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Interleukin-22 and Interleukin-22–Producing NKp44+ Natural Killer Cells in Subclinical Gut Inflammation in Ankylosing Spondylitis

Francesco Ciccia,[1] Antonina Accardo-Palumbo,[2] Riccardo Alessandro,[1] Aroldo Rizzo,[2] Simona Principe,[1] Sergio Peralta,[1] Francesca Raiata,[2] AnnaRita Giardina,[1] Giacomo De Leo,[1] and Giovanni Triolo[1]

Objective. **The intestinal inflammation observed in patients with ankylosing spondylitis (AS) is characterized by an overexpression of interleukin-23 (IL-23). IL-23 is known to regulate IL-22 production through lamina propria NKp44+ natural killer (NK) cells, which are thought to be involved in protective mucosal mechanisms. This study was undertaken to evaluate the frequency of NKp44+ NK cells and the expression of IL-22 in the ileum of AS patients.**

Methods. **Tissue NKp44+ NK cells, NKp46+ NK cells, and IL-22–producing cells were analyzed by flow cytometry. Quantitative gene expression analysis of IL-22, IL-23, IL-17, STAT-3, and mucin 1 (MUC-1) was performed by reverse transcriptase–polymerase chain reaction on ileal samples from 15 patients with AS, 15 patients with Crohn's disease (CD), and 15 healthy controls. NKp44, pSTAT-3, and IL-22 expression was analyzed by immunohistochemistry.**

Results. **The frequency of NKp44+ but not NKp46+ NK cells was increased in the inflamed ileum of AS patients compared to CD patients and controls. The frequency of NKp46+ NK cells was significantly increased only in CD patients. Among CD4+ lymphocytes and NKp44+ NK cell subsets, the latter were the major source of IL-22 on lamina propria mononuclear cells from AS patients. Significant up-regulation of IL-22, IL-23p19, MUC-1, and STAT-3 transcripts in the terminal ileum of patients with AS was observed. Immunohistochemical analysis confirmed the increased IL-22 and pSTAT-3 expression in inflamed mucosa from AS and CD patients.**

Conclusion. **Our findings indicate that overexpression of IL-22, together with an increased number of IL-22–producing NKp44+ NK cells, occurs in the gut of AS patients, where it appears to play a tissue-protective role.**

The asymptomatic ileal inflammation observed in patients with ankylosing spondylitis (AS) is immunologically characterized by the overexpression of interleukin-23 (IL-23). This overexpression, however, does not appear to be sufficient to induce Th17 polarization (1). Protective immunologic mechanisms, such as the expansion of IL-10–producing Treg cells, could, at least in part, explain the absence of a clear Th17 response despite the high levels of IL-23 observed in AS patients (2).

Although the primary focus of investigations of IL-23 function has been its effect on the adaptive immune system through the regulation of the Th17 pathway, recent studies of the gut suggest an important role for IL-23 in regulating innate immune responses (3–8). These studies have in fact identified a subset of IL-23–responsive human lamina propria natural killer (NK) cells that contribute to intestinal mucosa immunity. Lamina propria NK cells comprise 2 main populations, interferon-γ (IFNγ)–producing NKp46+ NK cells and IL-22–producing NKp44+ NK cells, which are balanced in normal human intestinal mucosa (9). This balance is disrupted in the inflamed mucosa of patients

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[1]Francesco Ciccia, MD, PhD, Riccardo Alessandro, PhD, Simona Principe, MA, Sergio Peralta, MD, AnnaRita Giardina, MD, PhD, Giacomo De Leo, PhD, Giovanni Triolo, MD: Università di Palermo, Palermo, Italy; [2]Antonina Accardo-Palumbo, PhD, Aroldo Rizzo, MD, Francesca Raiata, MSBiol: Azienda Ospedaliera Ospedali Riuniti Villa Sofia-Cervello, Palermo, Italy.

Drs. Ciccia and Accardo-Palumbo contributed equally to this work.

Address correspondence to Giovanni Triolo, MD, Department of Internal Medicine, Division of Rheumatology, Piazza delle Cliniche 2, 90127 Palermo, Italy. E-mail: giovanni.triolo@unipa.it.

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Table 1. Baseline characteristics of the patients*

	AS patients with acute inflammation (n = 8)	AS patients with chronic inflammation (n = 7)	CD patients (n = 15)
Age, years	35 (20–44)	38 (20–48)	46 (18–50)
Sex, no. (%) male	6 (75)	4 (57)	9 (60)
Disease duration, months	18 (14–32)	20 (12–26)	7 (2–14)
CRP, mg/liter	1 (0.4–2)	1 (0.2–2)	5 (3–14)†
Axial involvement, no. (%)	8 (100)	7 (100)	NA
Peripheral arthritis, no. (%)	2 (25)	3 (43)	NA
Enthesitis/dactylitis, no. (%)	2 (25)	1 (14)	NA
Uveitis, no. (%)	0 (0)	1 (14)	NA
Concomitant medical treatment, no. (%)			
NSAIDs	4 (50)	3 (43)	0 (0)
Biologic agents	0 (0)	0 (0)	0 (0)
Immunosuppressants	0 (0)	0 (0)	0 (0)
CD Activity Index score‡	NA	NA	245 (156–580)
BASDAI score§	4.4 (4.2–6.6)	5 (4.2–7)	NA

* Except where indicated otherwise, values are the median (range). The control group consisted of 15 healthy controls (12 men) with a median age of 52 years (range 38–68 years). CRP = C-reactive protein; NA = not applicable; NSAIDs = nonsteroidal antiinflammatory drugs.
† $P < 0.0001$ versus patients with ankylosing spondylitis (AS) with acute inflammation and patients with AS with chronic inflammation.
‡ Scores for the Crohn's disease (CD) Activity Index range from 0 to 600, with higher scores indicating more severe disease.
§ Scores for the Bath AS Disease Activity Index (BASDAI) range from 0 to 10, with higher scores indicating more severe disease.

with Crohn's disease (CD), where protective IL-22–producing NKp44+ NK cells are markedly reduced, whereas pathogenic IFNγ-producing NKp46+ NK cells are significantly expanded (9). Interestingly, the production of IFNγ and IL-22 by NKp46+ and NKp44+ NK cells, respectively, appears to be highly dependent on IL-23 signaling.

