Arthritis & Rheumatology
DOI 10.1002/art.39649

IL-9 over-expression and Th9 polarization characterize the inflamed gut, the synovial tissue and the peripheral blood of patients with psoriatic arthritis

Francesco Ciccia[1]MD, Giuliana Guggino[1]MD, Angelo Ferrante[1]MD, Stefania Raimondo[2], Rodolfo Bignone[1]MD, Vito Rodolico[3]MD, Sergio Peralta[4]MD, Melissa Van Tok[5], Alessandra Cannizzaro[6], Claudia Schinocca[1]MD, Piero Ruscitti[7]MD, Paola Cipriani[7]MD, Roberto Giacomelli[7]MD, Riccardo Alessandro[2]PhD, Francesco Dieli[2]MD, Aroldo Rizzo[6]MD, *Dominique Baeten[5]MD and *Giovanni Triolo[1]MD

Running head: IL-9 in PsA patients **Word count: 3460**

[1]Dipartimento Biomedico di Medicina Interna e Specialistica, Sezione di Reumatologia, Università degli Studi di Palermo, Italy

[2]Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi, Università di Palermo, Italy

[3]Dipartimento di Scienze per la Promozione della Salute e Materno Infantile, Università di Palermo, Italy

[4]Dipartimento Biomedico di Medicina Interna e Specialistica, Sezione di Gastroenterologia, Università degli Studi di Palermo, Italy

[5]Amsterdam Rheumatology and Immunology Center, Department of Clinical Immunology and Rheumatology, Academic Medical Center/University of Amsterdam, Amsterdam, The Netherlands

[6]Azienda Ospedaliera Ospedali riuniti Villa Sofia-Cervello, Anatomia Patologica, Palermo, Italy

[7]Dipartimento di Scienze Cliniche Applicate e Biotecnologiche, Sezione di Reumatologia, Università di L'Aquila, Italy

Address correspondence and reprint requests to: Professor Giovanni Triolo, Department of Internal Medicine, Division of Rheumatology, Piazza delle Cliniche 2, 90127 Palermo, Italy; e-mail giovanni.triolo@unipa.it, Telephone and FAX: +390916552137

*DB and GT shared co-senior authorship.

Competing interests: none

Received: Jul 01, 2015; Revised: Nov 23, 2015; Accepted: Feb 16, 2016

Abstract

Objective: This study was undertaken 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, with HLA-27 positive-ankylosing spondylitis (AS), Crohn's disease (CD) and healthy controls. IL-23, IL-17, IL-22, IL-9 and IL-9R expression and tissue distribution were evaluated by immunohistochemistry and confocal microscopy. Flow cytometry was used to study the frequency of Th9 cells among peripheral blood (PBMC), lamina propria (LPMC) and synovial (SMC) mononuclear cells. The functional relevance of IL-9R expression on epithelial cells was assessed in functional *in vitro* studies. Th9 cells were also studied in synovial samples of PsA patients.

Results: subclinical gut inflammation of PsA patients was characterized by a clear Th17 and Th22, but not Th1, polarized immune responses. Unlike AS and CD, a strong and significant up-regulation of IL-9 immunologically was observed in PsA gut, especially among infiltrating mononuclear cells, high endothelial venules and Paneth cells (PC). IL-9 mononuclear cells were demonstrated to be in large part Th9 cells. IL-9 over-expression was accompanied by significant PC hyperplasia. PC strongly over-expressed IL-9R and stimulation of epithelial cells, isolated from PsA patients, with IL-9 resulted in over-expression of α-defensin5 and IL-23p19. Peripheral and synovial expansion of $\alpha 4\beta 7^+$ Th9 cells was also observed in patients with PsA. Increased expression of IL-9 and IL-9R was also found in synovial tissues.

Conclusions: A strong IL-9/Th9 polarization seems to be the predominant immunological signature of patients with PsA.

Key words: Psoriatic arthritis, gut inflammation, synovia, IL-9, Th9 cells

Introduction

For a long time the relationship between inflammatory bowel disease (IBD) and psoriasis has been investigated by epidemiologic, genetic and immunologic studies and more recently, the relative risk of incident Crohn's disease (CD) and ulcerative colitis (UC) in patients with psoriasis and psoriatic arthritis has been documented[1].

The role of the different subsets of T effector cells in PsA gut pathology has been not still now addressed. The pathogenesis of PsA seems to be multifactorial and immunologically driven by a mixed Th1 and IL-23/Th17 response[2-4]. Recently IL-9, a member of the IL-2 cytokine family, secreted by naïve $CD4^+$ T cells in response to TGF-β and IL-4[5], has been also 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 anti-TNF and Ustekinumab therapy on circulating Th9 cells.

