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Interleukin-9 Overexpression and Th9 Polarization Characterize the Inflamed Gut, the Synovial Tissue, and the Peripheral Blood of Patients With Psoriatic Arthritis

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Objective. To investigate the expression and tissue distribution of Th9-related cytokines in patients with psoriatic arthritis (PsA).

Methods. Quantitative gene expression analysis of Th1, Th17, and Th9 cytokines was performed in intestinal biopsy samples obtained from patients with PsA, HLA-B27–positive patients with ankylosing spondylitis (AS), patients with Crohn's disease (CD), and healthy controls. Expression and tissue distribution of interleukin-23 (IL-23), IL-17, IL-22, IL-9, and IL-9 receptor (IL-9R) were evaluated by immunohistochemistry and confocal microscopy. Flow cytometry was used to study the frequency of Th9 cells among peripheral blood, lamina propria, and synovial fluid mononu-

clear cells. The functional relevance of IL-9R expression on epithelial cells was assessed in functional in vitro studies. Th9 cells in synovial tissue from patients with PsA were also studied.

Results. Subclinical gut inflammation in PsA patients was characterized by a clear Th17 and Th22, but not Th1, polarized immune response. Unlike AS and CD, a strong and significant up-regulation of IL-9 was observed in PsA gut, especially among infiltrating mononuclear cells, high endothelial venules, and Paneth cells. IL-9–positive mononuclear cells were demonstrated to be in large part Th9 cells. IL-9 overexpression was accompanied by significant Paneth cell hyperplasia. Paneth cells strongly overexpressed IL-9R, and stimulation of epithelial cells, isolated from PsA patients, with IL-9 resulted in overexpression of α -defensin 5 and IL-23p19. Peripheral and synovial expansion of α 4 β 7+ Th9 cells was also observed in patients with PsA. Increased expression of IL-9 and IL-9R was also found in synovial tissue.

Conclusion. Strong IL-9/Th9 polarization seems to be the predominant immunologic signature in patients in PsA.

The relationship between inflammatory bowel disease and psoriasis has been investigated in epidemiologic, genetic, and immunologic studies for many years, and recently, the relative risk of incident Crohn's disease (CD) and ulcerative colitis in patients with psoriasis and psoriatic arthritis (PsA) has been described (1). However, to date the role of different T cell subsets in PsA gut pathology has not been addressed. The pathogenesis of PsA seems to be multifactorial, and immunologically driven by a mixed Th1 and interleukin-23

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(IL-23)/Th17 response (2–4). Recently IL-9, a member of the IL-2 cytokine family that is secreted by naive CD4+ T cells in response to transforming growth factor β (5), has been demonstrated to be involved in the pathogenesis of several autoimmune diseases, including psoriasis (6–9). In the present study we aimed to characterize the subclinical gut inflammation of PsA, to investigate the expression and tissue distribution of IL-9/Th9-related molecules, and to evaluate the effect of tumor necrosis factor inhibitor (TNFi) and ustekinumab therapy on circulating Th9 cells.

PATIENTS AND METHODS

Patients. Intestinal biopsy specimens were obtained from 25 patients with PsA fulfilling the criteria of the Classification of Psoriatic Arthritis Study Group (10) (11 with predominant axial involvement) and from 15 patients with HLA-B27-positive ankylosing spondylitis (AS) fulfilling the New York criteria (11), without gastrointestinal symptoms. Ten patients with CD and 20 healthy controls who underwent ileocolonoscopy for routine clinical evaluation were also included in the study. Synovial tissue samples were also obtained from an additional 5 PsA patients with active disease at the time of sample collection and from 5 patients with osteoarthritis (OA) (12). Baseline characteristics of the patients and controls are shown in Supplementary Table 1 (on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>). Disease activity in patients with PsA with axial involvement and patients with AS was assessed with the Bath Ankylosing Spondylitis Disease Activity Score (BASDAI) (13); the 28-joint Disease Activity Score (DAS28) (14) was used to evaluate disease activity in patients with PsA and peripheral disease, and the Crohn's Disease Activity Index (CDAI) (15) was used to assess patients with CD. The severity of psoriasis was evaluated by the Psoriasis Area and Severity Index (PASI) (16). Collection of samples was approved by the local ethics committee and the University of Palermo Institutional Review Board, and informed consent was obtained from all patients and controls.

Histomorphologic grading and immunohistochemistry analysis. Biopsy specimens were evaluated by 2 experienced pathologists with expertise in inflammatory bowel diseases (AR and AC) who were blinded with regard to clinical data. Interrater agreement between the 2 pathologists, calculated with Cohen's kappa coefficient, was 0.85.

Gut specimens from patients with PsA and AS were assessed histologically for the presence of inflammation and classified according to the stage of histologic severity, as follows (17): stage 0 = normal histopathologic findings; stage 1 = lymphoid hyperplasia with increased inflammatory cell content in the lamina propria without evidence of cryptitis or epithelial abnormalities; stage 2 = diffuse increase of inflammatory cells in the lamina propria with crypt distortion, hyperplasia of crypt cell epithelium, infiltration of neutrophils, and crypt abscesses; stage 3 = ulcerations with or without epithelioid granulomas. Lymphoid follicles were identified using CD3/CD19/CD21 immunostaining.

The degree of inflammation was also evaluated in synovial tissue obtained from 5 patients with PsA and, as controls, from 5 patients with end-stage OA who were undergoing knee joint replacement surgery. The grade of tissue inflammation was also assessed by evaluating the number of CD3+, CD19+, and CD68+ cells. Immunohistochemistry analysis was performed as previously described (18); antibodies used are listed in Supplementary Table 2 (<http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>). The number of immunoreactive cells and lymphoid follicles was determined by counting the reactive cells/lymphoid follicles seen on photomicrographs obtained from 3 randomly and independently selected high-power fields (hpf).

To specifically address the nature of IL-9-producing cells, double staining for Th2 (IL-4/IL-9), Th17 (IL-9/IL-17), and Paneth cells (IL-9/ α -defensin 5) and triple staining for Th9 cells (CD4/IL-9/PU.1) were performed on paraffin-embedded synovial and ileal sections. Sections were treated with fluorescein isothiocyanate-, rhodamine red-, or Cy5-conjugated anti-mouse or anti-rabbit antibodies (Invitrogen) plus RNase (200 ng/ml) and counterstained using TOTO-3 iodide (642/660; Invitrogen) or DAPI (Life Technologies). Confocal microscopy was used to assess fluorescence staining.