IL-22, released by NKp44+ NK cells upon IL-23 signaling, could act by constraining inflammation and protecting mucosal integrity. At mucosal sites, in fact, IL-22 acting through STAT-3 signaling increases the innate immune responses of tissue cells, protecting the epithelial barrier in the gut through the induction of goblet cell hyperplasia and mucin production (10,11), and enhancing tissue regeneration.

In inflammatory bowel diseases, IL-22 seems to play a local protective role in ulcerative colitis (10), and a systemic protective role in CD (12). Conversely, IL-22 seems to act as a proinflammatory cytokine in certain human T cell–mediated inflammatory diseases, such as psoriasis (13) and rheumatoid arthritis (14), and also to play a local pathogenic role in CD (15). Although it is likely that this pathogenic effect could reflect dysregulated duration of production and/or the concentration of IL-22, the functional outcomes of IL-22 expression seem to be governed by the coexpression of IL-17A. When coexpressed, IL-17A and IL-22 may act synergistically to promote chemokine expression, neutrophil recruitment, and inflammation, whereas IL-22 in the absence of IL-17A may promote essentially tissue-protective responses (16).

The aim of this study was to evaluate the expression of IL-22 at both the messenger RNA (mRNA) and protein levels, and to characterize the cellular source of IL-22 in the inflamed gut of AS patients. We also evaluated IL-22–dependent intestinal tissue–protective responses, such as epithelial expression of pSTAT-3, goblet cell hyperplasia, and increased mucin content.

PATIENTS AND METHODS

Patients. Multiple ileal biopsy samples from patients with AS without clinical symptoms of bowel inflammation, patients with CD, and normal controls were consecutively obtained. The demographic and clinical characteristics of the patients and controls are summarized in Table 1. The AS group consisted of 15 consecutive patients (10 men and 5 women), ages 20–48 years, who were diagnosed according to the modified New York criteria (17). All of the AS patients were HLA–B27 positive. Seven of the 15 AS patients were receiving nonsteroidal antiinflammatory drugs at the time of ileocolonoscopy. At the time mucosal biopsy specimens were obtained, none of the AS patients had ever received biologic treatment. The CD group consisted of 15 patients (9 men and 6 women) ages 18–50 years. At the time of sample collection, none of the patients with CD were receiving corticosteroids, immunosuppressants, or biologic agents. Biopsy specimens were obtained from ileal zones of active ulceration. The control group consisted of 15 normal subjects (12 men and 3