Patients and Methods

Patients

Multiple intestinal biopsy specimens were obtained after informed consent from 25 patients with PsA diagnosed according to the CASPAR criteria (11 patients with predominant axial involvement)[10] and 15 patients with HLA-27-positive ankylosing spondylitis (AS) diagnosed according to the New York criteria[11], without gastrointestinal symptoms. Ten patients with CD and 20 healthy controls (HC) who underwent ileocolonoscopy for routine clinical evaluation were also included in the study. Synovial samples were also obtained from additional 5 PsA patients with active disease at the time of sample collection and from 5 patients with osteoarthritis[12]. Table 1 shows the baseline characteristics of the patients and controls. Disease activity for PsA with axial involvement and AS was assessed with BASDAI[13]; DAS28 [14] was used to evaluate disease activity in patients with PsA and peripheral disease and CDAI [15] for patients with CD. The severity of psoriasis was evaluated by the psoriasis area severity index (PASI)[16]. Collection of samples was approved by the local ethical committee and the institutional review board of the University of Palermo and informed consent was obtained from each patient and controls.

Histomorphological grading and immunohistochemistry

Biopsy specimens were evaluated by two experienced pathologists (AR and AC) with an expertise in inflammatory bowel diseases without access to clinical data. The inter-rater agreement calculated by the Cohen's K coefficient for the two pathologists was 0.85. Gut specimens from patients with PsA and AS were histologically analyzed for the presence of inflammation and divided according to the histologic results in four stages as previously described [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 identification was performed by using CD3/CD19/CD21 immunostaining. Synovial samples obtained from additional 5 patients with PsA and, as control, from 5 OA patients with end-stage disease undergoing knee joint replacement surgery, were also evaluated for the degree of inflammation. Grade of tissue inflammation was assessed by evaluating also the number of $CD3^+$, $CD19^+$ and $CD68^+$ cells. Immunohistochemistry was performed as previously described [18] and a list of primary and secondary antibodies used is provided in table 2. The number of immuno-reactive cells and lymphoid follicles was determined by counting the reactive cells/lymphoid follicles on microphotographs obtained from three randomly and independently selected high-power microscopic fields (original magnification × 400). To specifically address the nature of IL-9-producing cells, double staining for Th2 (IL-4/IL-9) and Th17 (IL9/IL-17) and for PC (IL-9/α-defensin5) and triple for Th9 cells (CD4/IL-9/PU.1) were performed on paraffin-embedded synovial and ileal sections. Sections were treated with FITC-, Rhodamine Red or Cy-5-conjugated anti-mouse or anti-rabbit antibodies (Invitrogen) plus RNasi (200 ng/ml) and counterstained using Toto-3 iodide (642/660; Invitrogen), or DAPI (Life Technologies). Confocal analysis was used to acquire fluorescence staining.

RNA extraction and quantitative TaqMan real-time polymerase chain reaction (RT-PCR) for ileal biopsies

Total RNA was extracted using the Qiagen RNeasy Mini kit, with on-column DNase I digestion. A total of 1 µg of RNA was reverse-transcribed to complementary DNA (cDNA) using a ThermoScript First-Strand cDNA Synthesis kit (Invitrogen). For quantitative

TaqMan real-time PCR, sets of primers and probes were obtained from Applied Biosystems (table S2). Samples were run in triplicate using the Step-One Real-Time PCR system (Applied Biosystems). Relative changes in gene expression between controls and patients were determined using the ΔΔCt method as previously described. Final values were expressed as fold of induction.

Isolation of peripheral blood and synovial mononuclear cells and flow cytometry

Peripheral blood mononuclear cells (PBMC) were obtained as previously described [19] from 5 of the PsA patients in which ileocolonscopy was also performed and from 9 additional PsA patients [5 of them before and after TNFi and 4 before and after ustekinumab treatment], 10 AS and 10 CD patients and 10 healthy controls; synovial mononuclear cells were also obtained from 5 PsA, who underwent to ileocolonscopy, and 5 OA patients. Cell viability (trypan blue dye exclusion) was always >95%. Four-color flow cytometry analysis was performed using a FACSCalibur (BD Biosciences). At least 50.000 cells (events), gated on lymphocytes or monocytes/macrophages region, were acquired for each sample. A list of the antibodies used is listed in Table 2.

Cell cultures

Intestinal epithelial cells were isolated from ileal samples of 5 PsA patients and 5 controls as previously described [20] and cultured in 24 flat bottom plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of $1x10^6$ cells in 1 ml of RPMI 1640 medium with 10% FCS, 2 mM L-glutamine, 20 nM HEPES and 100 U/ml penicillin/streptomycin with or without recombinant IL-9 (rIL-9) (0.1 or 10 ng/ml) (R&D Systems, MN, USA). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 for 48 hours and then characterized by RT-PCR for IL-17, IL-22, IL-23, RORc and STAT3.