RNA extraction and quantitative TaqMan real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of ileal biopsy specimens. Total RNA was extracted using a Qiagen RNeasy Mini kit, with on-column DNase I digestion. A total of 1 μ g of RNA was reverse-transcribed to complementary DNA using a ThermoScript First-Strand cDNA Synthesis kit (Invitrogen). For quantitative TaqMan real-time PCR, sets of primers and probes (Supplementary Table 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>) were obtained from Applied Biosystems. Samples were run in triplicate using a Step-One Real-Time PCR system (Applied Biosystems). Relative differences in gene expression between controls and patients were determined using the $\Delta\Delta C_t$ method as previously described (9). Final values were expressed as fold induction compared to controls.

Peripheral blood mononuclear cell (PBMC) and synovial fluid mononuclear cell (SFMC) isolation and flow cytometry. PBMCs were obtained as previously described (19) from 5 of the PsA patients in whom ileocolonoscopy was also performed and from 9 additional PsA patients (before and after TNFi treatment in 5 and before and after ustekinumab treatment in 4), 10 AS patients, 10 CD patients, and 10 healthy controls. SFMCs were also obtained from 5 PsA patients who underwent ileocolonoscopy and 5 OA patients. Cell viability (assessed by trypan blue dye exclusion) was always >95%. Four-color flow cytometric analysis was performed using a FACSCalibur (BD Biosciences). At least 50,000 cells (events), gated on lymphocytes or monocyte/macrophage regions, were acquired for each sample. Antibodies used are listed in Supplementary Table 2 (<http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>).

Cell cultures. Intestinal epithelial cells were isolated from ileal samples from 5 PsA patients and 5 controls as previously described (20) and cultured in 24-well flat-bottomed plates (Becton Dickinson Labware) at a density of 1×10^6 cells in 1 ml RPMI 1640 medium with 10% fetal calf serum, 2 mM L-glutamine, 20 nM HEPES, and 100 units/ml penicillin/streptomycin with or without recombinant IL-9 (0.1 ng/ml or 10 ng/ml; R&D Systems). Cells were incubated at 37°C in a

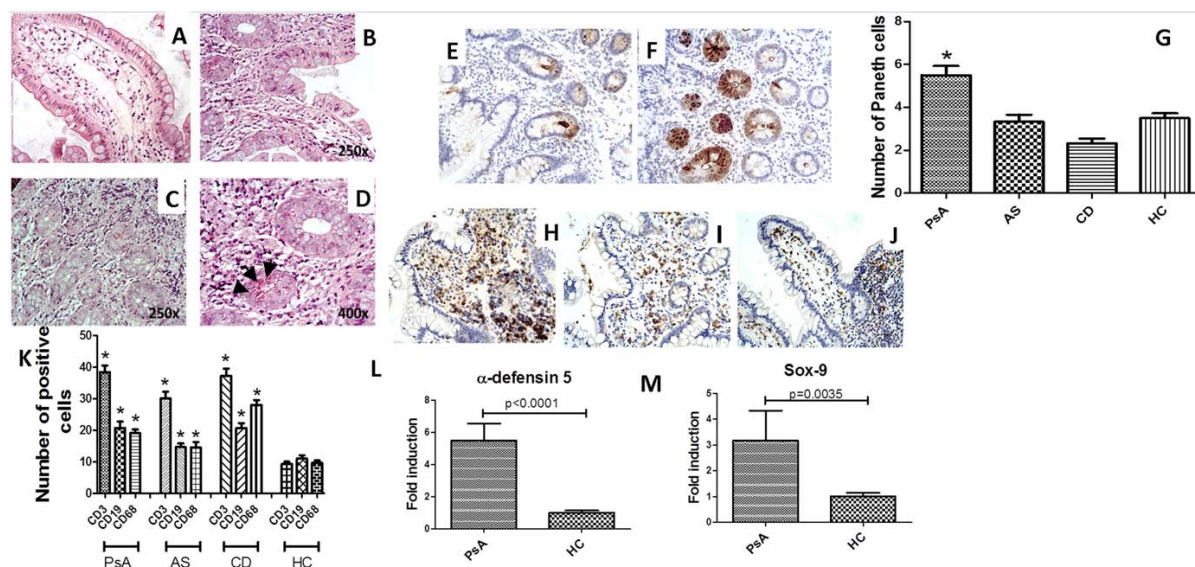


Figure 1. High levels of tissue inflammation and Paneth cell hyperplasia characterize subclinical gut inflammation in psoriatic arthritis (PsA). **A–D**, Representative images of hematoxylin and eosin–stained ileal sections from patients with PsA. **Arrowheads** in **D** indicate Paneth cells. **E** and **F**, Representative images of α -defensin 5 staining in samples from a healthy control (HC) (**E**) and a patient with PsA (**F**). **G**, Semiquantitative evaluation of Paneth cells (per high-power field [hpf]) in patients with PsA, ankylosing spondylitis (AS), and Crohn's disease (CD) and in healthy controls. **H–J**, Inflammatory infiltrating cells and CD3-positive cells (**H**), CD19-positive cells (**I**), and CD68-positive cells (**J**) in PsA gut. **K**, Semiquantitative evaluation of CD3-, CD19-, and CD68-positive cells (per hpf) in patients with PSA, AS, and CD and healthy controls. **L** and **M**, Relative levels of mRNA for α -defensin 5 (**L**) and SOX9 (**M**), assessed by quantitative reverse transcription–polymerase chain reaction, in ileal samples from PsA patients ($n = 25$) compared to healthy controls ($n = 20$). Values in **G** and **K–M** are the mean \pm SEM. * = $P < 0.05$ versus controls. Original magnification $\times 250$ in **A**, **E**, **F**, and **H–J**. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/journal/doi/10.1002/art.39649/abstract>.

humidified atmosphere containing 5% CO₂ for 48 hours, and levels of IL-17, IL-22, IL-23, retinoic acid receptor–related orphan nuclear receptor, and STAT-3 were then determined by RT-PCR.

Statistical analysis. The statistical significance of differences between groups was calculated by Student's *t*-test or the nonparametric Mann-Whitney test. Paired samples were analyzed by Wilcoxon's signed rank test. *P* values less than 0.05 were considered significant.

RESULTS

Subclinical gut inflammation in PsA patients is characterized by histologic inflammation and Paneth cell hyperplasia. All of the patients with CD had active disease (mean CDAI score 311 [range 200–430]). The endoscopic appearance of ileum seen on colonoscopy demonstrated an absence of macroscopic alterations in 10 of the PsA patients and 8 of the AS patients. The presence of mucosal erythema and erosions was observed in the remaining PsA, AS, and CD patients. Stage 0–1 lesions were observed in 6 PsA patients and 4 AS patients without macroscopic involvement (24% and 26.7% of the PsA group and the AS group, respec-

tively), stage 2 lesions were observed in 12 PsA patients (4 without macroscopic involvement) and 7 AS patients (4 without macroscopic involvement) (48% and 46.7% of the PsA and AS groups, respectively), and stage 3 lesions were observed in 7 PsA patients and 4 AS patients (28% and 26.7%, respectively).

