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Overexpression of Interleukin-23, but Not Interleukin-17, as an Immunologic Signature of Subclinical Intestinal Inflammation in Ankylosing Spondylitis

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Objective. **Subclinical gut inflammation is common in spondylarthritis, but the immunologic abnormalities underlying this process are undefined. Perturbation of the interleukin-23 (IL-23)/Th17 axis has emerged as a fundamental trigger of chronic inflammation. This study was undertaken to investigate the expression and tissue distribution of IL-23/Th17–related molecules in Crohn's disease (CD) and in subclinical gut inflammation in ankylosing spondylitis (AS).**

Methods. **Quantitative gene expression analysis of Th1/Th2 and IL-23/Th17 responses was performed in intestinal biopsy samples obtained from 12 patients with CD, 15 patients with AS, and 13 controls. IL-23 tissue distribution and identification of IL-23–producing cells were evaluated by immunohistochemistry.**

Results. **We demonstrated a strong and significant up-regulation of IL-23p19 transcripts in the terminal ileum in patients with AS and patients with CD. IL-23 was abundantly produced by infiltrating monocyte-like cells in inflamed mucosa from AS and CD patients. Notably, we also identified Paneth cells as a major source of IL-23 in patients with AS, patients with CD, and normal controls. Unlike CD, in AS patients, IL-23 was not associated with up-regulation of IL-17 and the IL-17–inducing cytokines IL-6 and IL-1β. Finally, while the Th1-related cytokines interferon-γ, IL-12p35, and IL-27p28 were overexpressed only in CD patients, IL-4, IL-5, and STAT-6 were also significantly increased in AS patients.**

Conclusion. **Our findings indicate that overexpression of IL-23, but not IL-17, is a pivotal feature of subclinical gut inflammation in AS. Identification of resident Paneth cells as a pivotal source of IL-23 in physiologic and pathologic conditions strongly suggests that IL-23 is a master regulator of gut mucosal immunity, providing a pathophysiologic significance to the reported association between IL-23 receptor polymorphisms and intestinal inflammation.**

Asymptomatic intestinal inflammation, usually involving the terminal ileum, has been demonstrated in a significant number of patients with spondylarthritis (SpA) (1). Historically, 2 types of inflammation have been observed in patients with SpA: acute inflammation as seen in infectious colitis and chronic inflammation resembling Crohn's disease (CD). Long-term evolution to overt CD has been described in 7% of patients with initial chronic gut inflammation (2).

Several immunologic similarities between gut inflammation in SpA and CD have been described, such as increased expression of the E-cadherin–catenin complex, up-regulation of $\alpha E\beta 7$ integrin on intraepithelial T cells, and increased numbers of macrophages expressing the scavenger receptor CD163, supporting the concept that this subgroup of SpA patients may be considered a model for early immune alterations related to CD (3).

T lymphocytes play an important role in the regulation of gut immune responses. In vivo and in vitro studies have demonstrated that dysregulated effector T

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cell responses are involved in the pathogenesis of intestinal inflammation (4). After antigenic stimulation, naive CD4+ T cells differentiate into various subsets that are characterized by the production of distinct cytokines with distinct effector functions. This process is governed by selective cytokines and intracellular pathways, such as STAT and suppressor of cytokine signaling (SOCS).

Historically, effector T cells have been categorized into 2 main subsets: Th1 cells, which produce large quantities of interferon-γ (IFNγ), and Th2 cells, which produce interleukin-4 (IL-4), IL-5, and IL-13. Th1 cells are proinflammatory cells linked to the induction and progression of Th1-related disorders such as CD (5,6).

Recently, a novel T cell subset characterized by IL-17 production (Th17) and capable of directly inducing tissue inflammation and autoimmunity has been shown to be involved in the pathogenesis of several autoimmune diseases (7). Although it is a subject of controversy, recent evidence indicates that Th17 cells in humans are differentiated from naive T cells by the combination of IL-1β, IL-6, and IL-23. Once differentiated, IL-23 triggers the proliferation of Th17 cells from activated memory T cells. Th17 cells produce IL-17A and IL-17F and, to a lesser extent, tumor necrosis factor (TNF) and IL-6 (7,8). Although the primary focus on IL-23 function has been the Th17 pathway and thus adaptive immune induction and function, a recent study (9) identified an important role for IL-23 in directly activating the innate intestinal immune system, suggesting a pivotal role of IL-23 in regulating local immune responses in the gut.

The limited data available on cytokine production in the gut of SpA patients indicate a small but significant impairment of Th1 cytokines in lymphocytes isolated from the colon of SpA patients (10). Activation of the IL-17/IL-23 and STAT pathways and the expression of negative regulator protein SOCS-3 in the intestine of SpA patients have not been evaluated as yet. The aim of this study was to evaluate the IL-23/Th17 axis, in parallel with Th1 and Th2 responses, in subclinical gut inflammation in patients with ankylosing spondylitis (AS). In addition, we aimed to identify the main cellular source(s) of IL-23 in normal intestinal mucosa and in the inflamed intestinal mucosa of patients with AS and patients with CD.

This is the first study to show that IL-23 is markedly up-regulated at the messenger RNA (mRNA) and protein levels in the terminal ileum in AS patients at levels similar to those found in CD patients. Notably, we demonstrate that, together with monocyte-like cells in the inflammatory infiltrates in AS and CD mucosa, resident Paneth cells are a major source of IL-23 in the intestinal mucosa under pathologic as well as physiologic conditions. This suggests a novel and critical role of Paneth cells in regulating mucosal immunity in the terminal ileum and in promoting local inflammation via dysregulated IL-23 expression.

PATIENTS AND METHODS

Patients and controls. Multiple biopsy specimens were obtained after informed consent from 15 patients with AS, 12 patients with CD, and 13 controls who underwent ileocolonoscopy for routine clinical evaluation. Independently of the presence of gastrointestinal symptoms, patients with AS were systematically referred by the rheumatologist for an ileocolonoscopy with biopsies.

