Expansion of Intestinal CD4+CD25^{high} Treg Cells in Patients With Ankylosing Spondylitis

A Putative Role for Interleukin-10 in Preventing Intestinal Th17 Response

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Objective. **Subclinical gut inflammation has been demonstrated in patients with ankylosing spondylitis (AS). This study was undertaken to determine the frequency of regulatory CD4+CD25^{high} T cells (Treg cells) and to evaluate Treg cell–related cytokines (interleukin-2 [IL-2], transforming growth factor β [TGFβ], and IL-10) and transcription factors (FoxP3 and STAT-5) in the ileum of patients with AS.**

Methods. **Quantitative gene expression analysis, by reverse transcriptase–polymerase chain reaction, of Treg-related cytokines (IL-2, TGFβ, and IL-10) and transcription factors (STAT-5 and FoxP3) was performed on ileal biopsy specimens from 18 patients with AS, 15 patients with active Crohn's disease (CD), and 15**

healthy subjects. Tissue and circulating Treg cells were also analyzed by flow cytometry.

Results. **A significant up-regulation of IL-2, TGFβ, FoxP3, STAT-5, and IL-10 transcripts in the terminal ileum of AS patients displaying chronic ileal inflammation was observed. Flow cytometric analysis of Treg cells showed significant peripheral expansion in both patients with AS and chronic inflammation and patients with CD (mean ± SD 1.08 ± 0.4% and 1.05 ± 0.3%, respectively) as compared with healthy subjects (0.25 ± 0.12%) ($P < 0.05$). Interestingly, a 5-fold increase in the proportion of Treg cells was observed in the gut of patients with AS (5 ± 3%) as compared with healthy subjects (1.2 ± 0.4%) ($P < 0.001$), with 70–80% of these cells also producing IL-10. In vitro studies showed that blocking IL-10 was sufficient to induce Th17 polarization on lamina propria mononuclear cells isolated from AS patients.**

Conclusion. **Our findings provide the first evidence that an active Treg cell response, mainly dominated by IL-10 production, occurs in the gut of AS patients and is probably responsible for the absence of a clear Th17 polarization in the ileum of AS patients.**

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Intestinal inflammation is a shared feature of ankylosing spondylitis (AS) and Crohn's disease (CD). Shared immunologic and genetic similarities, in both clinical and preclinical stages, and the observation that 7% of patients with AS and initial chronic gut inflammation develop overt CD strongly support the hypothesis that AS patients with more pronounced gut inflammation could be considered to have preclinical CD (1–3).

We have recently demonstrated that overexpres-

sion of interleukin-23 (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 transforming growth factor β (TGFβ), IL-1β, and IL-6, has been demonstrated to be involved in the differentiation of IL-17–producing CD4+ T cells, namely Th17 lymphocytes, that are highly pro-inflammatory and induce the severe autoimmunity (5). The participation of TGFβ in the differentiation of Th17 cells places them in close relationship with CD4+CD25^{high} Treg cells, since TGFβ, among its numerous properties, is able to induce the expression of FoxP3 in naive antigen-stimulated T cells in the peripheral immune compartment, leading to cells with regulatory or suppressor functions (6).

Given the high levels of TGFβ transcripts we observed in the gut of AS patients without concomitant overexpression of IL-1β and IL-6, we aimed to investigate whether the absence of a clear Th17 polarization, despite the high levels of IL-23 observed, was related to a Treg cell–mediated suppression in the gut of AS patients. Herein, we provide the first evidence that levels of CD4+CD25^{high} T cells are increased in the gut and in the peripheral blood of AS patients, with a concomitant significant intestinal increase in the levels of Treg cell–related transcripts (STAT-5, TGFβ, and IL-10), suggesting that an active Treg cell response, mainly dominated by IL-10 production, occurs in AS. Thus, we propose that the Treg cell response observed in the gut of AS patients, which is keeping autoreactive 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

Patients. Gut specimens and peripheral blood samples from patients with CD, patients with AS, and control subjects were consecutively obtained. The AS group consisted of 18 consecutive patients (11 men and 7 women), ages 19–48 years, who had no clinical symptoms of bowel inflammation. Patients were diagnosed according to the modified New York criteria (7), and all of the patients were HLA–B27 positive. Disease activity was evaluated using the Bath AS Disease Activity Index (BASDAI) (8), with active disease defined as a BASDAI score >4. The mean $\pm$ SD BASDAI score at the time mucosal biopsy specimens were obtained was 6.8 $\pm$ 2.5. Mean $\pm$ SD disease duration since diagnosis was 4 $\pm$ 2 months. At the time of sample collection, 11 of the 18 AS patients were taking nonsteroidal antiinflammatory drugs (NSAIDs).

The CD group consisted of 15 patients (9 men and 6 women), ages 20–50 years. Patients with pure colonic involvement were excluded from the study. Disease activity in each patient with CD was analyzed using the CD Activity Index (9)

and endoscopic and histopathologic data. Mean $\pm$ SD disease duration since diagnosis was 5 $\pm$ 1.5 months. At the time of sample collection, none of the patients in the CD group were receiving corticosteroids or immunosuppressants. The mean $\pm$ SD CD Activity Index score was 320 $\pm$ 70 at the time of enrollment.

The control group consisted of 15 normal subjects (12 men and 3 women), ages 41–68 years, who were undergoing ileocolonoscopy for routine evaluation. Specimens from 10 of the AS patients, 7 of the CD patients, and 7 controls in which sufficient messenger RNA (mRNA) for new experiments was available came from a previous study (4). Paired specimens for histologic analysis, quantitative polymerase chain reaction (PCR), and lamina propria mononuclear cell isolation for flow cytometric analysis and functional assays were obtained from new consecutively enrolled patients and controls (8 patients with AS, 8 patients with CD, and 8 controls). Collection of ileal biopsy specimens was approved by the ethics committee and the institutional review board of the University of Palermo.