Similar to findings in AS, PsA patient ileum exhibited an increased number of infiltrating inflammatory CD3+, CD19+, and CD68+ cells (Figures 1B, C, and H–K and Supplementary Table 3, on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>), an increased number of lymphoid follicles (mean \pm SEM 2.5 \pm 0.9/hpf in PsA patients compared to 1.4 \pm 0.6 in healthy controls; $P < 0.05$) and significant Paneth cell hyperplasia (Figures 1D and G and Supplementary Table 3), which correlated with the grade of intestinal inflammation (number of Paneth cells 6 \pm 0.72/hpf in histologic stage 3, 5.2 \pm 0.84 in stage 2, and 3.4 \pm 0.88 in stage 0–1). Paneth cell hyperplasia was also accompanied by increased expression of messenger RNA (mRNA) for Paneth cell–derived antimicrobial peptides, such as α -

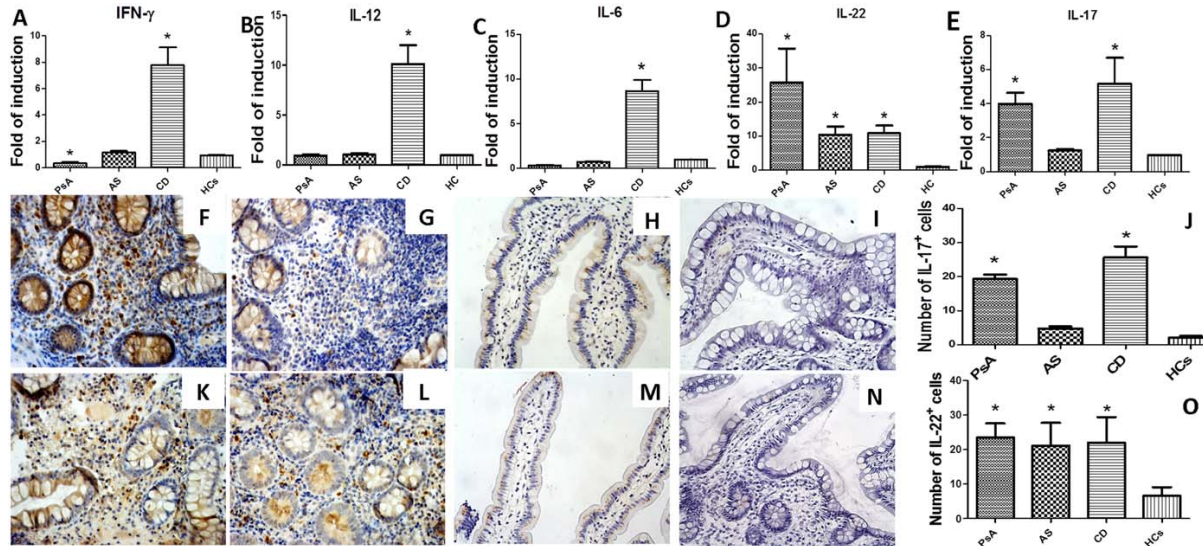


Figure 2. Th1 and Th17 cytokines in the gut of patients with PsA. **A–E**, Relative levels of mRNA for interferon- γ (IFN γ) (**A**), interleukin-12 (IL-12) (**B**), IL-6 (**C**), IL-22 (**D**), and IL-17 (**E**), assessed by quantitative reverse transcription–polymerase chain reaction, in ileal samples from patients with PsA, AS, and CD and healthy controls. **F–H**, Representative photomicrographs showing IL-17 immunostaining in samples from a patient with PsA (**F**), a patient with AS (**G**), and a healthy control (**H**). **J**, Semiquantitative evaluation of IL-17–positive cells (per hpf) in the ileum of patients with PsA, AS, and CD and healthy controls. **K–M**, Representative photomicrographs showing IL-22 immunostaining in samples from a patient with PsA (**K**), a patient with AS (**L**), and a healthy control (**M**). **I** and **N**, Representative photomicrographs showing isotype control staining for IL-17 (**I**) and IL-22 (**N**) in samples from patients with PsA. **O**, Semiquantitative evaluation of IL-22–positive cells (per hpf) in the ileum of patients with PsA, AS, and CD and healthy controls. Values in **A–E**, **J**, and **O** are the mean \pm SEM. * = $P < 0.05$ versus controls. Original magnification $\times 400$ in **F**, **G**, and **K**; $\times 100$ in **H** and **L**; $\times 250$ in **I**, **M**, and **N**. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/journal/doi/10.1002/art.39649/abstract>.

defensin 5, and of the transcription factor SOX9, which is involved in Paneth cell differentiation (21) (Figures 1L and M). No differences in the degree of tissue inflammation and/or number of infiltrating cells were observed between patients who were taking nonsteroidal antiinflammatory drugs (NSAIDs) and those who were not (data not shown). Collectively, these data confirm that in patients with PsA, similar to patients with AS, the ileum is characterized by subclinical histologic inflammation.

Th1 and Th17 polarization in the gut of patients with PsA. We next investigated the expression of cytokines involved in the Th1 and IL-23/Th17 axis. Unlike the findings in CD, and similar to those in AS, Th1 cytokines were not significantly overexpressed in PsA (Figures 2A–C). With regard to Th17 cells, a clear overexpression of IL-17 and IL-22 mRNA (Figures 2D and E) was demonstrated in PsA patients. Immunohistochemistry results confirmed the RT-PCR data demonstrating strong up-regulation of IL-17 (Figures 2F–H and J) and IL-22 (Figures 2K–M and O and Supplementary Table 3, on the *Arthritis & Rheumatology* web

site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>) in PsA patients, at levels similar to those observed in patients with CD.

IL-9 overexpression and Th9 polarization in the gut of patients with PsA. We next aimed to investigate in more detail the dominant cytokine pathways involved in subclinical gut inflammation in PsA. Based on recent reports indicating a key role of IL-9 in the pathogenesis of psoriasis (6,22), we studied the expression of IL-9 and Th9 in the gut of PsA patients. We first assessed ileal biopsy specimens by RT-PCR for IL-9 and Th9-related transcription factor mRNA expression. Levels of IL-9, PU.1, and interferon regulatory factor 4 mRNA were significantly up-regulated in the gut of PsA patients, especially in those with stage 2–3 lesions, but not in the gut of CD patients, AS patients, or controls (Figures 3A–C). In order to confirm IL-9 mRNA expression, paraffin sections of ileal specimens from patients and controls were stained with anti-IL-9 antibody. IL-9 immunoreactivity was observed among infiltrating mononuclear cells (Figures 3D–F), high endothelial venules (Figure 3F), and epithelial cells