Table 1 shows the baseline characteristics of the patients and controls. The AS group consisted of 9 men and 6 women with a median age of 41 years (range 38–65 years) who were diagnosed as having AS according to the modified New York criteria (11). Disease activity was evaluated using the Bath AS Disease Activity Index (BASDAI) (12), with a BASDAI score of $\geq$4 indicating active disease. All patients were HLA–B27 positive. At the time mucosal biopsy specimens were obtained, the mean $\pm$ SD BASDAI score was 6.7 $\pm$ 3.4, and none of the patients had ever received cytotoxic treatment. The CD group consisted of 8 men and 4 women with a median age of 50 years (range 48–60 years). The diagnosis of CD was made according to clinical parameters and findings of radiographic, endoscopic, and histopathologic analyses. Disease activity was evaluated using the CD Activity Index (13), with a score $>$150 indicating active disease, and endoscopic and histopathologic data. At the time of sample collection, the mean $\pm$ SD CD Activity Index score was 253 $\pm$ 51.5, and none of the patients had received steroids and/or cytotoxic drugs, immunosuppressive agents, or antibiotics. The control group consisted of 8 men and 5 women with a median age of 45 years (range 41–58 years) who were undergoing ileocolonoscopy for diagnostic purposes and in whom there was no evidence of underlying disease.

Two adjacent mucosal biopsy samples from each intestinal segment (rectum, descending colon, transverse colon, ascending colon, cecum, and terminal ileum) were obtained from each subject. One sample was used for immunohistology and the other for transcript analysis. In addition, small intestine biopsy specimens from 4 patients with ulcerative colitis (UC) and 2 with Behçet's disease (BD) were analyzed. The study was approved by the ethics committee and the institutional review board of the University of Palermo.

Quantitative TaqMan reverse transcriptase–polymerase chain reaction (RT-PCR) for Th1, Th2, and Th17 cytokines, STAT molecules, and SOCS-3. 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, sets of primers and probes were obtained from Applied Biosystems (Foster City, CA) (see Supplementary Table 1, available online at http://www3.interscience.wiley.com/journal/76509746/home). Samples were run in triplicate at 20 ng of

Table 1. Baseline characteristics of the patients and controls*

	CD patients (n = 12)	AS patients (n = 15)	Controls (n = 13)
Age, mean ± SD years	50 ± 10	41 ± 14	45 ± 13
Sex, no. (%) male	8 (66)	9 (60)	8 (62)
Disease duration, mean ± SD months	5 ± 1.5	6 ± 3.8	NA
Intestinal area involved, no. (%) of patients			
Ileum	12 (100)	NA	NA
Ileum and colon	5 (42)	NA	NA
CRP, mean ± SD mg/liter	22 ± 8	10 ± 6†	NA
Concomitant medical treatment, no. (%)			
Corticosteroids	0 (0)	0 (0)	NA
NSAIDs	0 (0)	7 (47)	NA
Immunosuppressants	0 (0)	0 (0)	NA
5-aminosalicylates	0 (0)	0 (0)	NA
CD Activity Index score, mean ± SD‡	253 ± 51.5	NA	NA
BASDAI score, mean ± SD§	NA	6.7 ± 3.4	NA

* AS = ankylosing spondylitis; NA = not applicable; CRP = C-reactive protein; NSAIDs = nonsteroidal antiinflammatory drugs.
† $P = 0.0005$ versus patients with Crohn's disease (CD).
‡ Scores for the CD Activity Index range from 0 to 600, with higher scores indicating more severe disease.
§ Scores for the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) range from 0 to 10, with higher scores indicating more severe disease.

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.

Histomorphologic grading. Tissue samples were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Sections (3 μm thick) were deparaffinized, rehydrated, and stained with hematoxylin and eosin. Specimens from patients with AS were divided into 3 subgroups as previously described (14): those with normal gut histology, those with acute inflammation, and those with chronic inflammation. Briefly, acute inflammation was defined by the preservation of normal architecture, with the presence of neutrophils and/or eosinophils in the crypt and villus epithelium. Chronic inflammation was defined by alterations of the mucosal architecture, with crypt distortion and atrophy in the colon, and villous blunting and fusion in the ileal mucosa, with an active granulocytic infiltrate in the epithelium accompanied by an increased mononuclear cell infiltrate and formation of basal lymphoid aggregates in the lamina propria.

Immunohistochemical localization and identification of IL-23– and IL-17–producing cells in the intestinal mucosa. Immunohistochemistry for IL-23 and IL-17 was performed on 3-μm–thick paraffin-embedded sections. Following rehydration, antigen was unmasked for 45 minutes at 95°C using Dako Target retrieval solution (pH 6; Dako, Carpinteria, CA). Endogenous peroxidase was blocked for 10 minutes with Dako peroxidase blocking reagent, and nonspecific binding was blocked for 20 minutes with Dako protein block. The primary antibodies mouse monoclonal anti-human IL-23p19 (IgG1, clone HLT2736; 1:50 dilution) (BioLegend, San Diego, CA) or rabbit anti-human IL-17 (1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) were added and incubated for 1 hour at room temperature. An isotype-matched irrelevant antibody was used as a negative control. Following 3 washes with Tris buffered saline, slides were incubated for 30 minutes with peroxidase-conjugated Dako EnVision polymer. After 3 further washings, peroxidase activity was visualized using diaminobenzidine chromogen (Dako), and slides were lightly counterstained with hematoxylin before dehydration and mounting in DePex (VWR International, Oslo, Norway). In order to identify Paneth cells (15) as a possible source of IL-23 in the intestinal crypts, double immunohistochemical staining for defensin 5 and IL-23 was performed using a Dako EnVision double staining kit. For defensin 5 detection, a mouse monoclonal antibody to human α-defensin NP5 (Abcam, Cambridge, UK) was incubated for 1 hour at room temperature at a 1:50 dilution, and staining was developed as described above.