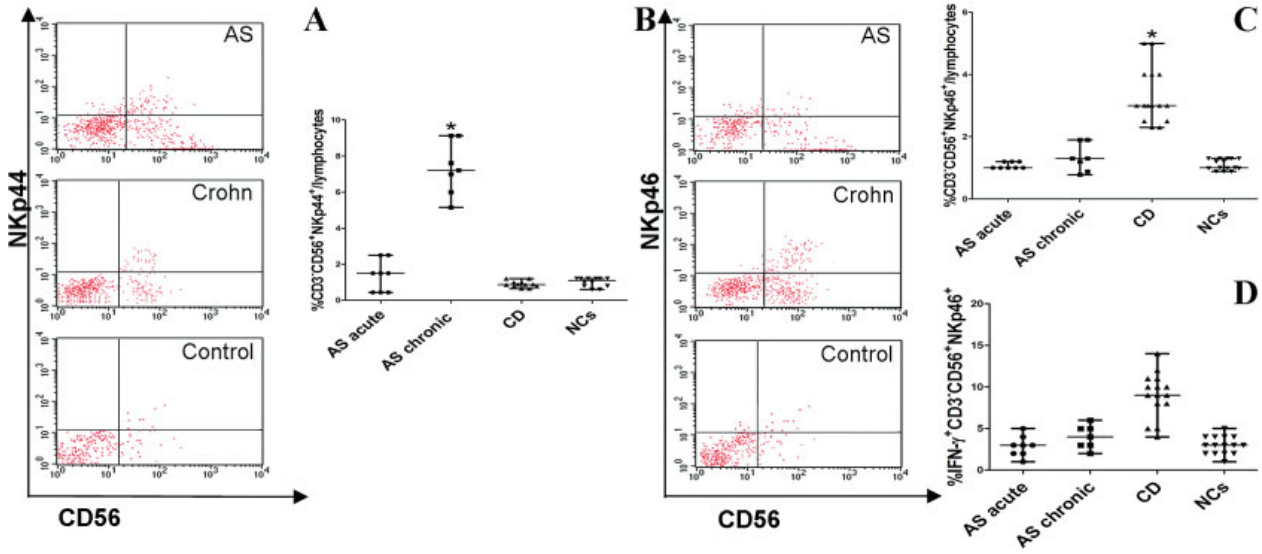

Figure 1. Increased frequency of CD3−CD56+NKp44+NKp46− cells in isolated lamina propria mononuclear cells (MNCs) from patients with ankylosing spondylitis (AS). Freshly isolated lamina propria MNCs from 15 patients with AS, 15 patients with Crohn's disease (CD), and 15 normal controls (NCs) were analyzed by flow cytometry. **A**, Representative dot plot of NKp44-expressing versus CD56-expressing cells among lamina propria MNCs from patients and controls (left) and percentages of CD3−CD56+NKp44+NKp46− cells among isolated lamina propria MNCs from patients and controls (right). $* = P < 0.05$ versus all other groups**. B**, Representative dot plot of NKp46-expressing versus CD56-expressing cells among lamina propria MNCs from patients and controls. **C**, Percentages of CD3−CD56+NKp44−NKp46+ cells among lamina propria MNCs from patients and controls. $* = P < 0.05$ versus all other groups. **D**, Percentages of interferon-γ (IFNγ)–producing CD3−CD56+NKp44−NKp46+ cells among lamina propria MNCs from patients and controls. $P < 0.001$, CD versus AS acute; $P < 0.05$, CD versus AS chronic; $P < 0.001$, CD versus normal controls. In **A**, **C**, and **D**, symbols represent individual patients, horizontal lines represent the median, and whiskers represent the range. NS = not significant. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

women), ages 38–68 years, who were undergoing ileocolonoscopy for routine evaluation. Paired specimens for histologic analysis, reverse transcriptase–polymerase chain reaction (RT-PCR), and lamina propria mononuclear cell (MNC) isolation for flow cytometric analysis and functional assays were obtained from 15 patients with AS, 15 patients with CD, and 15 healthy subjects. Written informed consent was obtained from all subjects, and the study protocol was approved by the local ethics committee of the University of Palermo.

Histomorphologic grading, goblet cell count, and evaluation of mucin content. Tissue samples were processed as previously described (1), and the degree of inflammation was evaluated by an experienced pathologist (AR). Specimens from patients with AS were divided into the following 3 subgroups, as previously described (1): 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 villous blunting and fusion in the ileal mucosa and an active granulocytic infiltrate in the epithelium accompanied by an increased MNC infiltrate and formation of basal lymphoid aggregates in the lamina propria. After standard hematoxylin and eosin staining, goblet cells were counted in sections from patients and controls. The number of goblet cells was assessed by counting the vacuoles in ≥3 crypts per slide. The number of goblet cells was normalized to the total number of epithelial cells (obtained by counting the nuclei) in the same crypt. Periodic acid–Schiff (PAS) and Alcian blue staining was used for the evaluation of intraepithelial mucin content.

Culture media, reagents, and antibodies. In all of the in vitro assays, cells were maintained in very low endotoxin RPMI 1640 medium (Sigma), 10 m*M* HEPES (EuroClone), 10% fetal bovine serum (EuroClone), 100 units/ml penicillin/streptomycin, and 0.05 m*M* 2-mercaptoethanol (Sigma). Phosphate buffered saline was obtained from EuroClone.

The following antibodies were used for flow cytometric analysis: goat anti-human IL-22 (R&D Systems), fluorescein isothiocyanate (FITC)–conjugated anti-goat IgG (Becton Dickinson), phycoerythrin (PE)–conjugated mouse anti-human CD4 (Becton Dickinson), allophycocyanin (APC)–conjugated anti-human IL-17 (R&D Systems), PE-conjugated anti-human IFNγ (Becton Dickinson), PE-conjugated mouse anti-human NKp44, FITC-conjugated mouse anti-human NKp46 (BioLegend), APC-conjugated mouse anti-human CD3 (Becton Dickinson), and PerCP-conjugated mouse anti-human CD56 (Becton Dickinson). Isotype-matched irrelevant antibodies were used as a negative control.

Isolation of lamina propria MNCs. Lamina propria MNCs were isolated from the gut biopsy specimens of 15

patients with AS, 15 patients with CD, and 15 healthy controls, as previously described (2). The isolated cells were counted and checked for viability using 0.1% trypan blue (viability ranged from 90% to 94%). Lamina propria MNCs were gated on a lymphocyte gate based on forward and side scatter patterns.

Flow cytometric analysis. Cell surface fluorescence intensity was assessed using a FACSCalibur analyzer and CellQuest software (Becton Dickinson). For analysis of intracellular IL-17, IL-22, and IFNγ production, cells were stimulated with 1 μg/ml phorbol myristate acetate, 0.5 μg/ml ionomycin, and 10 μg/ml brefeldin A (all from Sigma-Aldrich) for 4 hours. Intracellular staining was performed using Cytofix/Cytoperm reagent (Becton Dickinson) followed by goat anti-human IL-22, APC-conjugated anti-human IL-17, and PE-conjugated anti-human IFNγ antibodies.

RNA extraction and quantitative TaqMan RT-PCR of ileal biopsy specimens for IL-17, IL-22, IL-23, IL-17, STAT-3, and MUC-1. Quantitative real-time PCR analysis of target gene expression in the intestinal biopsy samples was performed as previously described (1,2). For quantitative TaqMan real-time PCR, Master Mix and TaqMan gene expression assays for GAPDH control and for IL-22 (Hs01574154_m1), IL-23 (Hs00372324_m1), IL-17 (Hs00174383_m1), STAT-3 (Hs00234174_m1), and mucin 1 (MUC-1; Hs00904324_m1) were obtained from Applied Biosystems. Samples were run in triplicate using the One-Step real-time PCR system (Applied Biosystems). Relative changes in gene expression between control and inflamed ileal samples were determined using the $\Delta\Delta C_t$ method. Levels of the target transcript were normalized to a GAPDH endogenous control, expressed as a constant (ΔC_t) in both groups. For $\Delta\Delta C_t$ values, additional subtractions were performed between ΔC_t values for ileal samples from patients and those from controls. Final values were expressed as the fold induction.