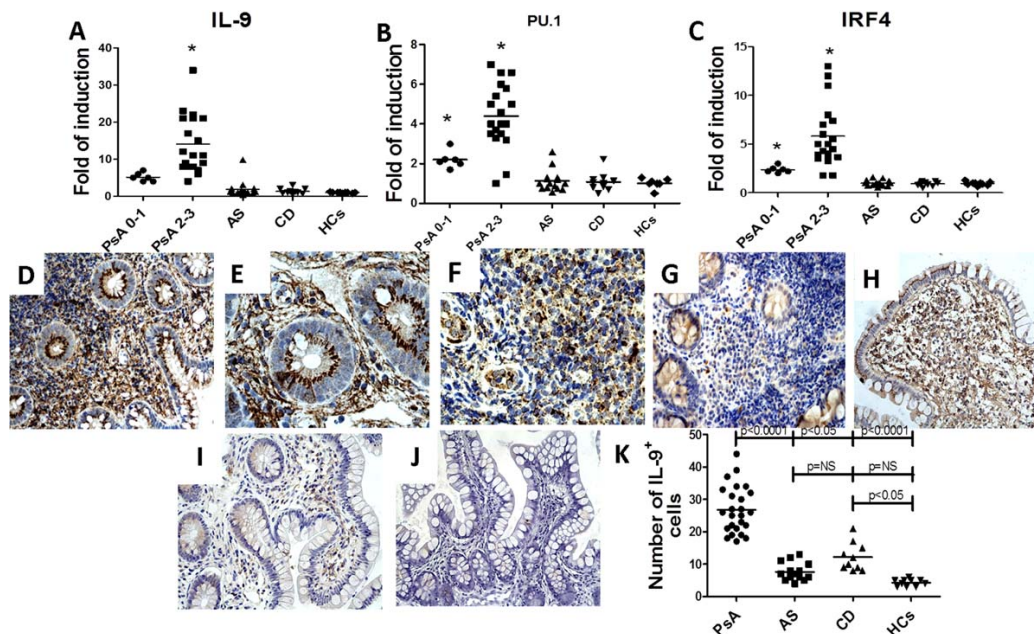


Figure 3. Interleukin-9 (IL-9) in the gut of patients with PsA. **A–C**, Relative levels of mRNA for IL-9 (**A**), PU.1 (**B**), and interferon regulatory factor 4 (IRF4) (**C**), assessed by quantitative reverse transcription–polymerase chain reaction, in ileal samples from patients with PsA and an ileal histologic score of 0 or 1 ($n = 6$), PsA and an ileal histologic score of 2 or 3 ($n = 19$), AS ($n = 15$), and CD ($n = 10$) and healthy controls ($n = 20$). $*$ = $P < 0.05$ versus controls. **D–I**, Representative photomicrographs showing IL-9 immunostaining in samples from patients with PsA (**D–F**), AS (**G**), and CD (**H**) and a healthy control (**I**). **J**, Representative photomicrograph showing isotype control staining for IL-9 in a sample from a patient with PsA. **K**, Semiquantitative evaluation of IL-9–positive cells (per hpf) in the ileum of patients with PsA, AS, and CD and healthy controls. In **A–C** and **K**, each symbol represents an individual subject; horizontal bars show the mean. Original magnification $\times 250$ in **D–J**. NS = not significant (see Figure 1 for other definitions).

located at the bottom of intestinal crypts highly resembling Paneth cells (Figures 3D and E). IL-9 immunostaining was also observed in ileal biopsy specimens from patients with AS (Figure 3G) and CD (Figure 3H) and healthy controls (Figure 3I), albeit at a much lesser degree. Quantification of the immunostaining confirmed increased IL-9 staining in PsA patients versus controls ($P < 0.0001$), with a similar trend in patients with CD ($P < 0.05$), but not in those with AS (Figure 3K and Supplementary Table 3, <http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>). The number of IL-9–expressing cells correlated with the degree of intestinal inflammation (Supplementary Figure 1A, <http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>).

Additionally, we characterized by confocal microscopy the main intestinal cellular source of IL-9 among infiltrating mononuclear cells and epithelial cells in PsA patients. CD4⁺ cells were the major source of IL-9 in the gut of patients with PsA. These cells were demonstrated to be in large part IL-9+PU.1+ (Th9) cells (Figures 4A–D), with double IL-17/IL-9–expressing

(Th17) cells being only rarely present (Figures 4E–G) and IL-4/IL-9 (Th2)–positive cells virtually absent (Figures 4H–J). Paneth cells were also demonstrated to overexpress IL-9 in PsA patients, by colocalization of IL-9 and α -defensin 5 (Figures 4K–M). Because the functional role of IL-9 depends on the expression of IL-9 receptor (IL-9R), we next studied the expression and tissue distribution of IL-9R. Significantly increased IL-9R expression was observed, at both the mRNA level (Figure 5A) and the protein level (Figures 5B–H and J and Supplementary Table 3, <http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>) in the gut of PsA patients compared to healthy controls, patients with AS, and patients with CD. Three main patterns of expression were observed: on the surface of infiltrating inflammatory mononuclear cells (Figures 5E and F), among intraepithelial lymphocytes (Figure 5G), and in the context of epithelial cells located at the bottom of intestinal crypts, resembling Paneth cells (Figure 5H).

To assess the potential functional consequences of increased IL-9 expression in the gut of PsA patients,