Histomorphologic grading. Tissue samples were processed as previously described (4) and divided into 2 main subgroups according to histologic features: samples with normal gut histologic features or minor inflammatory changes (acute lesions) and samples with chronic inflammation.

Quantitative TaqMan reverse transcriptase–PCR (RT-PCR) for Treg cytokines, FoxP3, and STAT-5 molecules in ileal biopsy specimens. Total RNA was extracted using the Qiagen RNeasy Mini kit, 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). 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), IL-6 (Hs00174131_m1), IL-10 (Hs00174086_m1), IL-17A (Hs00174383_m1), TGFβ1 (Hs00171257_m1), IL-23p19 (Hs00372324_m1), retinoid–related orphan receptor C (RORC) (Hs01076112_m1), FoxP3 (Hs01085835_m1), and STAT-5A (Hs00559647_m1) were obtained from Applied Biosystems. Samples were run in triplicate at 20 ng of cDNA 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 C_t method.

Immunohistochemistry for phospho–STAT-5. Immunohistochemistry for phospho–STAT-5 was performed on 3-μm–thick paraffin-embedded sections of intestinal biopsy specimens obtained from patients and controls as previously described (4). Briefly, following rehydration, antigen was unmasked using the Dako Target retrieval solution (pH 6) for 45 minutes at 95°C. Endogenous peroxidase was blocked for 10 minutes with Dako Peroxidase blocking reagent, and nonspecific bindings were blocked for 20 minutes with Dako Protein Block. The primary antibody, phosphorylated rabbit monoclonal anti-human STAT-5 (Tyr^{694}) (IgG; Cell Signaling) was added (at a 1:300 dilution in Dako diluent) and incubated for 1 hour at room temperature. An isotype-matched irrelevant antibody was used as a negative control. Slides were then incubated for 30 minutes with peroxidase-conjugated Dako EnVision polymer, peroxidase activity was visualized using diaminobenzidine-positive chromogen (Dako), and slides were lightly counterstained with hematoxylin before dehydration and mounting in DePex (VWR International). The number of

phospho–STAT-5–expressing cells was determined by counting the immunoreactive cells on photomicrographs obtained from 3 random high-power microscopic fields (400× magnification) under a Leica DM2000 optical microscope using a Leica DFC320 digital camera. Results are reported as the mean ± SD.

Culture media, reagents, and antibodies. In all in vitro assays, cells were cultured in very low endotoxin medium RPMI 1640 (Sigma), 10 mM HEPES (EuroClone), 10% fetal bovine serum (EuroClone), 100 units/ml of penicillin/streptomycin, and 0.05 mM 2-mercaptoethanol (Sigma). Phosphate buffered saline (PBS) was obtained from EuroClone. Purified recombinant human IL-6 (R&D Systems), phorbol myristate acetate (PMA), and ionomycin (Sigma-Aldrich) were used for in vitro culture assays.

The following antibodies were used for flow cytometric analysis: fluorescein isothiocyanate (FITC)–conjugated anti-CD4, FITC-conjugated anti-CD68, FITC-conjugated anti-CD20, phycoerythrin (PE)–conjugated anti–IL-10, PE-conjugated IgG isotype control monoclonal antibodies (all from Becton Dickinson), allophycocyanin-conjugated anti–IL-17 (Santa Cruz Biotechnology), and FITC-conjugated anti–Ki-67 (Biocompare).

Isolation of lamina propria mononuclear cells and peripheral blood mononuclear cells (PBMCs). Lamina propria mononuclear cells were isolated from 8 gut biopsy specimens from patients with AS, patients with CD, and healthy controls as described by Van Damme et al (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 1 mM EDTA and stirred for another 60 minutes at 37°C to remove intraepithelial cells. Subsequently, lamina propria mononuclear cells were obtained by cutting the biopsy into fragments of ~1–5 mm^3, which were then incubated for 3 hours at 37°C in type IV collagenase (25 units/ml; Sigma). No further purification of the cell populations was performed, in order 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 analyzed by fluorescence-activated cell sorting (FACS) 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 analyzed by flow cytometry. In some experiments, cells were stimulated with cytokines in the 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 from all patients and controls by density-gradient centrifugation (Histopaque; Sigma). Cell viability (trypan blue dye exclusion) was always >95%. Fresh cell suspension was either immediately analyzed by FACS analysis or cultured for 7 days in RPMI complete medium.

Flow cytometric analysis of surface and intracellular antigens. Total cells were incubated with monoclonal antibodies for 30 minutes on ice and washed twice in PBS containing 0.1% (weight/volume) NaN$_3$. After staining, the cells were fixed with 1% (w/v) paraformaldehyde (Sigma) in PBS for 30 minutes at room temperature before flow cytometric analysis. For intracellular staining, cells were stimulated with PMA (1 μg/ml) plus ionomycin (0.5 μg/ml) for 4 hours. After 2 hours, 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-color flow cytometry was performed using a FACSCalibur instrument (Becton Dickinson). At least 50,000 cells (events) were acquired for each sample. Lamina propria mononuclear cells were expressed as the percentage of cells within the lymphocyte gate. The acquired data were analyzed using the CellQuest software program (Becton Dickinson).