Immunohistochemical analysis for NKp44, IL-22, and pSTAT-3. Immunohistochemical analysis for NKp44, IL-22, and pSTAT-3 was performed on 5-μm–thick paraffin-embedded intestinal biopsy sections and lymph node sections (used as positive controls) from patients and controls, as previously described (1). The primary antibodies, rabbit anti-human NKp44 (1:100 dilution; BioLegend), rabbit anti-human IL-22 (IgG, NB100-737; 1:100 dilution) (Novus Biologicals), and phosphorylated rabbit monoclonal anti-human pSTAT-3 (Tyr705) (IgG, D3A7; 1:50 dilution) (Cell Signaling Technology), were added and incubated for 1 hour at room temperature. Ileal and lymph node sections incubated with an isotype-matched control antibody were used as a negative control. The numbers of NKp44–, IL-22–, and pSTAT-3–expressing cells were determined by counting the immunoreactive cells on photomicrographs obtained from 3 random high-power microscopic fields (original magnification $\times$ 400) under a Leica DM2000 optical microscope using a Leica DFC320 digital camera.

Statistical analysis. Statistical analysis was performed using the Kruskal-Wallis nonparametric test, with Dunn's post hoc test. Pearson's correlation was used to quantify the expression associations between the genes of interest. *P* values less than 0.05 were considered significant. All data are expressed as the median (range).

RESULTS

Histomorphologic grading. On the basis of gut histology as defined above and as previously described (1), we identified 2 main groups of AS patients: patients displaying normal histology or minor inflammatory signs (acute lesions) (n = 8) and patients with chronic inflammation (n = 7). As shown in Table 1, the 2 groups of AS patients did not significantly differ with regard to demographic characteristics, clinical presentation, or medications received.

Expansion of CD3−CD56+NKp44+NKp46− NK cells in the inflamed ileum of AS patients. Two distinct populations of intestinal NK cells, CD3−CD56+NKp44+NKp46− and CD3−CD56+NKp44−NKp46+, classified on the basis of NKp44 and NKp46 surface expression, were observed. The proportion of CD3−CD56+NKp44+NKp46− cells was significantly increased in AS patients compared to CD patients and normal controls (Figure 1A), while the proportion of CD3−CD56+NKp44−NKp46+ cells was significantly increased in CD patients compared to AS patients and normal controls (Figures 1B and C). CD56+NKp44+NKp46− and CD56+NKp44−NKp46+ cell numbers were balanced in the ileal mucosa of normal controls (Figures 1A–C). The increase in the numbers of NKp44+ cells in AS patients was confirmed by immunohistochemical analysis performed on paraffin-embedded ileal sections from patients and controls. (Results are available from the author upon request.) Even though CD3−CD56+NKp44−NKp46+ cells were not increased in AS, we next evaluated the percentage of IFNγ-producing NKp46+ cells in all groups of subjects. As shown in Figure 1D, the percentage of IFNγ-producing CD3−CD56+NKp44−NKp46+ cells was significantly increased in CD patients but not in AS patients and controls. (Representative dot plots of isotype control antibodies are available from the author upon request.)

CD56+NKp44+NKp46− NK cells are the main source of IL-22 in the gut of AS patients. We next evaluated the cellular source of IL-22 among isolated lamina propria MNCs from AS patients. To date, the best-characterized human mucosal cells that secrete IL-22 are known to be CD4+IL-22–expressing T cells (Th1, Th17, and Th22) and NKp44+ NK cells (for review, see ref. 18). Different results were observed in the groups studied. CD3−CD56+ cells coexpressing NKp44 were the main producers of IL-22 in AS patients with chronic intestinal inflammation (median 3% [range 3–4%]), compared to AS patients with acute inflammation (median 1.3% [range 0.88–1.7%]), CD patients