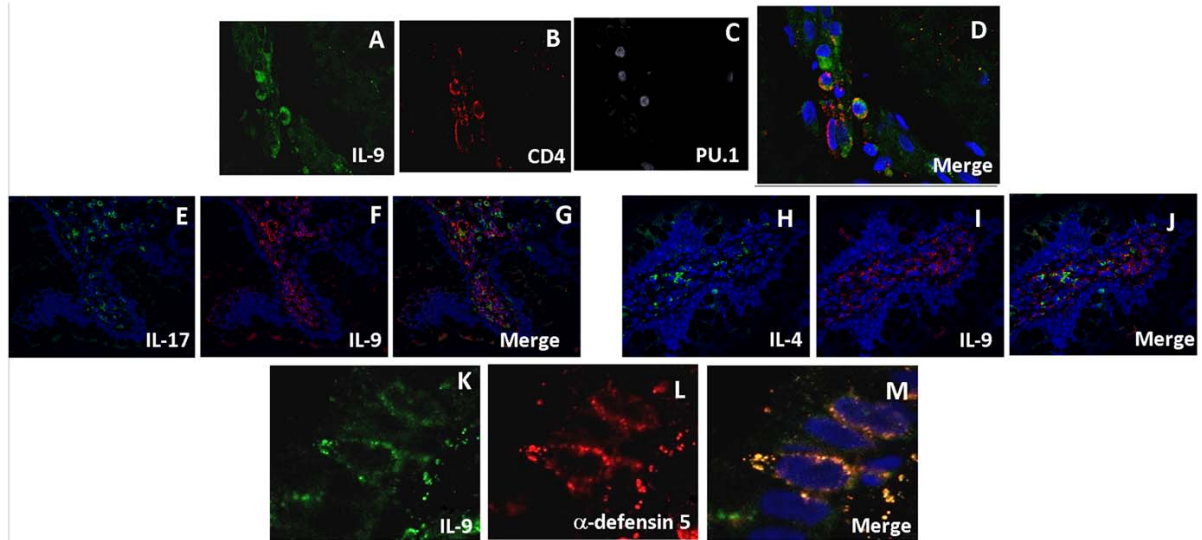


Figure 4. Production of interleukin-9 (IL-9) by Th9 cells, but not Th17/Th2 cells, in the gut of patients with psoriatic arthritis (PsA). **A–D**, Th9 cells. Representative images show single staining for IL-9 (**A**), CD4 (**B**), and PU.1 (**C**) and merged triple staining for IL-9, CD4, and PU.1 (**D**) in PsA gut samples. **E–G**, Th17 cells. Representative images show single staining for IL-17 (**E**) and IL-9 (**F**) and merged double staining for IL-17 and IL-9 (**G**) in PsA gut samples. **H–J**, Th2 cells. Representative images show single staining for IL-4 (**H**) and IL-9 (**I**) and merged double staining for IL-4 and IL-9 (**J**) in PsA gut samples. **K–M**, Paneth cells. Representative images show single staining for IL-9 (**K**) and α -defensin 5 (**L**) and merged double staining for IL-9 and α -defensin 5 (**M**) in PsA gut samples. Original magnification $\times 630$ in **A–D** and **K–M**; $\times 250$ in **E–J**. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/journal/doi/10.1002/art.39649/abstract>.

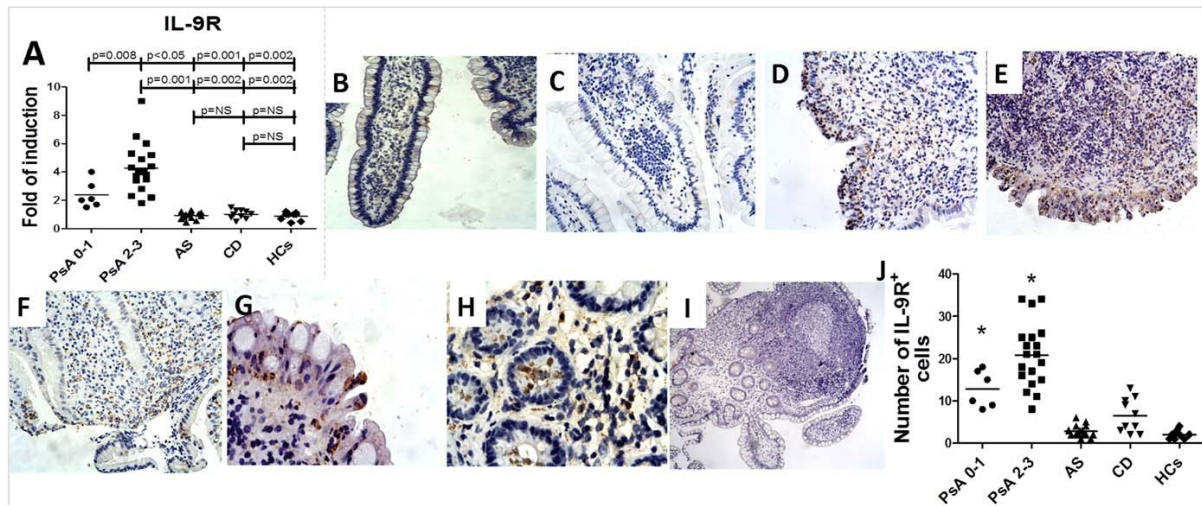


Figure 5. Interleukin-9 receptor (IL-9R) in the gut of patients with PsA. **A**, Relative levels of mRNA for IL-9R, assessed by quantitative reverse transcription–polymerase chain reaction, in ileal samples from patients with PsA ($n = 25$), AS ($n = 15$), and CD ($n = 10$) and healthy controls ($n = 20$). **B–H**, Representative photomicrographs showing IL-9R immunostaining in samples from a healthy control (**B**) and from patients with AS (**C**), CD (**D**), and PsA (**E–H**). **I**, Representative photomicrograph showing isotype control staining for IL-9R in a sample from a patient with PsA. **J**, Semiquantitative evaluation of IL-9R–positive cells (per hpf) in the ileum of patients with PsA and an ileal histologic score of 0 or 1, PsA and an ileal histologic score of 2 or 3, AS, and CD and healthy controls. $*$ = $P < 0.05$ versus controls. In **A** and **J**, each symbol represents an individual subject; horizontal bars show the mean. Original magnification $\times 250$ in **B–F**; $\times 400$ in **G**; $\times 630$ in **H**; $\times 100$ in **I**. NS = not significant (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/journal/doi/10.1002/art.39649/abstract>.