Th17 and Treg quantification. To determine the percentage of Treg cells, PBMCs and lamina propria mononuclear cells were stained using FITC-labeled anti-CD4 and peridinin chlorophyll A protein (PerCP)–labeled anti-CD25 and then analyzed using a FACSCalibur instrument. Results were expressed as the percentage of CD4+CD25^{high} lymphocytes. As suggested by Cao et al (11), Treg cells were defined as CD4+ lymphocytes that showed higher expression of CD25 than autologous CD8+ cells and were activated in vitro with phytohemagglutinin. To further characterize the phenotype and function of Treg cells, the intracellular production of IL-10 was investigated in CD4+CD25^{high} cells from the peripheral blood and lamina propria of patients and controls. IL-17–producing T cells were determined in lamina propria mononuclear cells by CD4 and IL-17 intracellular staining and subsequent flow cytometric analysis using a FACSCalibur instrument.

Cell cultures and stimulation assays. Lamina propria mononuclear cells (1 × 10^5) were cultured in RPMI 1640 complete medium. Where indicated, 100 or 500 ng/ml of IL-6 (R&D Systems) or 10 μg/ml of neutralizing anti–IL-10 (R&D Systems) was added to the cultures. For culture with Dynabeads CD3/CD28 T cell Expander (Invitrogen Dynal), lamina propria mononuclear cells 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 lamina propria mononuclear cells from patients with AS were separated into CD4+CD127−CD25+ and CD4+CD127−CD25− cells by double selection, using a Treg cell isolation kit for magnetic separation, according to the recommendations of the manufacturer (Miltenyi Biotec). The resulting purity of Treg 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 allogeneic CD4+CD127−CD25+ Treg cells in the presence of Dynabeads CD3/CD28 T cell Expander in complete medium. The cells were seeded at different T responder cell:Treg cell ratios (1:1 and 1:0.5) in triplicate. After 3 days of culture, cells were harvested and stained using PerCP-conjugated anti-CD25 and FITC-conjugated anti–Ki-67 antibodies to evaluate the percentage of proliferating cells. The data were acquired using a FACSCalibur instrument and analyzed by the CellQuest software program.

Statistical analysis. Student's t-test or the nonparametric Mann-Whitney test was used to calculate the statistical significance between groups. Spearman's rank correlation was performed to correlate TGFβ and FoxP3 expression levels in

both AS and CD patients. *P* values less than 0.05 were considered significant.

RESULTS

Prevalence and histomorphologic evaluation of subclinical intestinal inflammation in AS patients. Of the 18 patients with active AS (mean BASDAI 6.8) who underwent colonoscopy, 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 histologic features (12), patients with AS were divided into 2 main subgroups: those with normal histologic features or minor inflammation (acute lesions) (10 patients) and those with chronic inflammation (8 patients).

Overexpression of IL-23 mRNA in the gut of AS patients without a clear Th17 polarization. Overexpression of IL-23 and TGFβ, without a concomitant increase in other proinflammatory 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 TGFβ independent of the Th17 response. Subanalysis of AS patients showed no differences in IL-23 expression or Th17 polarization between patients who were taking NSAIDs and those who were not taking NSAIDs. Patients with CD displayed a marked increase in IL-17A, IL-6, IL-1β, and RORC expression as compared with controls (data not shown), confirming the coexistence of IL-23 up-regulation and Th17 responses in intestinal inflammation in CD.
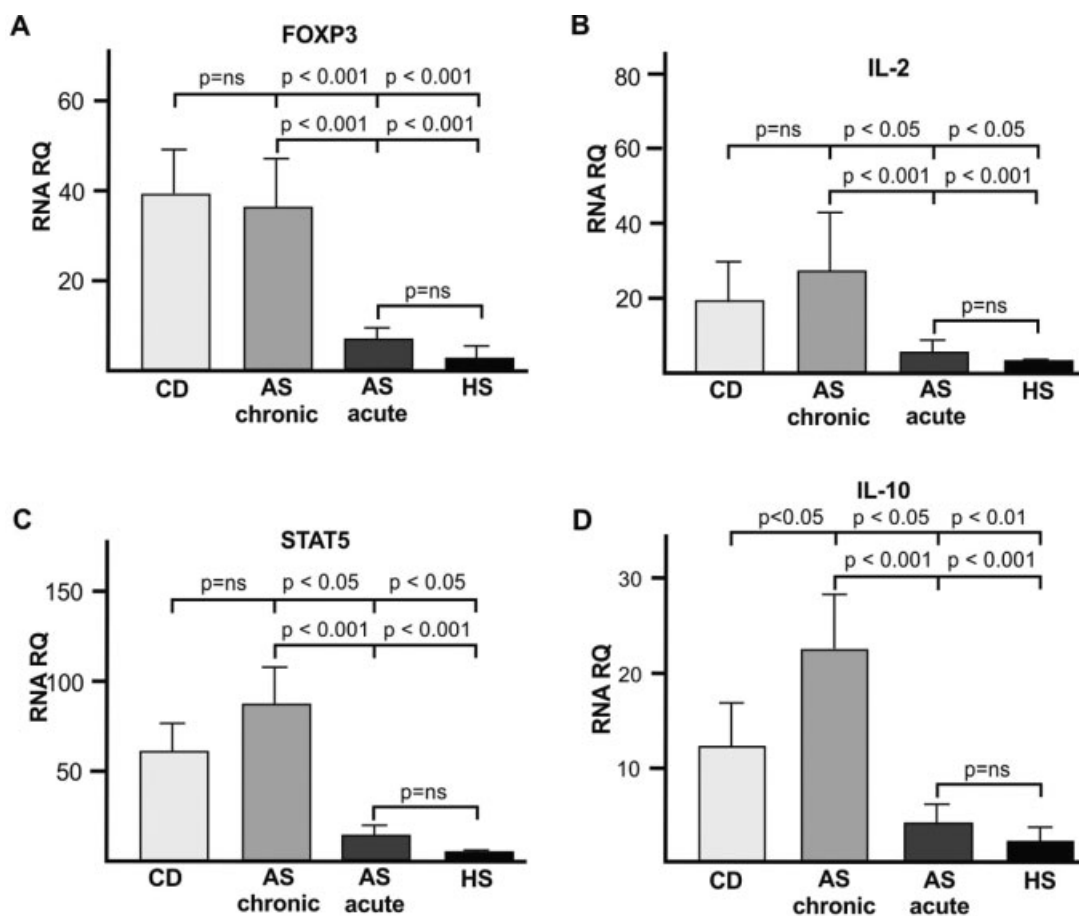