The number of IL-23– and IL-17–expressing cells was determined by counting IL-23– or IL-17–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 (Leica, Rijswijk, The Netherlands). Scoring was performed by 2 experienced pathologists (CT and VF) who were blinded with regard to subject group. Results are reported as the mean ± SD.

Statistical analysis. Statistical analysis of quantitative variables was performed using the Kruskal-Wallis nonparametric test, with Dunn's post test when analyzing more than 2 groups and the Mann-Whitney U test when analyzing 2 groups. P values less than 0.05 were considered significant.

RESULTS

Prevalence and histomorphologic evaluation of subclinical intestinal inflammation in AS patients. Of the 15 patients with active AS who underwent colonoscopy (mean BASDAI score 6.7), evidence of subclinical intestinal inflammation was observed in 11 (73%). In all cases, microscopic inflammatory lesions were observed only in the distal ileum and/or cecum, with other intes-

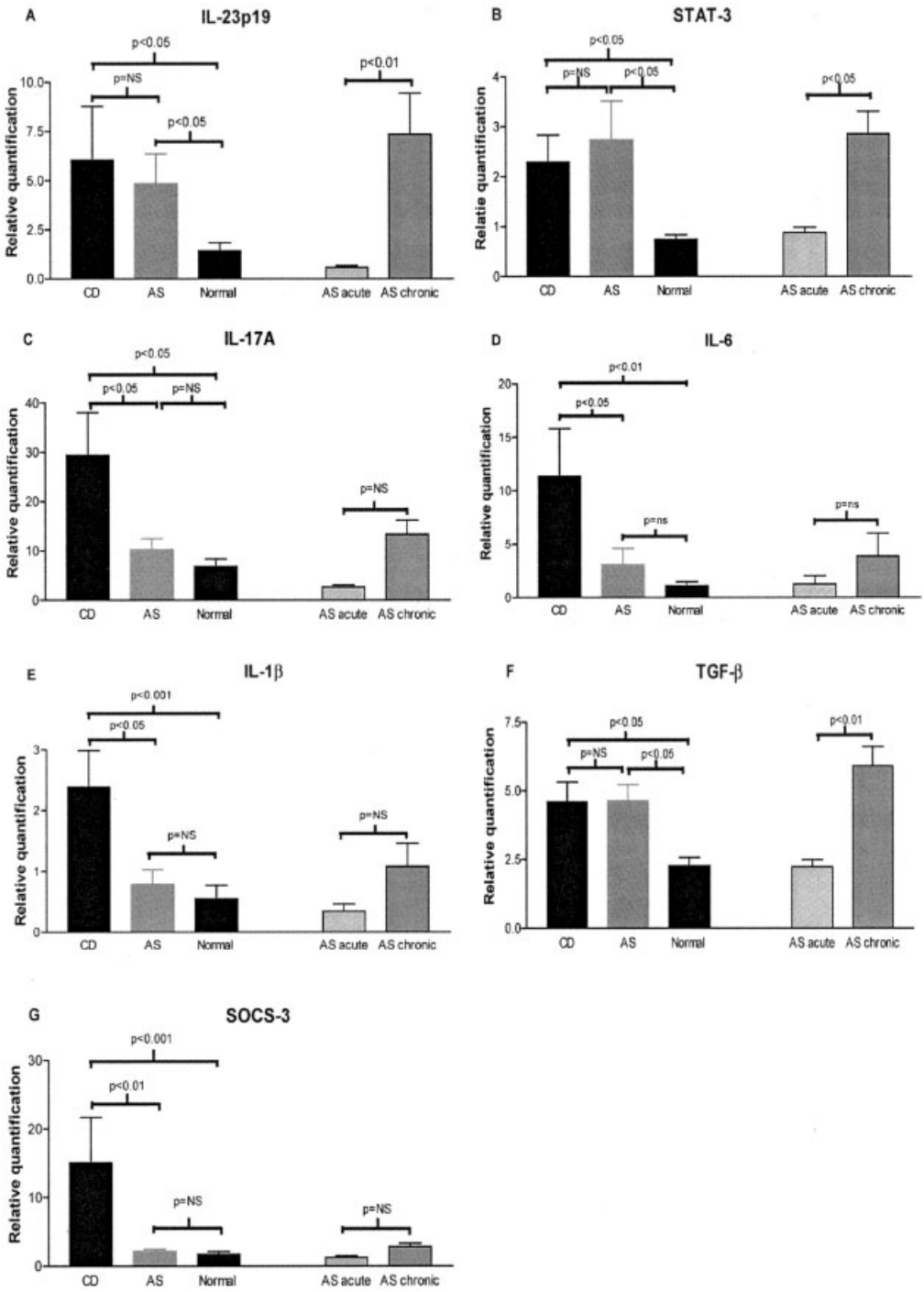

Figure 1. Interleukin-23 (IL-23)/Th17–related gene expression in mucosal biopsy specimens from patients with ankylosing spondylitis (AS), patients with Crohn's disease (CD), and normal controls. Relative mRNA quantification of IL-23/Th17–related cytokines and transcription factors IL-23p19 (**A**), STAT-3 (**B**), IL-17A (**C**), IL-6 (**D**), IL-1β (**E**), transforming growth factor β (TGFβ) (**F**), and suppressor of cytokine signaling 3 (SOCS-3) (**G**) was assessed by TaqMan real-time polymerase chain reaction in ileal/cecal biopsy specimens obtained from 12 patients with CD, 15 patients with AS, and 13 normal controls. Patients with AS were further divided into 2 groups: those with normal histologic findings or acute inflammation and those with chronic inflammation. Bars show the mean and SEM. NS = not significant.

tinal segments unaffected. On the basis of gut histology, as defined above and as previously described (14), patients with AS were divided into 2 main groups: those with normal histologic features or minor inflammation (acute lesions; 8 patients) and those with chronic inflammation (7 patients).