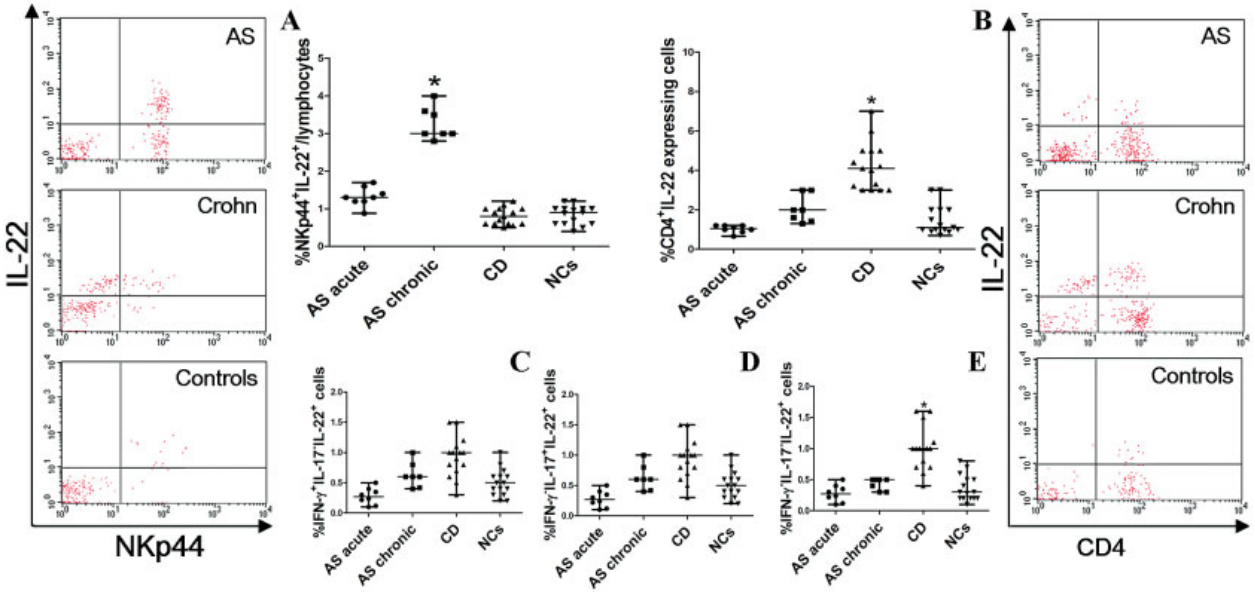

Figure 2. Interleukin-22 (IL-22)–producing cells in the gut of patients and controls. Freshly isolated lamina propria MNCs from 15 patients with AS, 15 patients with CD, and 15 healthy subjects were analyzed by flow cytometry. **A,** Representative dot plot of IL-22–expressing versus NKp44-expressing cells among lamina propria MNCs from patients and controls (left) and percentages of NKp44+IL-22+ cells among isolated lamina propria MNCs from patients and controls (right). $* = P < 0.05$ versus all other groups. **B,** Percentages of CD4+IL-22+ cells among isolated lamina propria MNCs from patients and controls (left) and representative dot plot of IL-22–expressing versus CD4-expressing cells among lamina propria MNCs from patients and controls (right). $* = P < 0.05$ versus all other groups. **C–E**, Percentages of different subsets of IL-22–producing Th1 (**C**), Th17 (**D**), and Th22 (**E**) cells among lamina propria MNCs from patients and controls. In **C** and **D**, $P < 0.0001$, CD versus AS acute; $P < 0.01$, CD versus normal controls. In **E**, $* = P < 0.05$ versus all other groups. Symbols represent individual patients, horizontal lines represent the median, and whiskers represent the range. 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.

(median 0.8% [range 0.55–1.2%]), and normal controls (median 0.9% [range 0.4–1.2%]) ($P < 0.05$, AS patients with chronic inflammation versus all other groups) (Figure 2A). Conversely, CD4+ T lymphocytes were the main source of IL-22 in CD patients (median 4.1% [range 3–7%]), compared to AS patients with chronic inflammation (median 2% [range 1.3–3%]), AS patients with acute inflammation (median 1.05% [range 0.66–1.2%]), and normal controls (median 1.1% [range 0.7–3%]) ($P < 0.05$, CD patients versus all other groups) (Figure 2B). Among CD4+ T cells, the percentage of IL-22–producing Th1, Th17, and Th22 cells was not significantly increased in AS patients, regardless of the degree of intestinal inflammation, compared to controls (Figures 2C–E). Conversely, the percentages of IL-22–producing Th1, Th17, and Th22 cells were significantly expanded in CD patients (Figures 2C–E).

Overexpression of IL-22 mRNA in the inflamed ileum of AS patients. The results described above strongly suggest that IL-22–producing CD3− CD56+NKp44+NKp46− NK cells are enriched and represent the main cellular source of IL-22 in the inflamed ileum of AS patients. We next assessed the expression of IL-22, IL-23, STAT-3, and MUC-1 mRNA in the inflamed ileum of AS patients, CD patients, and controls.

As previously described (1), IL-23 levels, but not IL-17 levels, were significantly increased in AS patients (data not shown). Because IL-22 has been demonstrated to be produced when IL-23 is stimulated, we next evaluated the expression of IL-22. As shown in Figure 3A, IL-22 was significantly overexpressed in the inflamed ileum of patients with chronic AS and patients with CD compared to patients with acute AS and controls. IL-22 levels were ~40-fold and ~50-fold higher in patients with chronic AS and patients with CD, respectively, than in controls.

Intestinal epithelial STAT-3 activation has been demonstrated to regulate immune homeostasis in the gut by promoting IL-22–dependent mucosal wound healing (19). Consistent with IL-22 up-regulation, STAT-3 was significantly increased in AS patients, to

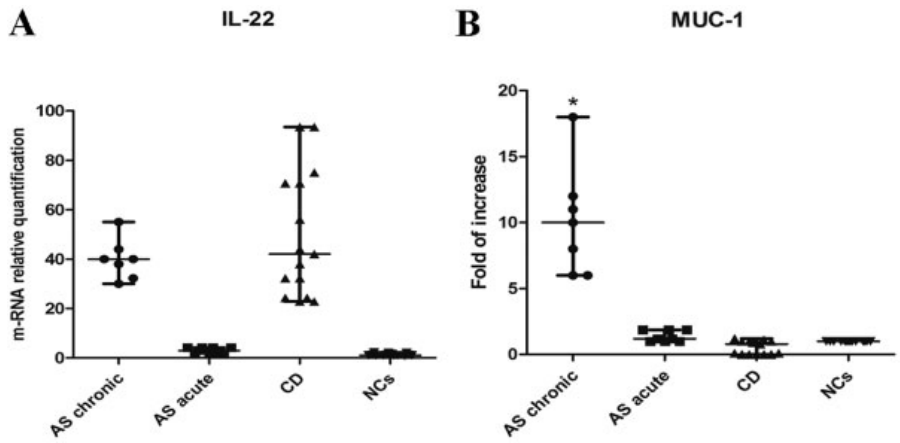

Figure 3. Interleukin-22 (IL-22) and mucin 1 (MUC-1) gene expression profiles in mucosal biopsy specimens from AS patients, CD patients, and normal controls. **A**, Relative mRNA quantification of IL-22, assessed by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR), in ileal biopsy specimens. $P < 0.05$, AS chronic versus AS acute; $P < 0.05$, AS chronic versus normal controls; $P < 0.0001$, CD versus AS acute; $P < 0.0001$, CD versus normal controls. **B**, Relative mRNA quantification of MUC-1, assessed by quantitative RT-PCR, in ileal biopsy specimens. $* = P < 0.0001$ versus all other groups. Symbols represent individual patients, horizontal lines represent the median, and whiskers represent the range. See Figure 1 for other definitions.

levels similar to those observed in CD patients (data not shown).