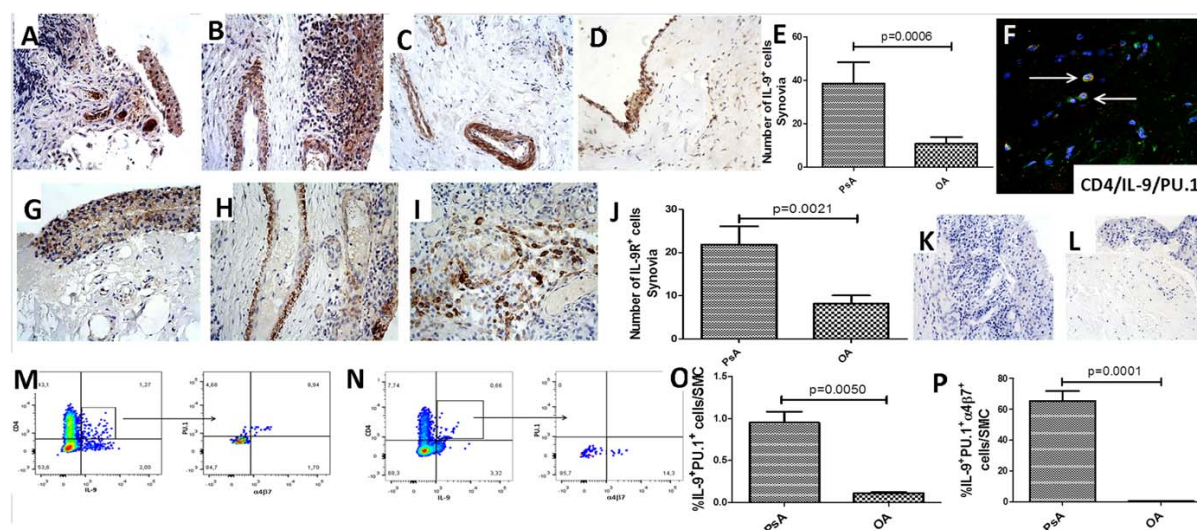


Figure 6. Th9 and interleukin-9 (IL-9)/IL-9 receptor (IL-9R) in the synovial compartment of patients with psoriatic arthritis (PsA). **A–D**, Representative photomicrographs showing IL-9 immunostaining in synovial tissue from patients with PsA (**A** and **B**) and patients with osteoarthritis (OA) (**C** and **D**). **E**, Semiquantitative evaluation of IL-9–positive cells (per high-power field [hpf]) in synovial tissue from patients with PsA and patients with OA. **F**, Merged double staining for IL-9 and PU.1 in synovial tissue from a representative PsA patient, demonstrating the presence of Th9 cells (**arrows**). **G–I**, Representative photomicrographs showing IL-9R immunostaining in synovial tissue from patients with PsA (**G** and **H**) and a patient with OA (**I**). **J**, Semiquantitative evaluation of IL-9R–positive cells (per hpf) in synovial tissue from patients with PsA and patients with OA. **K** and **L**, Representative photomicrographs showing isotype control staining for IL-9 (**K**) and IL-9R (**L**) in synovial tissue from patients with PsA. **M**, Representative dot plots showing Th9 cells in synovial fluid from 5 patients with PsA. A large percentage of Th9 cells also expressed $\alpha 4\beta 7$. **N**, Representative dot plots showing Th9 cells in synovial fluid from 5 patients with OA. **O** and **P**, Percentages of Th9 cells (**O**) and $\alpha 4\beta 7^+$ Th9 cells (**P**) in synovial fluid mononuclear cells (SMCs) from patients with PsA and patients with OA. Values in **E**, **J**, **O**, and **P** are the mean \pm SEM. Original magnification $\times 250$. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/journal/doi/10.1002/art.39649/abstract>.

we stimulated in vitro–isolated epithelial cells from PsA gut with IL-9. After IL-9 stimulation, we observed increased expression of mRNA for α -defensin 5, SOX9, and IL-23p19 only in isolated epithelial cells from patients with PsA (Supplementary Figures 1B–D, <http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>).

IL-9–secreting peripheral blood lymphocytes are expanded in PsA patients, express the gut homing marker $\alpha 4\beta 7$, and correlate with disease activity. We next evaluated the potential of peripheral blood lymphocytes from PsA patients to produce IL-9. As shown in Supplementary Figures 2A–C (<http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>), IL-9–producing CD4+ cells were significantly expanded in the peripheral blood of PsA patients compared to AS and CD patients and healthy controls, and expressed PU.1 and $\alpha 4\beta 7$. Their levels were significantly correlated with disease activity as assessed by the BASDAI and DAS28 (Supplementary Figure 2D). Although differences in the frequency of Th9 cells among patients with systemic lupus erythematosus have been reported to be sex-dependent

(23), we observed no difference between male and female patients with PsA (0.85% in female patients versus 0.81% in male patients; $P = 0.8$) (data not shown). We also found that a significantly increased proportion of peripheral Th9 cells from PsA patients expressed $\alpha 4\beta 7$ integrin (28% in PsA patients versus 4% in controls; $P < 0.05$) (Supplementary Figure 2C), suggesting a gut origin of circulating IL-9–expressing cells in PsA.

IL-9 and IL-9R expression and Th9 cells in PsA synovial samples. Since synovial tissue is one of the main targets of inflammation in PsA, we performed immunohistochemistry analysis to evaluate the expression of IL-9 and IL-9R in synovial fluid and synovial tissue specimens from PsA patients and controls. IL-9 and IL-9R were strongly expressed in the samples from PsA patients (Figures 6A and B) and observed rarely in samples from patients with OA (Figure 6C). IL-9 was observed in leukocytic infiltrates and in the synovial lining layer in patients with PsA, and mainly expressed in the synovial lining layer and among synovial vessels in patients with OA. Semiquantitative analysis of immuno-

histochemistry results revealed significantly higher expression of IL-9 in the synovium of patients with PsA compared to that of patients with OA (Figure 6E and Supplementary Table 3, <http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>). Expression of IL-9 by T cells was confirmed by confocal microscopy analysis (Figure 6F), demonstrating that Th9 cells are a source of IL-9 in PsA synovium. IL-9R was also overexpressed in leukocytic infiltrates and in the lining layer of PsA synovium and rarely observed in samples from patients with OA (Figures 6G–J and Supplementary Table 3). Flow cytometric analysis demonstrated that Th9 cells were significantly expanded in the synovial fluid of patients with PsA compared to that of patients with OA, and significantly overexpressed $\alpha 4\beta 7$ (Figures 6L–O).

Effects of TNFi and ustekinumab on circulating Th9 cells. TNFi treatment, and more recently, IL-23 inhibition, have been demonstrated to reduce inflammation in PsA (24). Nine of the 25 PsA patients enrolled in this study were treated with golimumab (a TNFi) ($n = 5$) or ustekinumab ($n = 4$), and the frequency of circulating Th9 cells was reassessed after treatment had been initiated. At baseline, 2 patients in the TNFi group and 1 patient in the ustekinumab group did not exhibit macroscopic alterations on ileocolonoscopy. As shown in Supplementary Figure 3 (<http://onlinelibrary.wiley.com/doi/10.1002/art.39649/abstract>), treatment with either TNFi or anti-IL-12/IL-23 therapies resulted in a significant reduction in circulating Th9 cells.

DISCUSSION

In this study, we confirmed the occurrence of subclinical gut inflammation in PsA patients and provide the first evidence of a specific histologic and immunologic signature characterized by pronounced Paneth cell hyperplasia with fully developed Th17 and Th9 responses. The latter was specific to PsA compared to AS and CD. We also demonstrated that IL-9 overexpression and Th9 polarization occur in inflamed synovial tissue and in the peripheral blood of PsA patients, leading us to hypothesize that there is a link between intestinal and synovial inflammation. Finally, clinical amelioration after treatment with TNFi and ustekinumab was associated with a significant reduction in circulating Th9 cells.

The presence of gut inflammation has been previously demonstrated in the ileal and colonic mucosa of patients with PsA without bowel symptoms, especially in those with axial involvement, even when mucosa appeared macroscopically normal (17,25,26). However, since in AS significant immune alterations were mainly

found, in earlier studies, at the ileal level (27), we focused our attention only on the ileal tract. In our study, both patients with axial involvement and those with peripheral involvement displayed subclinical ileal inflammation, with a higher frequency than that described in previous reports and with similar distribution between axial and peripheral disease.