IL-23 mRNA is strongly up-regulated in the intestinal mucosa of AS patients to levels similar to those found in CD patients, but is not associated with a well-defined Th17 response. We investigated whether subclinical gut inflammation in AS patients was characterized by up-regulation of cytokines and transcription factors involved in the IL-23/Th17 axis. Although it is a subject of controversy, the differentiation and expansion of human Th17 have recently been shown to be regulated by IL-23, IL-1β, and IL-6 (8,16). While transforming growth factor β alone stimulates the polarization of human CD4+ T cells into regulatory T cells by increasing FoxP3 (17), and suppressing the differentiation of Th1, Th2, and Th17 lymphocytes, the concomitant stimulation of IL-1β and IL-6 induces the generation of the proinflammatory Th17 phenotype (8). Once differentiated, Th17 cells need stimulation by IL-23 to expand and maintain the Th17 phenotype. The IL-23 signal seems to be mediated by STAT-3 and inhibited by SOCS-3 (18,19).

We first evaluated the mRNA expression levels of cytokines involved in Th17 immune responses in different intestinal segments in patients with AS, patients with CD, and histologically normal controls. The analysis among the different patient groups was performed by comparing the expression levels of each gene within the same intestinal segment. Differential expression of inflammatory cytokines in AS patients compared with normal controls was observed in the cecum and distal ileum but not in the rectum, descending colon, transverse colon, or ascending colon. Thus, analysis was performed comparing results obtained from cecal/ileal biopsy specimens only. In addition, a comparative analysis within AS patients was performed by subgrouping patients with AS with chronic inflammation versus those with acute inflammation or normal histologic findings.

As shown in Figure 1A, a marked and significant increase in ileal expression of IL-23p19 transcripts was observed in AS patients (~4-fold compared with control intestinal biopsy specimens), to levels similar to those seen in CD patients. The increase in IL-23p19 mRNA expression was particularly evident in mucosal biopsy specimens obtained from AS patients with chronic as compared with acute inflammation. Of relevance, an abundant (compared with most cytokines tested) basal expression of IL-23p19 mRNA was observed in the ileum of normal controls, suggesting a constitutive expression of IL-23 in this segment. Notably, up-regulation of IL-23p19 was not observed in the ileum of 4 patients with UC and 2 patients with BD (data not shown).

Consistent with IL-23p19 up-regulation, STAT-3, which mediates IL-23 signaling upon IL-23 receptor (IL-23R) ligation, was significantly increased in AS patients, to levels similar to those found in CD patients (Figure 1B). Despite the strong up-regulation of IL-23 mRNA detected, we observed only a modest and not statistically significant increase in IL-17A mRNA in the same segments (Figure 1C). Accordingly, cytokines involved in Th17 differentiation, such as IL-6 and IL-1β, were only modestly increased or not increased in AS patients (Figures 1D and E). However, trends (although not statistically significant) toward higher levels of IL-17, IL-6, and IL-1β mRNA were detected in AS patients with chronic lesions versus those with acute lesions. In contrast to patients with AS, patients with CD displayed a marked increase in IL-17A, IL-6, and IL-1β expression compared with controls (Figures 1C–E), confirming the coexistence of IL-23 up-regulation and Th17 responses in intestinal inflammation in CD. SOCS-3 was overexpressed in CD patients (~10-fold increase compared with control levels), but not in AS patients (Figure 1G).

Expression of Th1 and Th2 cytokines and related STAT genes in the intestinal mucosa of AS patients. We next evaluated the mRNA expression levels of multiple cytokines involved in Th1 and Th2 immune responses. As shown in Figure 2, intestinal biopsy specimens from AS patients, analyzed independently of the degree of intestinal inflammation, did not show any significant increase in the expression levels of IFNγ, IL-12p35, IL-27p28, or the Th1-related signaling molecule STAT-1 (Figures 2A–D) as compared with controls. Conversely, as expected, evident overexpression (>10-fold increase) of IFNγ was detected in patients with CD. Consistent with IFNγ up-regulation, concomitant overexpression of IL-12p35 (2-fold), IL-27p28 (5-fold), and STAT-1 (2-fold) was observed in samples from patients with CD. Interestingly, a trend toward an increase in Th1-related genes was observed in patients with AS displaying chronic inflammation as compared with those with normal histologic findings or acute inflammation, although the differences were not significant.

In patients with CD, a significant increase in ileal expression of TNFα transcripts was also observed as compared with controls (~4-fold), while no significant up-regulation of TNFα was detectable in AS (Figure 2G). When we analyzed AS patients with chronic inflammatory lesions alone, a more evident, although not