Because IL-22 has been demonstrated to induce tissue-protective responses through the production of mucins by intestinal epithelial cells (10), we also evaluated the expression of MUC-1 mRNA. As shown in

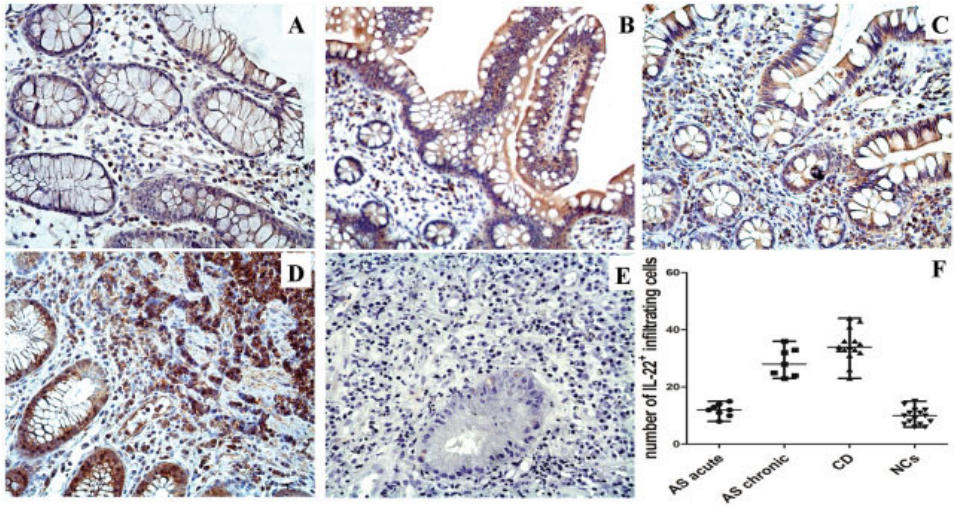

Figure 4. Immunohistochemical analysis of interleukin-22 (IL-22) expression in ileal samples from patients and controls. Five-micrometer–thick paraffin-embedded sections of ileal biopsy specimens obtained from patients and controls were treated with isotype control or anti–IL-22 antibodies. **A–D**, Staining for IL-22 in specimens from a normal control (**A**), an AS patient with acute inflammation (**B**), an AS patient with chronic inflammation (**C**), and a CD patient (**D**). IL-22–positive cells were rarely observed in normal controls and in AS patients with acute inflammation. Significant staining for IL-22 was observed only in AS patients with chronic inflammation and CD patients. Positively stained cells were observed among infiltrating MNCs and epithelial cells. Original magnification × 250. **E,** An ileal biopsy specimen from a CD patient, treated with isotype control antibody. Original magnification × 250. **F,** Quantification of IL-22+ cells in ileal biopsy specimens from patients and controls. Results are expressed as the number of positive cells per field. Symbols represent individual patients, horizontal lines represent the median, and whiskers represent the range. $P < 0.05$, AS acute versus AS chronic; $P < 0.01$, AS acute versus CD; $P < 0.01$, AS chronic versus normal controls; $P < 0.001$, CD versus normal controls. See Figure 1 for other definitions.

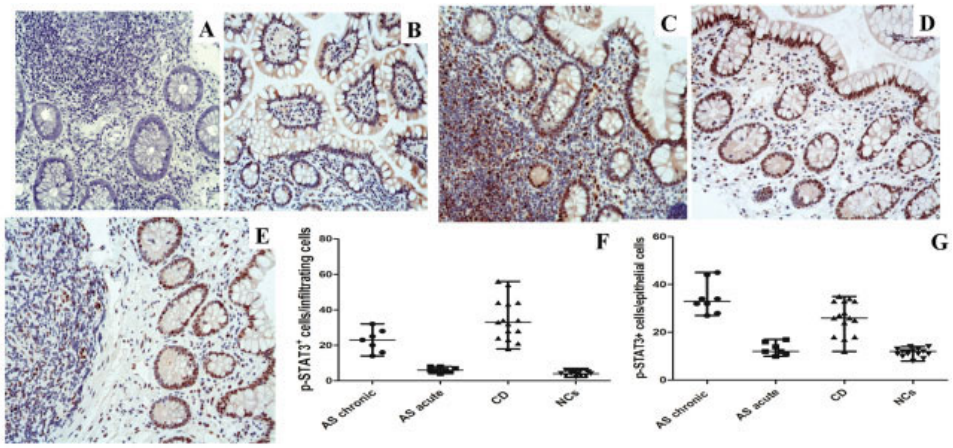

Figure 5. Immunohistochemical analysis of pSTAT-3 expression in ileal samples from patients and controls. Five-micrometer–thick paraffin-embedded sections of ileal biopsy specimens obtained from patients and controls were treated with isotype control or pSTAT-3 antibodies. **A**, An ileal biopsy specimen from a CD patient, treated with isotype control antibody. Original magnification × 250. **B–E,** Staining for pSTAT-3 in specimens from a normal control (**B**), a CD patient (**C**), an AS patient with acute inflammation (**D**), and an AS patient with chronic inflammation (**E**). Epithelial cells positive for pSTAT-3 were rarely observed in normal controls. Abundant pSTAT-3+ infiltrating inflammatory cells were observed in CD patients. CD patients, AS patients with acute inflammation, and AS patients with chronic inflammation showed intense epithelial pSTAT-3 positivity. Original magnification × 250. **F** and **G**, Quantification of pSTAT-3+ cells among infiltrating cells (**F**) and epithelial cells (**G**) in ileal biopsy specimens from patients and controls. Results are expressed as the number of positive cells per field. Symbols represent individual patients, horizontal lines represent the median, and whiskers represent the range. In **F**, $P < 0.01$, AS chronic AS acute; $P < 0.001$, AS chronic versus CD; $P < 0.05$, AS chronic versus normal controls; $P < 0.0001$, CD versus normal controls. In **G**, $P < 0.01$, AS chronic versus AS acute; $P < 0.0001$, AS chronic versus normal controls; $P < 0.05$ CD versus AS acute; $P < 0.0001$, CD versus normal controls. 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.