Statistical analysis

Student's t-test or the nonparametric Mann-Whitney test was used to calculate the statistical significance between groups. Paired samples were analyzed with the Wilcoxon signed-rank test. *p* values less than 0.05 were considered significant.

Results

Subclinical gut inflammation of PsA patients is characterized by histologic inflammation and Paneth cells hyperplasia

All the patients with CD had active disease with a CDAI of 311 (200-430). The endoscopic appearance of ileum with colonoscopy demonstrates in 10 PsA patients and 8 of AS patients the absence of macrospic alterations. 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 and 4 AS without macroscopic involvement (24% and 26.6%, respectively), stage 2 lesions were observed in 12 PsA and 7 AS patients (4 of them in both PsA and AS groups without macroscopic involvement; 48% and 46.6%, respectively) and stage 3 were observed in 7 PsA and 4 AS patients (28% and 26.6%, respectively). Similar to AS, PsA ileum showed an increased number of infiltrating inflammatory $CD3^+$, $CD19^+$ and $CD68^+$ cells (Figure 1B-C, H-K; Supplemental table 3), an increased number of lymphoid follicles (2.5±0.9 in PsA compared to 1.4±0.6 in HCs, $p<0.05$) and a significant Paneth cells hyperplasia (Figure 1D, arrows, and G; Supplemental Table 3) that correlated with the grade of intestinal inflammation (number of PC: 6±0.72 in stage 3, 5.2±0.84 in stage 2, and 3.4±0.88 in stage 0-1). PC hyperplasia was also accompanied by an increased mRNA expression of PC-derived antimicrobial peptides such as α-defensin-5 and of the transcription factor SOX9 that is involved in PC differentiation (Figure 1L-M)[21]. No differences in the degree of tissue inflammation and/or number of infiltrating cells were observed between patients who were taking NSAIDs compared to those assuming anti-inflammatory drugs (data not shown). Collectively, these data confirm that the ileum of PsA patients, similar to AS, is characterized by sub-clinical histologic inflammation.

Th1 and Th17 polarization in PsA gut

We next investigated the expression of cytokines involved in the Th1 and IL-23/Th17 axis. Differently from CD and similarly to AS, Th1 cytokines were not significantly over-expressed in PsA (Figure 2A-C). Regarding Th17 cells, a clear IL-17 and IL-22 (Figure 2D-E) mRNA over-expression was demonstrated in PsA patients. By IHC we confirm RT-PCR data demonstrating a strong up-regulation of IL-17 (Figure 2F-H, J) and IL-22 (Figure 2 K-M, O; Supplemental table 3) in PsA patients at levels similar to those observed in CD patients (not shown).

IL-9 over-expression and Th9 polarization in PsA gut

We next aimed to investigate in more detail the dominant cytokine pathways steering 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 biopsies by RT-PCR for IL-9 and Th9-related transcription factors mRNA expression. Levels of IL-9, PU.1 and IRF4 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, AS patients and controls (Figure 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 immuno-reactivity was observed among infiltrating mononuclear cells (Figure 3D-F), high endothelial venules (Figure 3F) and epithelial cells located at the bottom of intestinal crypts highly resembling Paneth cells (Figure 3D-E). IL-9 immunostaining was also observed in ileal biopsies of AS (Figure 3G), CD (Figure 3H), and HC (Figure 3I), albeit at a much lesser degree. Quantification of the immunostaining confirmed increased IL-9 staining in PsA versus HC ($p<0.0001$), with a similar trend in CD ($p<0.05$), but not in AS (Fig 3K) (Supplemental Table 3). The number of IL-9-expressing cells correlated with the degree of intestinal inflammation (Supplemental Figure 1A). Furthermore, 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 PsA patients. These cells were demonstrated to be in large part $IL\text{-}9^+PU.1^+$ cell (Th9 cells) (Figure 4A-D), being only rarely present double IL-17/IL9 expressing cells (Th17) (Figure 4E-G) and virtually absent IL-4/IL-9 (Th2) positive cells (Figure 4H-J). PC were also demonstrated to over-express IL-9 in PsA patients by co-localization of IL-9 and α-defensin5 (Figure 4K-M). Because the functional role of IL-9 depends on the expression of IL-9R, we next studied the expression and tissue distribution of IL-9R. As shown in figure 5, a significantly increased expression of IL-9R was observed, at both m-RNA (Figure 5A) and protein level (Figure 4D-H, J; Supplemental Table 3), in the gut of PsA patients compared to healthy (Figure 5B, J; Supplemental Table 3), AS (Figure 5C, J; Supplemental Table 3) and CD (Figure 5D, J; Supplemental Table 3) subjects. Three main patterns of expression were observed: the first, present on the surface of infiltrating inflammatory mononuclear cells (Figure 5E-F), the second among intraepithelial lymphocytes (Figure 5G) and the third observed 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, we stimulated *in vitro* isolated epithelial cells from PsA gut with IL-9. We observed an increased mRNA expression of α-defensin5, SOX9 and IL-23p19 only in PsA isolated epithelial cells after IL-9 stimulation (Supplemental Figure 1B-D).

