cells. T_{reg} play a fundamental role in the maintenance of intestinal hyporesponsiveness to luminal antigens through IL-10- and TGF-β-dependent mechanisms (13). In the presence of a breakdown of this tolerance, as a result of impaired T_{reg} function or increased proinflammatory stimuli, inflammation may occur. In inflammatory bowel diseases, for example, the frequency of $CD4^+CD25^{high}$ cells varies with disease activity, with active IBD being associated with a contraction of the peripheral blood T_{reg} pool and an only moderate expansion in intestinal lesions, numerically insufficient however to compensate local inflammation (14).

We have recently demonstrated that over expression of IL-23 and TGF-β, but not IL-17, is a pivotal feature of gut inflammation in ankylosing spondylitis (AS). TGF-β regulates both the differentiation of inflammatory Th17 cells and suppressive T_{reg} subsets, with the concomitant presence of proinflammatory cytokines favouring Th17 cell differentiation (5;15;16). On the basis of these findings we were tempted to speculate if the absence of a clear Th17 polarization, despite the high levels of IL-23 observed, could be due to a T_{reg}-mediated suppression in the gut of AS patients.

This is the first study to show that T_{reg}-related cytokines and transcription factors expression is markedly up-regulated at mRNA level in intestinal inflammation in patients with AS. IL-2 mRNA overexpression in CD and in AS patients was associated with up-regulation of STAT-5 (the main transducer of IL-2 signaling upon IL-2R ligation), FOXP3 and IL-10, indirectly suggesting that the IL-2/STAT5 axis is functional in AS and CD patients.

The interaction of interleukin-2 (IL-2) with the interleukin-2 receptor (IL-2R) is critically required to promote thymic and peripheral T_{reg} development, in part by up-regulation of FOXP3 and CD25 (17;18). IL-2-signaling via STAT5 has been demonstrated to constrain Th17 differentiation (19). Interestingly, although over-expression of Foxp-3 was observed in both AS and CD patients, a significant positive correlation between TGF-β and Foxp3 was found only in AS patients, strongly suggesting that the high levels of TGF-β observed in AS could function as a T_{reg} cytokine in the inflamed ileum. The different inflammatory milieu occurring in AS and CD ileum, despite the expression levels of TGF-β was similar in AS and CD patients, could justify a divergent immunomodulatory mechanism for TGF-β in AS and in CD, being pro-inflammatory in the latter. TGF-β is able to induce differentiation of naïve T cells into either Th17 or T_{reg} cells in a dose-dependent manner, with high levels of TGF-β inhibiting Th17 response (15). In particular it has been recently demonstrated that functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta-dependent mechanism (20). We however, cannot exclude that TGF-β produced by regulatory T cells themselves, could contribute to the increased expression levels observed in inflamed ileum. In this regard, however, the specific role of the dendritic cells of AS patients in inducing T_{reg} differentiation and the contribution of single cell types in TGF-β production need to be better investigated.

In this study, we have further expanded the understanding of the immunoregulatory properties of $CD4^{+}CD25^{high}$ T_{reg} cells by demonstrating an increased number of T_{reg} cells, with preserved immunosuppressant activity, together with over-expression of T_{reg}-related cytokines and transcription factor at m-RNA level in the inflamed ileum of AS patients. The increased prevalence of T_{reg} cells, together with the demonstration of the absence of a clear Th1 or Th17 polarization strongly indicates the importance of T_{reg} cells in the maintenance of intestinal homeostasis in an early stage of chronic gut inflammation.

The importance of either interleukin-10 or interleukin-10 receptor 2 have been demonstrated in both murine and human model of colitis (21-24). Cure of murine colitis by $CD4^{+}CD25^{high}$ T_{reg} cells has also been shown to be dependent on IL-10 with IL-10-producing $CD4^{+}CD25^{high}$ T_{reg} cells selectively enriched within the colonic lamina propria suggesting compartmentalization of the regulatory T cell response at effector sites (25).