Figure 3B, MUC-1 expression was increased (8 fold) in AS patients with chronic inflammation compared to controls. MUC-1 mRNA expression was decreased in the involved ileal mucosa of patients with Crohn's disease when compared to controls. The difference, however, was not significant and was presumably the result of loss of epithelial integrity due to inflammatory processes.

IL-22 and pSTAT-3 expression in ileal specimens from patients with AS with chronic inflammation. Given the strong up-regulation of IL-22 mRNA in mucosal biopsy specimens from patients with AS and patients with CD, we next investigated its protein expression. IL-22–positive cells were rarely observed in normal controls (Figure 4A) or AS patients with acute inflammation (Figure 4B). Immunohistochemical analysis of all inflamed specimens from AS patients and CD patients showed a higher expression of IL-22 in AS patients with chronic inflammation (Figure 4C) and patients with CD (Figure 4D), confirming the up-regulation of IL-22 mRNA observed in the RT-PCR assay. Cells with positive staining were observed among MNCs distributed within the lymphomonocytic inflammatory infiltrates and epithelial cells (Figures 4C and D). Terminal ileal biopsy specimens from AS patients with chronic inflammatory lesions and from CD patients showed the highest fraction of IL-22–expressing cells, which was significantly higher than that in controls (Figure 4F).

Both IL-23 and IL-22 signal through STAT-3 in different cellular targets, the former mainly in lymphoid cells and the latter mainly in epithelial cells. To assess whether IL-22 and IL-23 signals were active, we looked for the activation of the downstream signaling pathway by evaluating STAT-3 expression. Since the function of STAT molecules is not reflected by transcript levels but rather by their phosphorylation status, we next investigated pSTAT-3 protein expression in ileal specimens from patients and controls. As shown in Figure 5B, pSTAT-3–expressing cells were rarely detected in normal controls. Immunohistochemical analysis of samples from AS patients and CD patients demonstrated 2 different patterns of pSTAT-3 expression. The first pattern, prominent in CD patients, was characterized by a large number of pSTAT-3–expressing cells within the lymphomonocytic inflammatory infiltrates (Figure 5C). The second pattern of pSTAT-3 expression, characterized by abundant epithelial expression of pSTAT-3, was detected in the terminal ileum of both CD and AS

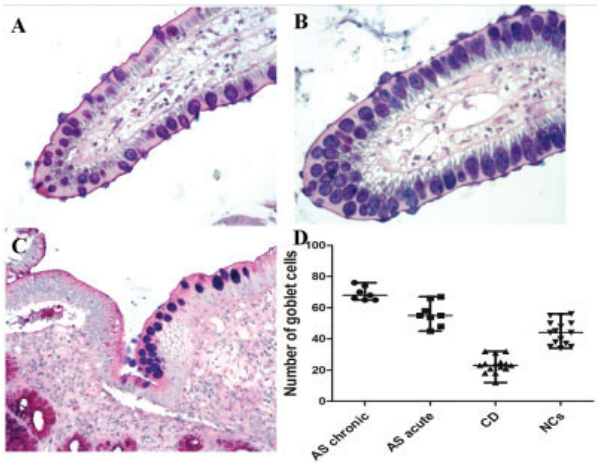

Figure 6. Mucin content and number of goblet cells in ileal samples from patients and controls. **A–C**, Representative photomicrographs showing 5-μm–thick paraffin-embedded sections of ileal biopsy specimens obtained from a normal control (**A**), an AS patient (**B**), and a CD patient (**C**), stained with periodic acid–Schiff and Alcian blue for evaluation of mucin content. Increased mucin content and increased numbers of mucin-positive epithelial cells were observed in AS patients compared to healthy controls and CD patients. Original magnification $\times$ 100. **D,** Number of goblet cells in the mucosa. Results are expressed as the number of positive cells per field. Symbols represent individual patients, horizontal lines represent the median, and whiskers represent the range. $P < 0.0001$, AS chronic versus CD; $P < 0.0001$, AS acute versus CD; $P < 0.05$, AS chronic versus normal controls; $P < 0.001$, CD versus normal controls. 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.

patients irrespective of the presence or absence of inflammatory lesions (Figures 5C–E).

We then compared the numbers of pSTAT-3–positive cells in samples from AS patients, CD patients, and normal controls. As shown in Figures 5F and G, terminal ileal biopsy specimens from AS patients with chronic inflammatory lesions and CD patients showed the highest fraction of pSTAT-3–expressing cells among infiltrating cells (median 26% [range 17–28%] and 34% [range 18–56%], respectively) and epithelial cells (median 33% [range 27–46%] and 26% [range 12–35%], respectively) compared to controls (median 4% [range 2–6%] in infiltrating cells and 11% [range 6–14%] in epithelial cells). In AS patients with acute inflammation, pSTAT-3 expression in infiltrating cells (median 6% [range 4–8%]) and epithelial cells (median 12% [range 11–17%]) was not significantly different from that in controls.

Interestingly, in AS patients with chronic inflammation but not in CD patients, the number of IL-22+ cells was significantly correlated with the number of pSTAT-3–positive epithelial cells. (Results are available from the author upon request.) This suggests a prominent role of pSTAT-3 in mediating IL-22 signaling at this site in AS patients.

Goblet cell count and evaluation of mucin content in subclinical gut inflammation in AS patients. In experimental models of intestinal inflammation, IL-22 enhances STAT-3 activation specifically within colonic epithelial cells and induces both STAT-3–dependent expression of mucus-associated molecules and restitution of mucus-producing goblet cells (10). In this regard, we next considered the frequency of mucin-producing goblet cells and the mucin content in the ileum of patients and controls.