Figure 1. Overexpression of Treg cell–related genes in mucosal biopsy specimens from patients with ankylosing spondylitis (AS). Relative quantification (RQ) of mRNA for FoxP3 (**A**), interleukin-2 (IL-2) (**B**), STAT-5, (**C**), and IL-10 (**D**) was assessed by TaqMan real-time polymerase chain reaction in ileal biopsy specimens obtained from 18 patients with Crohn's disease (CD), 15 patients with AS (further divided into patients with no inflammation or acute inflammation versus patients with chronic inflammation), and 15 healthy subjects (HS). Bars show the mean and SD. NS = not significant.
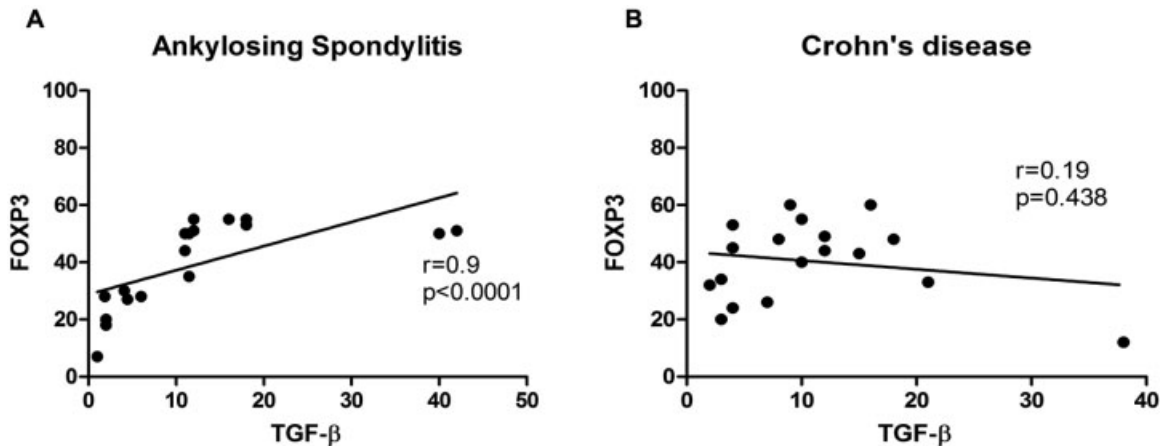
Figure 2. Correlation of levels of transforming growth factor β (TGFβ) with FoxP3 in patients with AS (**A**) and patients with CD (**B**). There was a significant correlation between TGFβ and FoxP3 levels in AS patients, but not in CD patients, by Spearman's rank correlation test. See Figure 1 for other definitions.

Increased expression of mRNA for IL-2, IL-10, TGFβ, FoxP3, and STAT-5 in ileal biopsy specimens from AS patients. Given the strong up-regulation of TGFβ in AS patients without a concomitant Th17 polarization, we next investigated whether an up-regulation of cytokines and transcription factors involved in Treg cell immune responses could occur in the inflamed ileum of AS patients. As previously demonstrated (4), significant up-regulation of TGFβ was observed in AS patients with chronic ileal inflammation (4-fold increase compared with controls; $P < 0.01$) and in CD patients (3-fold increase compared with controls; $P < 0.05$) (data not shown). Overexpression of FoxP3 (Figure 1A) was also observed in patients with AS and
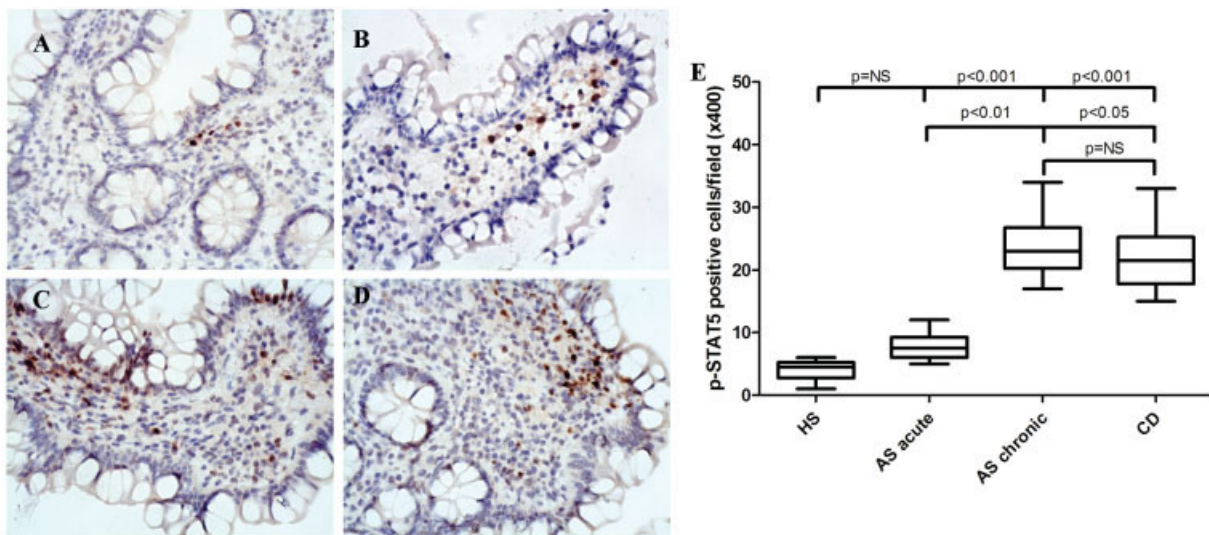