The evidence of IL-2, IL-10 and STAT5 overexpression in mucosal biopsy specimens from patients with AS and to a lesser extent CD, observed in the present study, together with the observation that the 70 to 80% of LP T_{reg} cells are also IL-10 producing cells, suggests a key role of this cytokine in modulating intestinal immune response, insufficient to control inflammation in CD, but indispensable in AS. Furthermore, our demonstration of a significant Th17 expansion from isolated LPMC of AS patients, in the presence of IL-10 inhibition, strongly supports the key role of IL-10 in preventing the onset of a clear Th17 polarization.

We don't know actually the exact mechanism by which a breakdown in immune regulatory networks, leads to chronic inflammatory diseases in the intestine of AS patients. We hypothesize that in genetically prone subjects an unknown infective stimuli could induce the production of pro-inflammatory cytokines, such as IL-6 or IL-1, down-regulating T_{reg} response with the occurrence of active differentiation of pathogenic Th17 cells. In this context the high levels of IL-23 occurring in the gut of AS patients, could sustain the Th17 commitment, leading to the development of gut inflammation.

In summary, this is the first study to demonstrate that T_{reg} cells are activated in the terminal ileum in AS patients. This response is probably responsible for the absence of a clear Th17 polarization despite the high levels of IL-23 observed in AS intestine. Up-regulation of IL-2, STAT-5 and IL-10 confirms a key role of IL-10 in the maintenance of gut immune

homeostasis strongly suggesting the potential of IL-10 pathway as a strategy to restore intestinal homeostasis in human intestinal inflammation.

Legend to Figures

Figure 1. T_{reg}-related genes are over-expressed in mucosal biopsy specimens from patients with AS. Relative mRNA quantification of TGFβ (A), FOXP3 (B), IL-2 (C), STAT5 (D) and IL-10 (E), was assessed by TaqMan real-time polymerase chain reaction in ileal biopsy specimens obtained from 18 patients with Crohn's disease (CD), 15 patients with Ankylosing Spondylitis (AS) (further divided in normal/acute vs chronic inflammation), and 15 healthy controls (HS). Bars show the mean and SEM. NS=not significant.

Figure 2. Correlation plots of TGF-β against FOXP3 in AS (A) and CD patients (B). The Spearman's rank correlation showed a significant correlation between TGF-β and Foxp3 in AS (R=0.9, p<0.001) but not in CD (R=0.19, p=0.438) patients.

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Figure 5. LP $CD4^{+}CD127^{-}CD25^{+}$ T cells from AS patients suppress the proliferation of allogenic PB $CD4^{+}CD25^{-}$ Tcells in vitro. Isolated allogenic PB $CD4^{+}CD25^{-}$ T responder cells of healthy controls were stimulated with anti-CD3/CD28 microbeads alone or in the presence of LP $CD4^{+}CD127^{-}CD25^{+}$ cells of AS and CD patients and controls at different ratio as indicated. After 5 days, $CD4^{+}$ T-cell proliferation was evaluated by flow cytometry. Results are representative are representative of five independent experiments performed by using isolated LPMC from 3 AS (A), 3 CD (B) patients and 3 controls (C) and PBMC from 3 controls. Bars indicate average proliferation ± SEM of responder (R) cells cultured alone or in the presence of T_{regs} (S) at 0.5:1 and 1:1 ratio. *Indicates a $p<0.05$.

Figure 6. Block of IL-10 induces expansion of intestinal Th17 population in AS patients. LPMC isolated from AS patients were considered pre-activated by TGF-β and IL-23 and cultured in the presence of IL-6 at concentration of 100 and 500 ng/ml, with or without a neutralizing mouse anti-human IL-10 antibody. Results are representative of six independent experiments. Addition of IL-6 at 100 ng/ml did not modify the frequency of