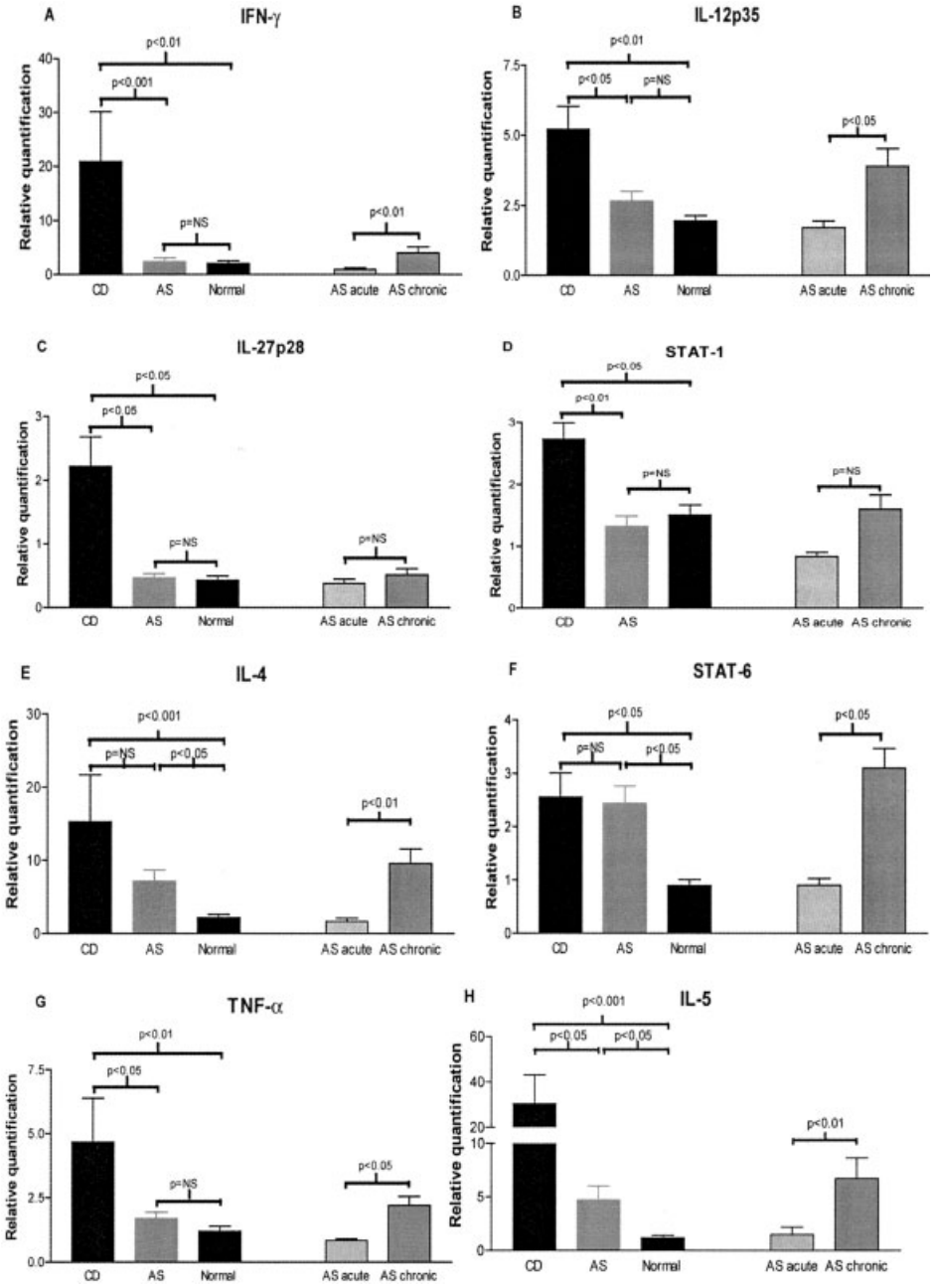

Figure 2. Characterization of Th1- and Th2-related gene expression profiles in mucosal biopsy specimens from patients with AS, patients with CD, and normal controls. Relative mRNA quantification of Th1- and Th2-related cytokines and transcription factors interferon-γ (IFNγ) (**A**), IL-12p35 (**B**), IL-27p28 (**C**), STAT-1 (**D**), IL-4 (**E**), STAT-6 (**F**), tumor necrosis factor α (TNFα) (**G**), and IL-5 (**H**) was assessed by TaqMan real-time polymerase chain reaction in ileal/cecal biopsy specimens obtained from 12 patients with CD, 15 patients with AS, and 13 normal controls. Patients with AS were further divided into 2 groups: those with normal histologic findings or acute inflammation and those with chronic inflammation. Bars show the mean and SEM. See Figure 1 for other definitions.

statistically significant, increase in TNFα mRNA levels (~2-fold) was detected.

We analyzed the Th2 profile of intestinal ileal/cecal biopsy specimens from patients with AS. Interestingly, increases in IL-4 and IL-5 mRNA were observed in patients with AS as well as in patients with CD. In AS

patients, IL-4 mRNA expression was increased ~3.5-fold and IL-5 mRNA expression was increased ~5-fold, and in CD patients, IL-4 mRNA expression was increased ~5.5-fold and IL-5 expression was increased 30-fold, compared with controls (Figures 2E and H). Similarly, STAT-6, which mediates IL-4 signaling upon IL-4R ligation, was also increased in AS patients (2-fold compared with controls), to levels similar to those found in patients with CD (Figure 2F). Interestingly, both in AS and CD, patients with high levels of IL-4 mRNA displayed low IFNγ expression, suggesting the presence of different stages of evolution of the inflammatory lesions (data not shown). In contrast, IL-13 mRNA expression was detectable in only 2 of 15 patients with AS and 2 of 12 patients with CD.

Identification of Paneth cells and infiltrating monocyte-derived cells as the cellular sources of IL-23 in the terminal ileum of patients with AS and patients with CD. Given the strong up-regulation of IL-23 mRNA in mucosal biopsy specimens from patients with AS or CD, we next investigated its protein expression and distribution pattern. Immunohistochemical analysis of samples from AS patients and CD patients demonstrated 2 different patterns of IL-23 expression in the terminal ileum. The first pattern was a unique feature of CD and AS with chronic inflammatory lesions and was characterized by a large number of IL-23–producing cells within the lymphomonocytic inflammatory infiltrates (Figures 3A–C). IL-23+ infiltrating cells were never observed in normal controls and were only detectable in and around inflammatory aggregates in patients with AS or CD. The morphologic appearance of IL-23+ infiltrating cells was mainly characterized by a large cytoplasm and often dendritic morphology, consistent with a monocyte-derived cell lineage (i.e., macrophages/dendritic cells). This expression pattern was similar to that observed in human lymph nodes (results not shown).

A second pattern of IL-23 expression was detectable in the terminal ileum of CD patients, in AS patients irrespective of the presence or absence of inflammatory lesions, and in normal controls. This second pattern was characterized by abundant expression of IL-23 in the crypts where IL-23 was produced by epithelial cells localizing deep within the crypts (Figures 3D–F).