Eight PsA patients and 3 AS patients in our study were taking NSAIDs at the time of ileocolonoscopy. NSAID-associated ileitis has been described in a significant proportion of long-term NSAIDs users. Histologically, however, it is not easy to distinguish between NSAID-associated ileitis and CD or AS-associated gut inflammation. It has been reported that a type of stricture known as “diaphragm disease” is pathognomonic for NSAID-induced damage (28). We obviously cannot exclude the possibility that the PsA and AS patients who were taking NSAIDs had NSAID-related ileitis, although no stricture were observed in any of the patients. PsA patients enrolled in our study had high PASI scores and a relatively short duration of disease (~40 months in our study, compared to 40 years in the Belgian study [17]). The proportion of PsA patients with axial involvement displaying ileal subclinical inflammation was comparable to that in patients with AS. Unlike in AS, however, PsA gut inflammation was characterized by higher numbers of infiltrating inflammatory cells, frequently organized in lymphoid follicles, at levels similar to those observed in patients with CD.

An increased number of Paneth cells was also observed in PsA patients. Paneth cells are highly specialized epithelial cells of the small intestine that reside at the base of small intestinal crypts involved in the synthesis and secretion of antimicrobial peptides required for maintenance of crypt sterility and regulation of the balance of colonizing microbiota and enteric pathogens (29). Paneth cells originate from the stem cell zone located in the lower portion of the crypt of Lieberkühn, and their differentiation/hyperplasia seem to be dependent on proinflammatory cytokines such as colony-stimulating factor 1 (30), IL-9, and IL-13 (31), highlighting the role of inflammation in modulating Paneth cell differentiation. On the other hand, Paneth cells have been also demonstrated to participate in the regulation of several pathways of innate and adaptive immunity, including that of the IL-23/IL-17 axis (27,32). The presence of significant intestinal dysbiosis, different from AS dysbiosis (33), was recently demonstrated in PsA patients (34). In this context, the increased number of Paneth cells and the increased levels of antimicrobial peptides, such as α -defensin 5, demonstrated in the present study may suggest the activation of innate defensive

responses in the PsA gut in response to intestinal dysbiosis.

Our immunologic characterization of the inflamed gut of PsA patients demonstrated a distinctive constellation of immune responses. Similar to previous findings in AS (27), we did not observe significant up-regulation of Th1-related cytokines. On the other hand, different from findings in AS and similar to those in CD, a fully developed Th17 response, characterized by strong up-regulation of both IL-17 and IL-22, appears to be present in the gut of patients with PsA.

Beyond the IL-23/Th17 axis, however, a newly recognized subset of effector T cells—so-called Th9 cells—has been suggested to play a role in the pathogenesis of human inflammatory diseases, and in particular, psoriasis (35). Although Th9 cells have been shown to be involved in the pathogenesis of psoriasis, their developmental origin remains elusive (6,22). In this study we demonstrated for the first time that IL-9 is strongly overexpressed by infiltrating inflammatory cells in the gut of patients with PsA. Among inflammatory cells, Th9 lymphocytes were the major source of IL-9 in PsA, highlighting the role of adaptive immunity in the production of IL-9. Of note, IL-9 was also produced by Paneth cells, and these cells also expressed IL-9R. These data, together with the evidence that stimulation of epithelial cells with IL-9 up-regulates the expression of antimicrobial peptides and of cytokines, such as IL-23, may also suggest the possibility of a functional auto-crine loop involving IL-9/IL-9R.

Both peripheral arthritis and spine inflammation occur in patients with PsA. We therefore also evaluated PsA synovial samples for IL-9 and IL-9R expression, and demonstrated that both were significantly overexpressed. In PsA synovial tissue synovial fibroblasts and synovial vessels, together with Th9 cells, were found to be the main source of IL-9. Constitutive production of IL-9 was also observed in OA synovial samples, in which endothelial cells and synovial fibroblasts were demonstrated to produce IL-9.

Th9 cells were also expanded in the peripheral blood and synovial fluid of PsA patients, and their levels were correlated with disease activity. The relatively low percentage of circulating Th9 cells (although significantly increased compared to controls) was similar to that described in other autoimmune diseases (36), indicating that these cells probably specifically recirculate at sites of inflammation. Notably, both circulating and synovial Th9 cells expressed relatively high levels of the intestinal homing receptor $\alpha 4\beta 7$, suggesting a predominant intestinal origin of these cells. Although only a small number of PsA patients were studied before and after

biologic treatment, in both TNFi- and ustekinumab-treated patients we observed a significant reduction of circulating Th9 cells occurring together with clinical improvement.

In conclusion, specific histologic and immunologic features seem to characterize the subclinical gut inflammation in patients with PsA. We also demonstrated that Th1 is predominant in CD but not in spondyloarthritis (AS and PsA), that Th17/22 expression is increased in all 3 diseases, and most importantly, that only Th9 cells are specific to PsA. These findings suggest that IL-9 and Th9 cells may play a role in driving local and systemic inflammation in PsA and might be considered as a potential future therapeutic target.

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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. Ciccia 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, Guggino, Baeten, Triolo.
Acquisition of data. Ciccia, Guggino, Ferrante, Raimondo, Bignone, Rodolico, Peralta, Van Tok, Cannizzaro, Schinocca, Ruscitti, Cipriani, Rizzo.

Analysis and interpretation of data. Ciccia, Guggino, Ferrante, Raimondo, Bignone, Giacomelli, Alessandro, Dieli, Rizzo, Baeten, Triolo.

REFERENCES

- Li WQ, Han JL, Chan AT, Qureshi AA. Psoriasis, psoriatic arthritis and increased risk of incident Crohn's disease in US women. *Ann Rheum Dis* 2013;72:1200–5.
- Suzuki E, Mellins ED, Gershwin ME, Nestle FO, Adamopoulos IE. The IL-23/IL-17 axis in psoriatic arthritis. *Autoimmun Rev* 2014;13:496–502.
- Noordenbos T, Yeremenko N, Gofita I, van de Sande M, Tak PP, Cañete JD, et al. Interleukin-17–positive mast cells contribute to synovial inflammation in spondylarthritis. *Arthritis Rheum* 2012;64:99–109.
- Partsch G, Wagner E, Leeb BF, Bröll H, Dunky A, Smolen JS. T cell derived cytokines in psoriatic arthritis synovial fluids. *Ann Rheum Dis* 1998;57:691–3.
- Schmitt E, Klein M, Bopp T. Th9 cells, new players in adaptive immunity. *Trends Immunol* 2014;35:61–8.
- Schlapbach C, Gehad A, Yang C, Watanabe R, Guenova E, Teague JE, et al. Human T_H9 cells are skin-tropic and have autocrine and paracrine proinflammatory capacity. *Sci Transl Med* 2014;6:219ra8.
- Hughes-Austin JM, Deane KD, Derber LA, Kolfenbach JR, Zerbe GO, Sokolove J, et al. Multiple cytokines and chemokines are associated with rheumatoid arthritis-related autoimmunity in first-degree relatives without rheumatoid arthritis: Studies of the Aetiology of Rheumatoid Arthritis (SERA). *Ann Rheum Dis* 2013;72:901–7.