Figure 3. Phospho–STAT-5 expression by infiltrating immune cells within inflammatory lesions in the terminal ileum of patients with AS or CD. **A–D**, Representative photomicrographs showing 3-μm–thick paraffin-embedded sections of distal ileal biopsy specimens obtained from healthy subjects (**A**), patients with AS and normal histologic features or acute inflammation (**B**), patients with AS and chronic inflammation (**C**), and patients with CD (**D**), stained for phospho–STAT-5. Abundant phospho–STAT-5 expression was observed in a large number of mononuclear cells infiltrating the intestinal mucosa of samples from patients with AS and chronic inflammation and from patients with CD, but not in samples from normal subjects or patients with AS displaying normal histologic features or acute inflammation (original magnification $\times$ 400). **E**, Number of phospho–STAT-5–positive cells in the mucosa. Results are expressed as the number of positive cells per field. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. See Figure 1 for definitions.

chronic inflammation and patients with CD, but the correlation between TGFβ and FoxP3 was statistically significant only in AS patients (r = 0.9, P < 0.0001) (Figures 2A and B), strongly suggesting that the high levels of TGFβ observed could act as a Treg cytokine in the chronic inflamed ileum in AS.

We next evaluated IL-2 and STAT-5 mRNA expression. As shown in Figures 1B and C, significantly higher levels of IL-2 and STAT-5 mRNA transcripts were detected in both AS patients with chronic inflammation and CD patients when compared with patients with AS without inflammation, patients with AS with acute inflammation, or normal controls, suggesting that the IL-2/STAT-5 axis is functioning in AS and CD patients.

Finally, we investigated IL-10 mRNA expression. In fact, the regulatory mechanism in the intestine that has been best characterized to date seems to be IL-10 dependent. When compared with healthy subjects and patients with AS and acute inflammation or normal histologic features, IL-10 mRNA was significantly over-expressed in patients with AS and chronic inflammation and in patients with CD (Figure 1D) (P < 0.001 for healthy subjects versus patients with AS and chronic inflammation and P < 0.01 for healthy subjects versus patients with CD), suggesting that an active Treg cell response sufficient to overcome intestinal inflammatory responses apparently occurs in gut inflammation in AS but not in CD.

Increase in phospho–STAT-5–expressing cells in ileal specimens from patients with AS and chronic inflammation. STAT-5 signaling is important for FoxP3 expression and Treg cell function. Since this function of STAT molecules is not reflected by transcript levels but by their phosphorylation status, we next investigated phospho–STAT-5 protein expression in ileal specimens from patients with AS, patients with CD, and controls. As shown in Figure 3, phospho–STAT-5–expressing cells were rarely detected in the immune cell infiltrates in controls and AS patients with normal histologic features or acute inflammation (Figures 3A, B, and E) but were abundant in patients with AS with chronic inflammation and patients with CD (Figures 3C–E).

Expansion of IL-10–producing CD4+CD25^{high} Treg cells in the gut of AS patients. First, we evaluated the percentage of IL-10+ cells in lymphoid B and T cells and myeloid cells. As shown in Figure 4A, CD4+ T cells were the main cellular sources of IL-10 in patients with AS and chronic ileal inflammation. We next evaluated by flow cytometric analysis the percentage of CD4+CD25^{high} Treg cells occurring in lamina propria mononuclear cells in patients with AS, patients with CD, 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, lamina propria mononuclear cells that were isolated from gut biopsy specimens from AS patients with chronic ileal inflammation showed a significantly higher frequency of CD4+CD25^{high} Treg cells compared with patients with AS without inflammation, patients with CD, and healthy controls (mean $\pm$ SD 5 $\pm$ 3%; 1.12 $\pm$ 0.4%; 1.8 $\pm$ 0.2%; and 1.2 $\pm$ 0.4%, respectively; P < 0.001 for AS with chronic inflammation versus each of the other groups) (Figure 4B).

Considering the increase in IL-10 mRNA expression in ileal biopsy specimens from AS patients, we also evaluated IL-10 production by lamina propria–derived Treg cells. Lamina propria mononuclear cells were stained for CD4, CD25, and intracellular IL-10 after PMA stimulation. The percentage of IL-10–producing Treg cells was significantly higher in lamina propria mononuclear cells from AS patients with chronic intestinal inflammation (mean $\pm$ SD 70 $\pm$ 12%) than in patients with AS without inflammation, patients with CD, and healthy subjects (P < 0.01, P < 0.05, and P < 0.01, respectively) (Figure 4C).

Frequency of circulating CD4+CD25+ Treg cells in the peripheral blood of patients with AS. In order to determine whether an increased number of CD4+CD25+ Treg cells in the gut of AS patients reflects a systemic modulation of these cells in the peripheral circulation, we analyzed PBMCs from patients with AS, patients with CD, and healthy subjects. As shown in Figure 4D, flow cytometric analysis of Treg cells showed significant peripheral expansion in AS (mean $\pm$ SD 1.08 $\pm$ 0.4%) at levels similar to those observed in CD patients (1.05 $\pm$ 0.3%) as compared with healthy subjects (0.25 $\pm$ 0.12; P < 0.05), indicating that increased gut frequency of Treg in AS might not be related to a local compartmentalization.