We then compared the number of IL-23+ cells in samples from AS patients, CD patients, and normal controls. As shown in Figure 3G, terminal ileal biopsy specimens from AS patients with chronic inflammatory lesions showed the highest fraction of IL-23–expressing cells (mean ± SD 35.6 ± 7) at levels similar to those in patients with CD (29.4 ± 6.14) and significantly higher than in controls (16.8 ± 4.87), confirming the up-regulation of IL-23p19 mRNA detected by RT-PCR.

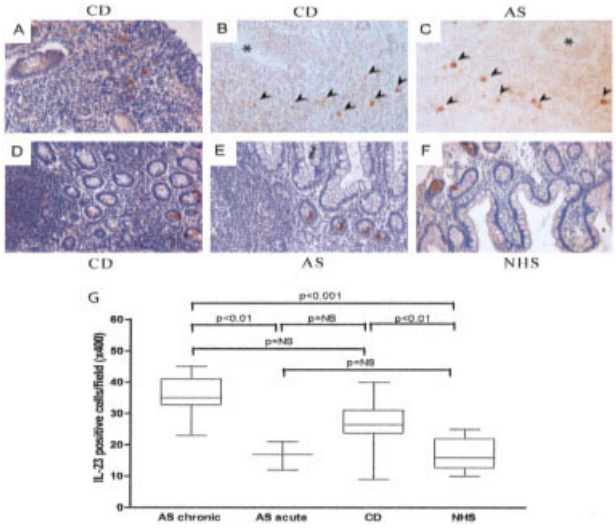

Figure 3. IL-23 expression by infiltrating immune cells within inflammatory lesions in the terminal ileum of patients with CD or AS. **A–F,** Representative photomicrographs showing 3-μm–thick paraffin-embedded sections of distal ileal biopsy specimens obtained from patients with CD (**A**, **B**, and **D**), patients with AS (**C** and **E**), and normal healthy subjects (NHS) (**F**), stained for IL-23p19. Abundant IL-23 expression was observed in a large number of mononuclear cells infiltrating the intestinal mucosa of samples from patients with CD and patients with AS (**A–C**), but not in normal subjects. In both CD and AS, IL-23 was expressed by immune cells (**arrowheads** in **B** and **C**) with a large cytoplasm and often dendritic morphology (**A**) localizing in proximity to the intestinal lumen (**asterisks** in **B** and **C**). Together with infiltrating cells, strong expression of IL-23 was invariably observed within epithelial crypts in close proximity to mononuclear inflammatory infiltrates in both CD patients (**D**) and AS patients (**E**). IL-23 was also observed in the crypts in normal controls (**F**), consistent with basal constitutive expression of the protein. (Original magnification × 200 in **A**, **D**, **E**, and **F**; × 400 in **B** and **C**.) **G,** Number of IL-23+ 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 other definitions.

Based on morphologic appearance and localization within the crypts, IL-23+ epithelial cells highly resembled Paneth cells. In order to confirm this observation, we performed sequential section analysis and double immunohistochemistry for IL-23 and defensin 5, a specific marker of Paneth cells (15). IL-23 and defensin 5 strictly colocalized within the deeper part of the crypts (Figure 4), allowing the precise identification of Paneth cells as IL-23–producing cells under pathologic and physiologic conditions.

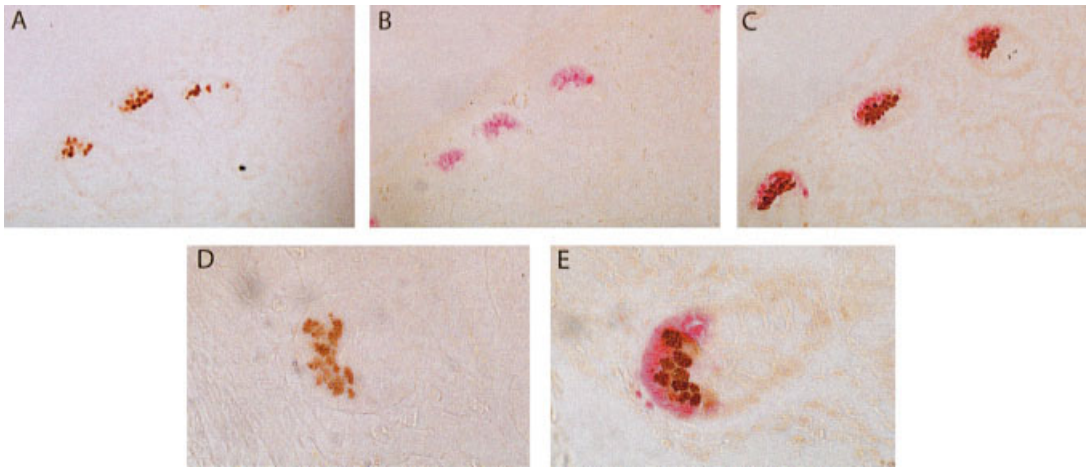

Figure 4. Characterization of Paneth cells as the source of IL-23 in intestinal crypts. Representative photomicrographs showing sequential analysis and double immunohistochemistry of 3-μm–thick paraffin-embedded tissue sections of terminal ileal biopsy specimens obtained from a patient with AS and single stained for anti–IL-23p19 (brown staining) (**A** and **D**) and defensin 5, a specific marker of Paneth cells (red staining) (**B**), and double stained for IL-23 and defensin 5 (**C** and **E**) are shown. Precise colocalization of IL-23 and defensin 5 staining was observed in the distal ileum in AS patients (as well as in CD patients and controls [results not shown]) at the base of the crypts, consistent with IL-23p19 expression by Paneth cells. Interestingly, while defensin 5 was expressed uniformly in the cytoplasm, IL-23 staining was mainly polarized toward the apical cytoplasm of Paneth cells. (Original magnification $\times$ 200 in **A–C**; $\times$ 400 in **D** and **E**.) See Figure 1 for definitions.