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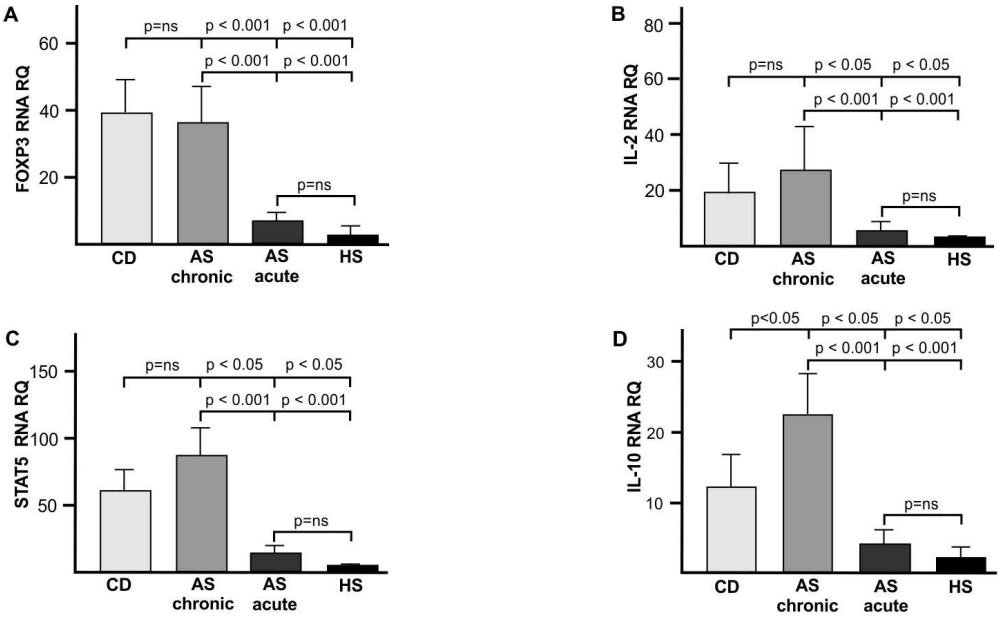

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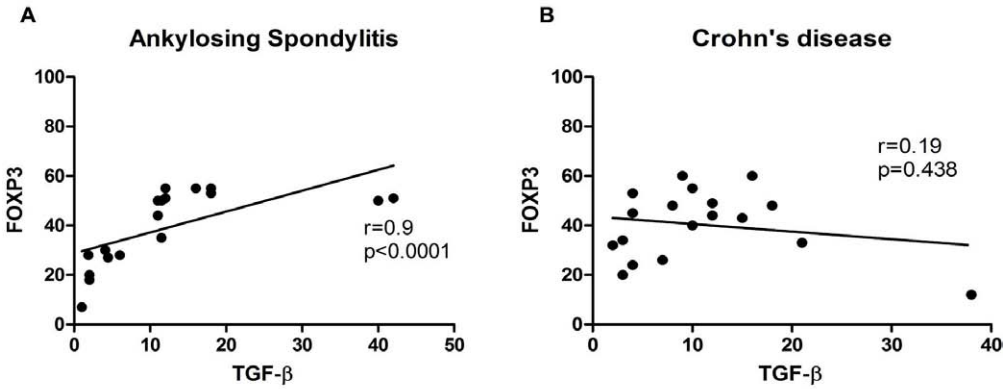

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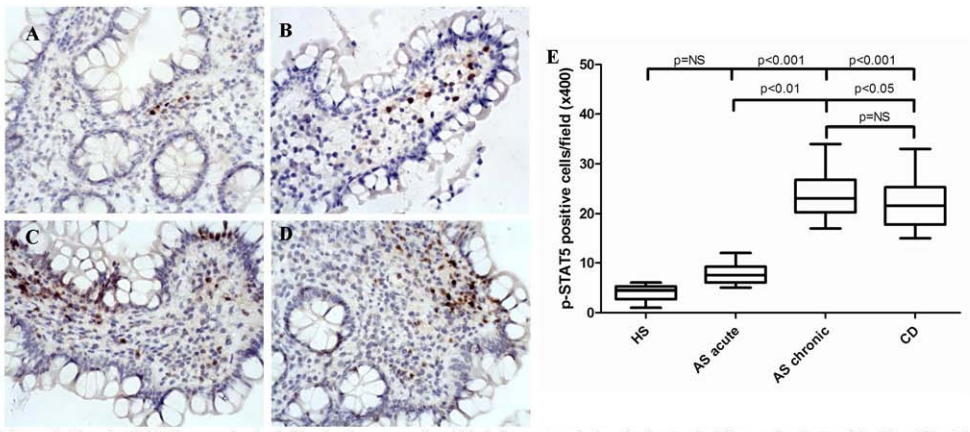