Lamina propria–derived CD4+CD127−CD25^{high} Treg cells from AS patients have a direct suppressive effect on T cells. We next investigated the functional properties of CD4+CD25^{high} Treg cells isolated from the ileum of 8 normal subjects and 8 AS patients by testing their ability to suppress the proliferative responses of allogeneic peripheral blood CD4+CD25− T responder cells from healthy subjects. Lamina propria CD4+CD127−CD25+ cells were selected by antibody-coated magnetic microbeads, and their ability to suppress the proliferative responses of allogeneic peripheral blood T responder cells to polyclonal CD3/CD28 T cell receptor stimulation was tested in vitro. Isolated CD4+CD127−CD25− T responder cells were stimulated with anti-CD3/CD28 microbeads alone or in the
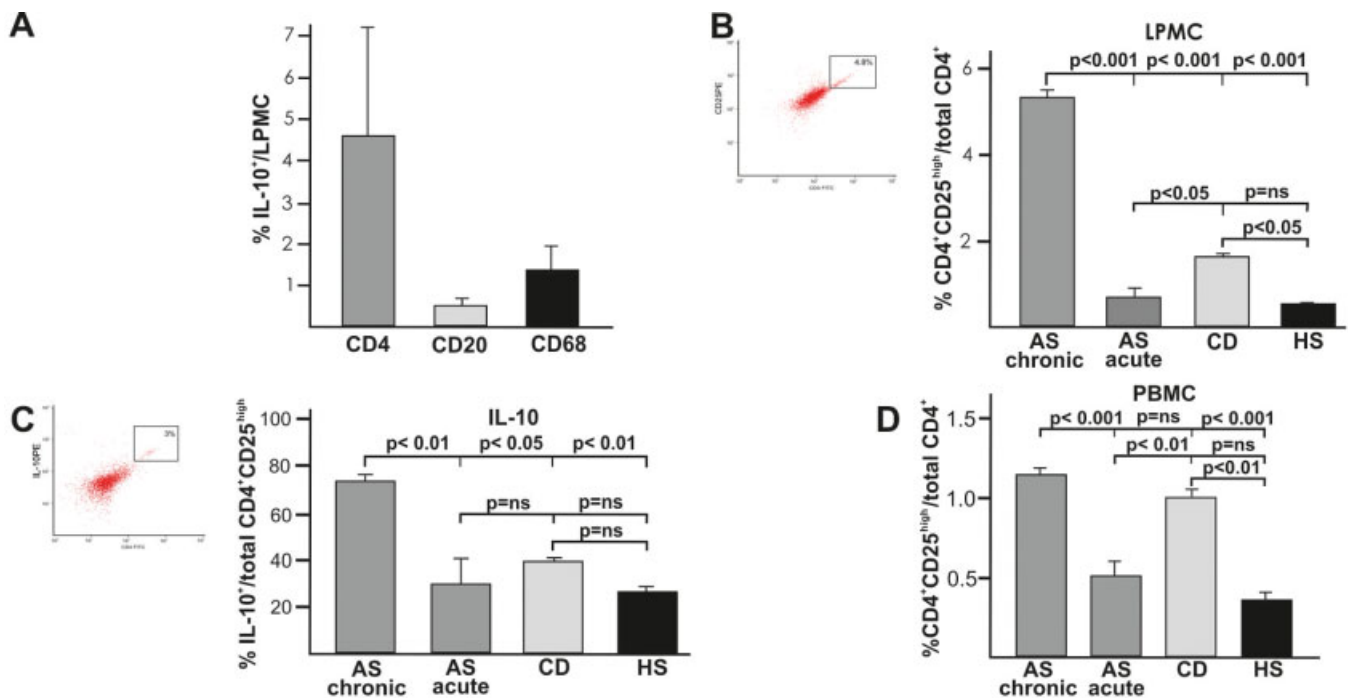
Figure 4. Increased frequency of CD4+CD25^{high} T cells in isolated lamina propria mononuclear cells (LPMCs) and peripheral blood mononuclear cells (PBMCs) from patients with AS. Freshly isolated lamina propria mononuclear cells and PBMCs from 8 patients with AS, 8 patients with CD, and 8 healthy subjects were stained with fluorescein isothiocyanate (FITC)–conjugated CD68, FITC-conjugated CD20, FITC-conjugated CD4, phycoerythrin (PE)–conjugated CD25, or PE-conjugated IL-10 and analyzed by flow cytometry. **A**, Percentages of IL-10–positive T and B lymphocytes and myeloid cells among isolated lamina propria mononuclear cells from AS patients. **B**, Representative dot plot of CD4 versus CD25 among isolated lamina propria mononuclear cells from AS patients and percentages of CD4+CD25^{high} cells among lamina propria CD4+ T cells isolated from patients and controls. **C**, Representative dot plot of CD4 versus IL-10 among lamina propria mononuclear cells from AS patients and percentages of IL-10–positive cells among lamina propria CD4+ cells isolated from patients and controls. **D**, Percentages of CD4+CD25^{high} cells among peripheral blood CD4+ T cells isolated from patients and controls. Bars show the mean and SD. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

presence of lamina propria CD4+CD127−CD25+ cells at different ratios. After 7 days, CD4+ T cell proliferation was evaluated by flow cytometry. Treg cells from patients with AS inhibited T responder cell proliferation by a mean ± SD of 81 ± 2.64% at a 1:1 ratio (Figure 5A). At a ratio of 0.5 Treg cells to 1 T responder cell, the percentage of inhibition was 56 ± 4% (Figure 5A). These data indicate that Treg cells from lamina propria of AS patients have a normal direct suppressive effect on the activation of T responder cells isolated from normal individuals. Treg cells from patients with CD inhibited T responder cell proliferation by an average of 77 ± 4% at a 1:1 ratio (Figure 5B). At a 0.5:1 ratio, the percentage of inhibition was 51 ± 6% (Figure 5B). Treg cells from controls inhibited T responder cell proliferation by an average of 78 ± 5% at a 1:1 ratio (Figure 5C). At a 0.5:1 ratio the percentage of inhibition was 60 ± 5.6% (Figure 5C).