8. Nalleweg N, Chiriac MT, Podstawa E, Lehmann C, Rau TT, Atreya R, et al. IL-9 and its receptor are predominantly involved in the pathogenesis of UC. *Gut* 2015;64:743–55.
9. Ciccia F, Rizzo A, Guggino G, Cavazza A, Alessandro R, Maugeri R, et al. Difference in the expression of IL-9 and IL-17 correlates with different histological pattern of vascular wall injury in giant cell arteritis. *Rheumatology (Oxford)* 2015;54:1596–604.
10. Taylor W, Gladman D, Helliwell P, Marchesoni A, Mease P, Mielants H, and the CASPAR Study Group. Classification criteria for psoriatic arthritis: development of new criteria from a large international study. *Arthritis Rheum* 2006;54:2665–73.
11. Van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis: a proposal for modification of the New York criteria. *Arthritis Rheum* 1984;27:361–8.
12. Baeten D, Van den Bosch F, Elewaut D, Stuer A, Veys EM, De Keyser F. Needle arthroscopy of the knee with synovial biopsy sampling: technical experience in 150 patients. *Clin Rheumatol* 1999;18:434–41.
13. Garrett S, Jenkinson T, Kennedy LG, Whitelock H, Gaisford P, Calin A. A new approach to defining disease status in ankylosing spondylitis: the Bath Ankylosing Spondylitis Disease Activity Index. *J Rheumatol* 1994;21:2286–91.
14. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* 1995;38:44–8.
15. Best WR, Becktel JM, Singleton JW, Kern F. Development of a Crohn's disease activity index: National Cooperative Crohn's Disease Study. *Gastroenterology* 1976;70:439–44.
16. Fredriksson T, Pettersson U. Severe psoriasis—oral therapy with a new retinoid. *Dermatologica* 1978;157:238–44.
17. Schattman L, Mielants H, Veys EM, Cuvelier C, De Vos M, Gyselbrecht L, et al. Gut inflammation in psoriatic arthritis: a prospective ileocolonoscopy study. *J Rheumatol* 1995;22:680–3.
18. Ciccia F, Bombardieri M, Rizzo A, Principato A, Giardina AR, Raiata F, et al. Over-expression of Paneth cell-derived antimicrobial peptides in the gut of patients with ankylosing spondylitis and subclinical intestinal inflammation. *Rheumatology (Oxford)* 2010;49:2076–83.
19. Ciccia F, Accardo-Palumbo A, Alessandro R, Rizzo A, Principe S, Peralta S, et al. Interleukin-22 and IL-22-producing Nkp44+ natural killer cells in subclinical gut inflammation in ankylosing spondylitis. *Arthritis Rheum* 2012;64:1869–78.
20. Van Damme N, De Vos M, Baeten D, Demetter P, Mielants H, Verbruggen G, et al. Flow cytometric analysis of gut mucosal lymphocytes supports an impaired Th1 cytokine profile in spondyloarthritis. *Ann Rheum Dis* 2001;60:495–9.
21. Bastide P, Darido C, Pannequin J, Kist R, Robine S, Marty-Double C, et al. Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. *J Cell Biol* 2007;178:635–48.
22. Singh TP, Schön MP, Wallbrecht K, Gruber-Wackernagel A, Wang XJ, Wolf P. Involvement of IL-9 in Th17-associated inflammation and angiogenesis of psoriasis. *PLoS One* 2013;8:e51752.
23. Ouyang H, Shi Y, Liu Z, Feng S, Li L, Su N, et al. Increased interleukin-9 and CD4+IL-9+ T cells in patients with systemic lupus erythematosus. *Mol Med Rep* 2013;7:1031–7.
24. Olivieri I, D'Angelo S, Palazzi C, Padula A. Advances in the management of psoriatic arthritis. *Nat Rev Rheumatol* 2014;10:531–42.
25. Scarpa R, Manguso F, D'Arienzo A, D'Armiento FP, Astarita C, Mazzacca G, et al. Microscopic inflammatory changes in colon of patients with both active psoriasis and psoriatic arthritis without bowel symptoms. *J Rheumatol* 2000;27:1241–6.
26. Scarpa R, Ayala F, Caporaso N, Olivieri I. Psoriasis, psoriatic arthritis, or psoriatic disease? *J Rheumatol* 2006;33:210–2.
27. Ciccia F, Bombardieri M, Principato A, Giardina AR, Tripodo C, Porcasi R, et al. Overexpression of interleukin-23, but not interleukin-17, as an immunologic signature of subclinical intestinal inflammation in ankylosing spondylitis. *Arthritis Rheum* 2009;60:955–65.
28. Lang J, Price AB, Levi AJ, Burke M, Gumpel JM, Bjarnason I. Diaphragm disease: pathology of disease of the small intestine induced by non-steroidal anti-inflammatory drugs. *J Clin Pathol* 1988;41:516–26.
29. Keshav S. Paneth cells: leukocyte-like mediators of innate immunity in the intestine. *J Leukoc Biol* 2006;80:500–8.
30. Huynh D, Dai XM, Nandi S, Lightowler S, Trivett M, Chan CK, et al. Colony stimulating factor-1 dependence of Paneth cell development in the mouse small intestine. *Gastroenterology* 2009;137:136–44.
31. Steenwinckel V, Louahed J, Lemaire MM, Sommereyns C, Warnier G, McKenzie A, et al. IL-9 promotes IL-13-dependent Paneth cell hyperplasia and up-regulation of innate immunity mediators in intestinal mucosa. *J Immunol* 2009;182:4737–43.
32. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* 2010;10:479–89.
33. Costello ME, Ciccia F, Willner D, Warrington N, Robinson PC, Gardiner B, et al. Intestinal dysbiosis in ankylosing spondylitis. *Arthritis Rheumatol* 2015;67:686–91.
34. Scher JU, Ubeda C, Artacho A, Attur M, Isaac S, Reddy SM, et al. Decreased bacterial diversity characterizes the altered gut microbiota in patients with psoriatic arthritis, resembling dysbiosis in inflammatory bowel disease. *Arthritis Rheumatol* 2015;67:128–39.
35. Kaplan MH, Hufford MM, Olson MR. The development and in vivo function of T helper 9 cells. *Nat Rev Immunol* 2015;15:295–307.
36. Leng RX, Pan HF, Ye DQ, Xu Y. Potential roles of IL-9 in the pathogenesis of systemic lupus erythematosus. *Am J Clin Exp Immunol* 2012;1:28–32.