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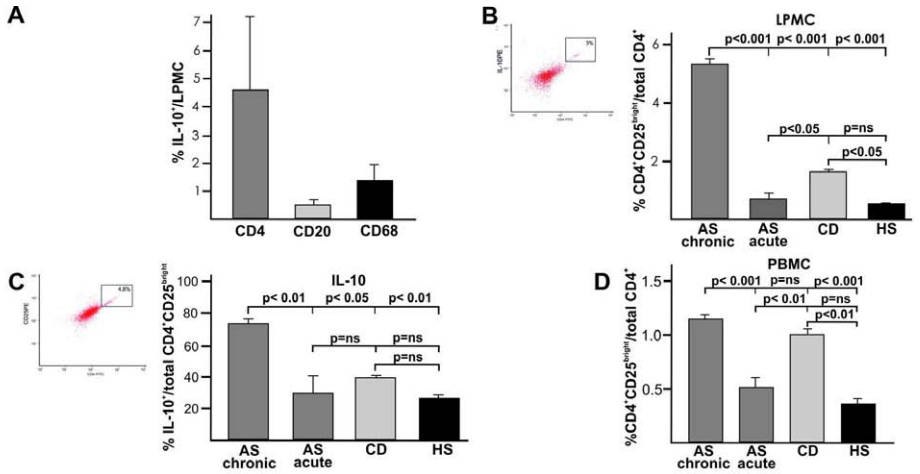

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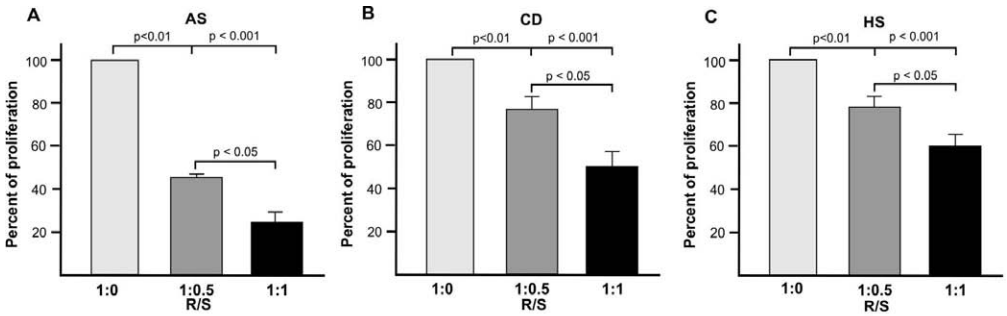

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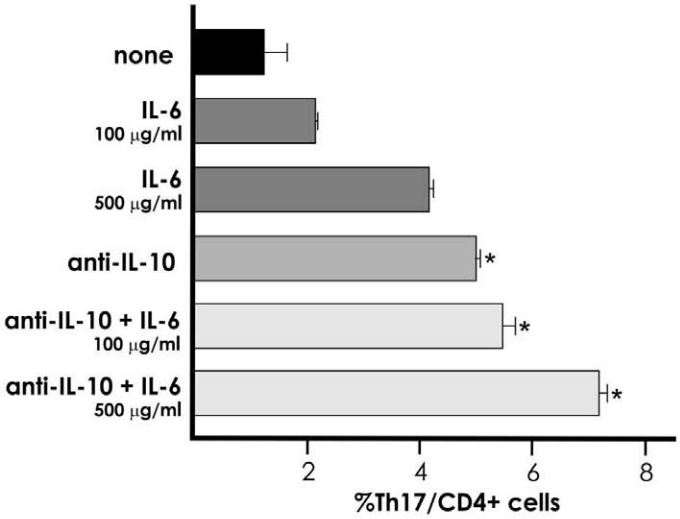

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