Expansion of the intestinal Th17 population after blockade of IL-10 in AS patients. Given the high transcript levels of IL-10 observed in AS patients, we evaluated the effect of blocking this cytokine on the intestinal Th17 population. Since the higher levels of TGFβ and IL-23 were observed in the gut of AS patients, we considered lamina propria mononuclear cells from AS patients that were preactivated by these cytokines.

The mean ± SD frequency of IL-17–producing CD4+ T cells among CD4+ T lymphocytes derived from lamina propria mononuclear cells from AS patients was 1.2 ± 0.35%. Lamina propria mononuclear cells 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 TGFβ with or without a neutralizing mouse anti-human IL-10 antibody. As shown in Figure 6, addition of IL-6 at 100
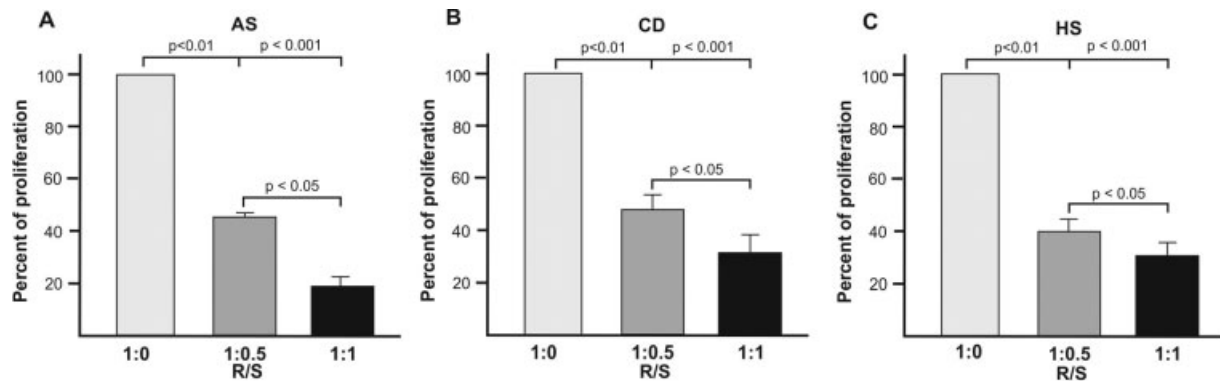
Figure 5. Lamina propria CD4+CD127−CD25+ T cells from patients with AS suppress the proliferation of allogeneic peripheral blood CD4+CD25− T cells in vitro. Isolated allogeneic peripheral blood CD4+CD25− T responder cells from healthy controls were stimulated with anti-CD3/CD28 microbeads alone or in the presence of lamina propria CD4+CD127−CD25+ cells from patients with AS, patients with CD, and controls at the indicated ratios. After 5 days, CD4+ T cell proliferation was evaluated by flow cytometry. Representative results of 5 independent experiments performed using isolated lamina propria mononuclear cells from 3 patients with AS (**A**), 3 patients with CD (**B**), and 3 controls (**C**) and peripheral blood mononuclear cells from 3 controls are shown. Bars show the mean and SD proliferation of responder (R) cells cultured alone or in the presence of Treg cells (S) at 1:0.5 and 1:1 ratios. See Figure 1 for other definitions.

ng/ml did not modify the frequency of Th17 cells, whereas at 500 ng/ml IL-6 was sufficient to overcome IL-10 blockade, inducing significant Th17 expansion. Inhibition of IL-10 induced a significant expansion of

Figure 6. Blocking of IL-10 induces expansion of the intestinal Th17 population in patients with AS. Lamina propria mononuclear cells isolated from patients with AS were considered preactivated by transforming growth factor β and IL-23 and cultured in the presence of IL-6 at concentrations of 100 and 500 ng/ml, with or without a neutralizing mouse anti-human IL-10 antibody. Addition of IL-6 at 100 ng/ml did not modify the frequency of Th17 cells, whereas 500 ng/ml of IL-6 was sufficient to induce significant Th17 expansion. Inhibition of IL-10 induced a significant expansion of Th17 cells at levels similar to those observed with the highest concentration of IL-6. An additional effect was observed when the highest concentrations of IL-6 were used together with IL-10 blocking. Bars show the mean and SD of 6 independent experiments. $*$ = $P < 0.05$ versus lamina propria mononuclear cells cultured alone. See Figure 1 for definitions.

Th17 cells ($5 \pm 0.25\%$; $P < 0.05$) at levels similar to those observed with the highest concentration of IL-6. An additional effect on Th17 expansion was observed when the highest concentrations of IL-6 were used together with IL-10 blockade.
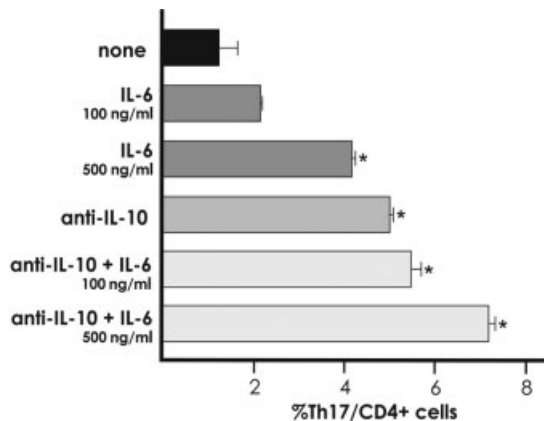
DISCUSSION

Regulation of immune intestinal homeostasis is now thought to be dependent upon the functions of CD4+CD25+ Treg cells. Treg cells play a fundamental role in the maintenance of intestinal hyporesponsiveness to luminal antigens through IL-10– and TGFβ-dependent mechanisms (13). In the presence of a breakdown of this tolerance, as a result of impaired Treg cell function or increased proinflammatory stimuli, inflammation may occur. In inflammatory bowel diseases, for example, the frequency of CD4+CD25high cells varies with disease activity, with active IBD being associated with a contraction of the peripheral blood Treg cell pool and an only moderate expansion in intestinal lesions, which is numerically insufficient to compensate for local inflammation (14).