An increased number of IL-17–producing cells characterizes the terminal ileum in CD patients but not AS patients. We used immunohistochemistry to detect IL-17+ cells in the terminal ileum of AS patients and CD patients. As shown in Figure 5, IL-17–producing cells were rarely detected in the immune cell infiltrates in AS but were abundant in CD patients (Figures 5A and B). Accordingly, quantitative analysis showed that the number of IL-17+ cells in AS patients was not increased as compared with normal controls, although a trend toward an increase in IL-17 cells in chronic AS lesions was apparent (Figure 5C).

DISCUSSION

The presence of a shared pathogenesis between SpA and CD has been suggested by evidence of similar arthropathy and intestinal inflammation in patients with either disease (3). Interestingly, long-term evolution to overt CD has been described in 7% of patients with SpA with initial chronic gut inflammation (2). Moreover, several immunologic similarities between SpA with gut inflammation and CD have been demonstrated, such as increased expression of the E-cadherin–catenin complex, up-regulation of $\alpha E\beta 7$ integrin on intraepithelial T cells, and increased numbers of macrophages expressing the scavenger receptor CD163, supporting the concept of preclinical CD in patients with SpA with gut inflammation (3). However, the immunologic abnormalities underlying the inflammatory process in the terminal ileum of SpA patients are poorly understood.

This is the first study to show that IL-23 expression is markedly up-regulated at both the mRNA and protein levels in subclinical intestinal inflammation in patients with AS, to levels similar to those found in patients with CD. Interestingly, IL-23 mRNA overexpression in AS was associated with up-regulation of STAT-3, the main transducer of IL-23 signaling upon IL-23R ligation, indirectly suggesting that the IL-23 axis is functional in AS and CD patients. Conversely, SOCS-3, an inhibitor of IL-23 activation, was increased in CD patients only, suggesting that an imbalance in the ratio of IL-23 to SOCS-3 may characterize subclinical gut inflammation in AS.

Based on recent findings, it is becoming increasingly clear that IL-23 exerts an essential pathogenetic role in promoting autoimmunity and chronic inflammation in several models of autoimmune diseases, such as experimental inflammatory arthritis (20) and experimental colitis (21). Our original demonstration of local IL-23 up-regulation in the terminal ileum of patients with AS and patients with CD strongly suggests that this cytokine might also play a pivotal role in promoting intestinal inflammation and inflammatory bowel disease.

IL-23 is a member of the IL-12 family that drives

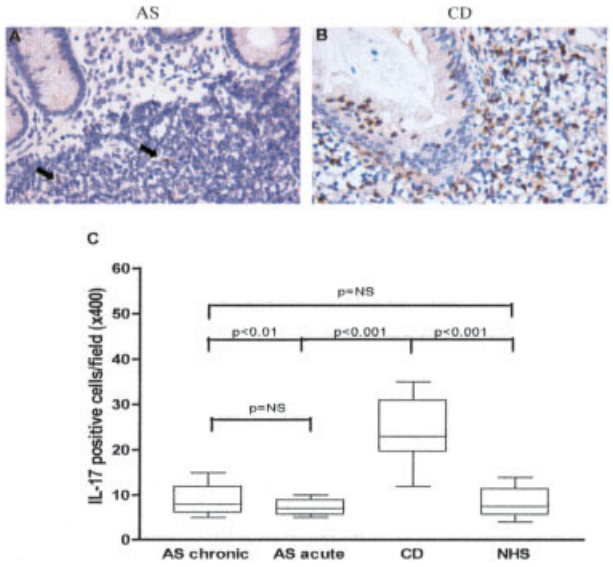

Figure 5. An increased number of IL-17–producing cells characterizes intestinal inflammation in CD but not in AS. **A** and **B**, Immunohistochemistry for IL-17 (brown staining) in ileal inflammatory lesions in patients with AS (**A**) and patients with CD (**B**). While detection of IL-17–producing cells (**arrows**) was relatively rare in AS, abundant expression of this cytokine was clearly observed in CD. (Original magnification × 200.) **C**, Number of IL-17 +cells in the mucosa in AS patients with chronic inflammation, AS patients with acute inflammation, CD patients, and normal healthy subjects (NHS). 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 outside the boxes represent the 10th and the 90th percentiles. Lines inside the boxes represent the median. See Figure 1 for other definitions.

a highly pathogenetic T cell population involved in the initiation of autoimmune diseases. IL-23 is a heterodimeric proinflammatory cytokine composed of a p19 subunit and the p40 subunit; the latter also forms part of the IL-12 heterodimer with IL-12p35 (22). Recent evidence suggests that IL-23 acts as a regulator of the maturation of autoreactive T cells producing IL-17 (Th17) and promotes chronic inflammation dominated by IL-17, IL-6, IL-8, and TNFα, as well as by neutrophils and macrophages (23–27). Dysregulation of the IL-23/Th17 axis has been proposed as a major pathogenetic mechanism leading to the development of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease, rheumatoid arthritis (7), and psoriasis (28). However, while patients with CD demonstrated a fully developed Th17 response with high levels of IL-17 and of the IL-17–inducing cytokines IL-6 and IL-1β, this was not the case in patients with AS, in whom marginal up-regulation of IL-17 was observed.

Nevertheless, up-regulation of IL-23 is likely to have profound relevance in regulating intestinal inflammation even independently of IL-17. In this regard, recent evidence (9) indicates an important role of IL-23 in promoting intestinal inflammation by directly activating innate immune cells. In an experimental model characterized by injection of an agonistic anti-CD40 monoclonal antibody into T cell– and B cell–deficient mice, neutralization of IL-12 resulted in the reversal of systemic inflammation, with no effect on mucosal immunopathology, while neutralization of IL-23 resulted in prevention of CD40-induced colitis without affecting wasting disease or serum cytokines, supporting a specific role of IL-23 in local mucosal inflammation.