Specimens from AS patients with chronic inflammation showed an increased percentage of goblet cells (64%) compared to controls (47%) ($P < 0.05$) (Figures 6A, B, and D). A significantly reduced percentage of goblet cells was observed in CD patients (23%; $P < 0.001$ versus controls), probably due to the inflammatory process–related loss of epithelial integrity (Figures 6C and D).

A strong Alcian blue PAS-positive reaction was seen in AS patients compared to controls and CD patients (Figures 6A–C).

DISCUSSION

This is the first study to show that NKp44+ NK cells are increased in the gut mucosa of patients with AS exhibiting subclinical chronic intestinal inflammation. We also demonstrate that IL-22 is overexpressed in the ileum of AS patients, and that NKp44+ NK cells represent its major source. Finally, increased IL-22 expression is accompanied by goblet cell hyperplasia and increased mucus production, suggesting that it plays a tissue-protective rather than a proinflammatory role in AS.

Natural killer cells are innate lymphocytes involved in innate and adaptive immune responses. Although classified into 2 main subsets on the basis of the level of expression of the adhesion molecule CD56, NK cells have long been considered to be a homogeneous population with stereotyped roles in immune responses. Recently, several studies have demonstrated significant diversification of NK cell function in the intestinal mucosa (4–8), identifying a rare mucosal NK cell subpopulation expressing the nuclear transcription factor retinoic acid receptor–related orphan nuclear receptor (RORγt) (which is not expressed by classic NK cells) and the surface marker NKp44 (which is expressed by

activated classic NK cells). Although the nature of NKp44+ non-T cells and their relationship with classic NK cells is currently a subject of controversy, lamina propria NK cells have been classified, on the basis of the expression of the 2 natural cytotoxic receptors NKp44 and NKp46, into 2 main subsets, NKp44+ and NKp46+ NK cells, which are balanced in normal human intestinal mucosa (9). Although NKp44+ and NKp46+ NK cells seem to play divergent roles in the gut, the former being essentially protective through the release of IL-22, and the latter being essentially pathogenic through the production of IFNγ, both are controlled by IL-23 signaling (4,9).

IL-22 is a member of the IL-10 family of cytokines (for review, see ref. 18). The direct effects of IL-22 are restricted to nonhematopoietic cells, since its receptor is expressed on the surface of epithelial cells and some fibroblasts in various organs, including parenchymal tissue of the gut, lung, skin, and liver. All studies investigating IL-22–induced signal transduction in cells with endogenous receptor expression demonstrated tyrosine phosphorylation of STAT-3 (4,19–22).

Although IL-22 has been demonstrated to ameliorate intestinal inflammation through the induction of mucin expression and the restitution of goblet cells during recovery from acute intestinal injury in a mouse model of ulcerative colitis (10), the exact role of IL-22, whether protective or inflammatory, in human inflammatory bowel disease is still poorly understood. Although it is likely that the pathogenic effect of IL-22 could reflect dysregulated duration of production and/or the concentration of IL-22, the functional outcomes of IL-22 expression seem to be governed by coexpression of IL-17A. When coexpressed, IL-17A and IL-22 act synergistically to promote chemokine expression, neutrophil recruitment, and inflammation, whereas IL-22 in the absence of IL-17A promotes essentially tissue-protective responses (16).

In the present study, IL-22 expression, as demonstrated by both RT-PCR and immunohistochemistry, was more pronounced in AS patients with chronic intestinal inflammation and CD patients and was accompanied by increased IL-23 but not IL-17 levels in AS patients. Intense positive staining for IL-22 was observed among the MNCs infiltrating the inflamed ileal biopsy specimens of both AS patients and CD patients. Although it is arduous to immunohistochemically distinguish between surface staining and intracellular staining, we also observed strong staining for IL-22 in the epithelium, which we believe is produced by epithelial cells. Indeed, expression of IL-22 has recently been reported in epithelial cells from the salivary glands of patients with Sjögren's syndrome (23).

Interestingly, in AS patient samples, IL-22 expression at the protein level was significantly correlated with the increased epithelial expression of pSTAT-3. The absence of concomitant IL-17 expression, taken together with STAT-3 mRNA overexpression and the observation that the active phosphorylated form of STAT-3 resides almost exclusively in the epithelial cells of the ileum of AS patients, could suggest a prevalent role of pSTAT-3 in regulating immune homeostasis in the gut by promoting IL-22–dependent mucosal wound healing. In this regard, expansion of IL-22–producing NKp44+ cells in AS patients was associated with increased MUC-1 transcript levels and an increased number of mucin-producing goblet cells, supporting the notion of a protective phenotype in the ileum of AS patients.

This study suggests that intestinal overexpression of IL-23 in AS patients could be oriented toward a protective rather than a pathogenic role in intestinal inflammation. IL-23 overexpression could play a central role in modulating IL-22 production in NKp44+IL-22+ cells, generating an intestinal milieu rich in STAT-3, which favors the expression of RORγt in lymphoid cells of the lamina propria (24), the expression of IL-22, and, under certain conditions (such as the concomitant presence of proinflammatory cytokines), the expression of IL-17.

In conclusion, this is the first study to demonstrate that IL-22+NKp44+ NK cells are significantly expanded in the inflamed ileum of AS patients. Differential temporal and spatial coexpression of IL-22 and IL-17 may determine the balance between the protective and proinflammatory roles of IL-22 in the intestinal mucosa in AS patients and the eventual evolution toward overt Crohn's disease. The factors conditioning this evolution remain to be elucidated.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. 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 conception and design. Ciccia, Accardo-Palumbo, Rizzo, Principe, Peralta, Raiata, De Leo, Triolo.

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