We have recently demonstrated that overexpression of IL-23 and TGFβ, but not IL-17, is a pivotal feature of gut inflammation in AS (4). TGFβ regulates both the differentiation of inflammatory Th17 cells and suppressive Treg cell subsets, with the concomitant presence of proinflammatory cytokines favoring Th17 cell differentiation (5,15,16). On the basis of these findings, we investigated whether the absence of a clear

Th17 polarization, despite the high levels of IL-23 observed, could be due to a Treg cell–mediated suppression in the gut of AS patients.

This is the first study to show that Treg cell–related cytokines and transcription factor expression are markedly up-regulated at the mRNA level in intestinal inflammation in patients with AS. IL-2 mRNA overexpression in CD patients and in AS patients was associated with the up-regulation of STAT-5 (the main transducer of IL-2 signaling upon IL-2 receptor [IL-2R] ligation), FoxP3, and IL-10, indirectly suggesting that the IL-2/STAT-5 axis is functional in AS and CD patients.

The interaction of IL-2 with IL-2R is critically required to promote thymic and peripheral Treg development, in part by up-regulation of FoxP3 and CD25 (17,18). IL-2 signaling via STAT-5 has been demonstrated to constrain Th17 differentiation (19). Interestingly, although overexpression of FoxP3 was observed in both AS and CD patients, a significant positive correlation between TGFβ and FoxP3 was found only in AS patients, strongly suggesting that the high levels of TGFβ observed in AS could function as a Treg cytokine in the inflamed ileum. The different inflammatory milieus occurring in the ileum of AS and CD patients, despite the similar levels of TGFβ in these patients, could support divergent immunomodulatory mechanisms for TGFβ in AS and in CD, being proinflammatory in the latter. TGFβ induces differentiation of naive T cells into either Th17 cells or Treg cells in a dose-dependent manner, with high levels of TGFβ inhibiting Th17 response (15). In particular, it has recently been demonstrated that a functionally specialized population of mucosal CD103+ dendritic cells (DCs) induces FoxP3+ Treg cells via a TGFβ-dependent mechanism (20). However, we cannot exclude the possibility that TGFβ produced by Treg cells themselves could contribute to the increased expression observed in the inflamed ileum. In this regard, the specific role of the DCs of AS patients in inducing Treg cell differentiation and the contribution of single cell types in TGFβ production need to be better investigated.

In this study, we have further expanded the understanding of the immunoregulatory properties of CD4+CD25high Treg cells by demonstrating that there are increased numbers of Treg cells, with preserved immunosuppressant activity, together with overexpression of Treg cell–related cytokines and transcription factors at the mRNA level in the inflamed ileum of AS patients. The increased prevalence of Treg cells, together with the demonstration of the absence of a clear Th1 or Th17 polarization, strongly indicates the importance of Treg cells in the maintenance of intestinal homeostasis in an early stage of chronic gut inflammation.

The importance of either IL-10 or IL-10R2 has been demonstrated in both human colitis and murine models of colitis (21–24). Cure of murine colitis by CD4+CD25high Treg cells has also been shown to be dependent on IL-10, with IL-10–producing CD4+CD25high Treg cells selectively enriched within the colonic lamina propria, suggesting compartmentalization of the Treg cell response at effector sites (25).

The evidence of IL-2, IL-10, and STAT-5 overexpression in mucosal biopsy specimens from patients with AS and, to a lesser extent, patients with CD observed in the present study, together with the observation that 70–80% of the lamina propria Treg cells are also IL-10–producing cells, suggests a key role of IL-10 in modulating intestinal immune response, which is insufficient to control inflammation in CD, but is indispensable in AS. Furthermore, our demonstration of a significant Th17 expansion from isolated lamina propria mononuclear cells obtained from 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.

The exact mechanism by which a breakdown in immune regulatory networks leads to chronic inflammatory diseases in the intestines of AS patients is currently unknown. We hypothesize that in genetically prone subjects, an unknown infective stimulus could induce the production of proinflammatory cytokines, such as IL-6 or IL-1, down-regulating Treg cell responses 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 Treg 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 the intestine of AS patients. 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 strategies involving the IL-10 pathway to restore intestinal homeostasis in human intestinal inflammation.

AUTHOR CONTRIBUTIONS

data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ciccia, Accardo-Palumbo, Giardina, Triolo.

Acquisition of data. Ciccia, Accardo-Palumbo, Di Maggio, Bombardieri, Rizzo, Alessandro, Ferrante, Principe, Peralta, Drago, Triolo.

Analysis and interpretation of data. Ciccia, Accardo-Palumbo, Giardina, Di Maggio, Principato, Bombardieri, Rizzo, Alessandro, Ferrante, Principe, Conte, Drago, Craxì, De Leo, Triolo.

ROLE OF THE STUDY SPONSOR

BioNat Italy purchased the reagents for RT-PCR experiments, performed RT-PCR experiments, collected and analyzed data, and provided writing assistance for the corresponding text in the Methods section of the article. Publication of the article was contingent upon the approval of BioNat Italy.

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