The evidence of IL-23 overexpression in mucosal biopsy specimens from patients with CD and AS observed in the present study has particular relevance given the recent demonstration of the association between the IL-23R gene and inflammatory bowel diseases, with the finding that several independent functional single-nucleotide polymorphisms of the gene and its neighboring region conferred either strong protection against or marked susceptibility to CD (29). Recently, it was also demonstrated that the IL-23R gene could be involved in genetic predisposition to AS (30,31). However, the underlying pathogenetic mechanisms related to the aberrant expression of IL-23 in the gut of SpA patients need to be investigated. Constitutive p40 promoter activation and IL-23 production by murine ileal dendritic cells have been demonstrated, suggesting a predisposition of this segment to developing chronic inflammatory lesions through IL-23 and providing a molecular explanation for the preferential clinical manifestation of CD in this segment (32).

The results of this study contribute to the understanding of the immunoregulatory properties of IL-23 in the gut by providing the novel and extremely relevant observation that, in addition to IL-23 expression by infiltrating monocyte-like cells in inflamed AS and CD mucosa, Paneth cells represent a major cellular source of IL-23 in the terminal ileum in physiologic and pathologic conditions. Paneth cells are secretory epithelial cells located at the base of the crypts of Lieberkühn in the small intestine that act as resident host-defense cells by secreting antimicrobial peptides. Recently, there has been increasing interest, originally raised by the evidence that these cells produce TNFα in the terminal ileum (33), in the role of Paneth cells in contributing to intestinal immunoregulation and promoting mucosal inflammation (for review, see ref. 34).

In this study, we have further expanded the understanding of the immunoregulatory properties of

Paneth cells by demonstrating their constitutive expression of IL-23 under physiologic conditions and their overexpression of IL-23 in AS and CD. Our findings suggest that these cells play a fundamental role in regulating local mucosal immune responses via IL-23. The identification of basal IL-23 production by Paneth cells also explains the strong constitutive expression of mRNA for IL-23p19 in the terminal ileum of normal individuals. Overall, this suggests that dysregulation of the IL-23/IL-23R axis (i.e., via IL-23 overexpression and/or altered IL-23R expression or function) might be specifically responsible for the increased incidence of inflammatory lesions observed in this segment. In this regard, since Paneth cells have been shown to express NOD-2 (35) and since NOD-2 dysregulation is clearly implicated in the pathogenesis of CD (36,37), it would be extremely interesting to investigate whether abnormalities in NOD-2 might be responsible for dysregulated IL-23 expression in Paneth cells.

As briefly discussed above, IL-23 overexpression in AS was not associated with a fully developed Th17 response, as demonstrated by the relatively low levels of IL-17 we found. This is possibly related to the low expression of the IL-17–inducing cytokines IL-6 and IL-1β in AS compared with their expression in CD. In this regard, whereas IL-23 triggers the proliferation of Th17 cells from activated memory T cells, IL-23 alone is a relatively ineffective inducer of IL-17 and requires the combination of IL-6 and IL-1β to induce differentiation of Th17 cells from human naive T cells (8). Conversely, we confirmed previous reports of elevated IL-17 expression in the terminal ileum of patients with CD (38). Evidence of increased Th17 responses in CD is of particular interest because of the nonredundant role of Th17 lymphocytes in promoting autoimmunity and chronic inflammation.

Interestingly, analysis of the kinetics of cytokine expression in the central nervous system in EAE has demonstrated that IL-17 peaks before IFNγ, while IFNγ persists in the target tissue even after IL-17 expression has disappeared. In this regard, while Th17 cells might be generated more rapidly than Th1 cells during inflammation and direct the initial acute inflammation, Th1 cells might function in perpetuating tissue inflammation (39).

Consistent with the results of previous studies (10), we demonstrated that Th1 cytokines are not overexpressed in the gut of AS patients without clinical inflammation, while in CD patients, high levels of all Th1 cytokines were observed as expected. Although a trend toward an increase in IFNγ and TNFα mRNA levels was detected in intestinal biopsy specimens from AS patients, particularly in those with chronic inflammatory lesions, these differences were not significant. In addition, increased levels of IL-4 and IL-5 mRNA were detected in mucosal biopsy specimens from some AS patients. Interestingly, patients with AS (and patients with CD) displaying high IL-4 or IL-5 levels were characterized by low IFNγ expression and vice versa, possibly suggesting different stages of the inflammatory response. This evidence strengthens the findings of previous studies showing that increased expression of Th2 cytokines characterized different stages of the inflammatory lesions in CD patients (40,41). It would be of interest to investigate whether AS patients displaying higher levels of IFNγ are more prone to develop overt CD.

In summary, this is the first study to demonstrate that IL-23 is abundantly expressed in the terminal ileum in AS patients with subclinical gut inflammation at levels comparable to those found in CD patients. We have also shown that IL-23 up-regulation is not accompanied by a defined polarization toward a Th1, Th2, or Th17 phenotype, suggesting that the inflammatory lesions in AS represent an early, possibly preclinical stage of CD. Finally, we provide evidence that infiltrating cells in inflammatory lesions in AS and CD and Paneth cells under pathologic and physiologic conditions are the main sources of IL-23 in the gut. Overall, these results strongly support a fundamental role of IL-23/IL-23R dysregulation as a pathogenetic player and immunologic marker of subclinical gut inflammation in AS patients.

AUTHOR CONTRIBUTIONS

Dr. Triolo had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Ciccia, Bombardieri, AnnaRita Giardina, Pitzalis, Triolo.

Acquisition of data. Ciccia, Bombardieri, Principato, AnnaRita Giardina, Peralta.

Analysis and interpretation of data. Ciccia, Bombardieri, Principato, AnnaRita Giardina, Tripodo, Porcasi, Franco, Ennio Giardina, Craxi, Pitzalis, Triolo.

Manuscript preparation. Ciccia, Bombardieri, Triolo.

Statistical analysis. Ciccia.

Performance of colonoscopies. Peralta.

Overall study supervision. Pitzalis, Triolo.

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