IL-9-secreting peripheral blood lymphocytes are expanded in PsA patients, express the gut homing markers $\alpha 4\beta 7$ and are correlated with the disease activity

We next evaluated the potential of peripheral blood lymphocytes of PsA patients to produce IL-9. As shown in Supplemental figure 2A, IL-9 producing $CD4^+$ cells were significantly expanded, and express PU.1 and $\alpha 4\beta 7$, in the peripheral blood of PsA patients compared to AS and CD patients and controls (Supplemental Figure 2B and 2C).

They were significantly correlated with disease activity as assessed by BASDAI and DAS28 (Supplemental Figure 2D). Although differences in the frequency of Th9 cells have been reported to be gender-dependent in patients with systemic lupus erythematosus[23], no difference was observed between male and female patients (0.85% in female vs 0.81% in male patients, p=0.8, data not shown) We also found that a significant high proportion of peripheral Th9 cells in PsA expressed $\alpha 4\beta 7$ integrin (Supplemental Figure 2C, 28% in PsA vs 4% in controls, $p<0.05$) suggesting a gut origin for 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 evaluated by IHC the expression of IL-9 and IL-9R in the synovial fluid and synovial specimens of PsA patients and controls. A strong expression of IL-9 and IL-9R was found in PsA patients (Figure 6A-B) and rarely observed in the samples of OA patients (Figure 6C). Expression of IL-9 was observed in the leukocytic infiltrates and in the lining layers (Figure 6A-B). In OA patients IL-9 was mainly expressed in synovial layer and among synovial vessels (Figure 6C-D) The semiquantitative analysis of IHC revealed a significantly higher expression of IL-9 in PsA than in OA (Figure 6E; Supplemental Table 3). Expression of IL-9 by T cells was finally confirmed by confocal microscopy analysis demonstrating that Th9 cells are a source of IL-9 in PsA synovium (Figure 6F). IL-9R was also over-expressed in the leukocytic infiltrates and in the lining layers of PsA synovium (Figure 6G-H, J; Supplemental Table 3) and rarely observed in the samples of OA patients (Figure 6I-J; Supplemental Table 3). Flow cytometric analysis demonstrated that Th9 cells were significantly expanded in PsA (Figure 6M,O) compared to OA (Figure 6N,O) synovial fluids and significantly over-expressed $\alpha 4\beta 7$ (Figure 6L-M, O).

Effects of TNF-inhibitors and ustekinumab on circulating Th9 cells

TNF-blocking agents and, more recently, IL-23 inhibition have been demonstrated to reduce the inflammation in PsA patients. Nine out of the 25 PsA patients enrolled in the study were treated with Golimumab (n=5) and with Ustekinumab (n=4) and re-assessed for the frequency of circulating Th9 cells. Two patients in the anti-TNF group and 1 patient in the Ustekinumab group did not show at baseline ileocolonscopic macroscopic alterations. As shown in Supplemental Figure 3, either anti-TNF (Supplemental Figure 3A, C) and anti-IL12/IL23 therapies (Supplemental Figure 3B, C) resulted in a significant reduction of 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 a pronounced PC hyperplasia with a fully developed Th17 and Th9 responses. The latter was specific for PsA compared to AS and CD. We also demonstrated that IL-9 over-expression and Th9 polarization occur in inflamed synovial tissues and in the peripheral blood of PsA patients, leading us to hypothesize a link between intestinal and synovial inflammation. Finally, clinical amelioration after treatment with TNF-i 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, 24, 25]. However, since that in AS significant immune alterations were mainly found, in previous studies, at ileal level [26] we focused our attention only on ileal tract. In our study, both the patients with axial and peripheral involvement displayed subclinical ileal inflammation with a higher frequency of that described in previous studies and with similar distribution between axial and peripheral disease. Eight PsA patients and 3 AS partients in our study were taking NSAIDs at the time of ileocolonscopy. NSAIDs ileitis has been described in a significant proportion of long-term NSAIDs users. Histologically, however, is not easy to distinguish between NSAIDs ileitis and Crohn's disease or AS-associated gut inflammation. It has been reported that a type of stricture known as "diaphragm disease" is pathognomonic for NSAID-induced damage [27]. We obviously can't exclude that the PsA and AS patients who were taking NSAIDs should have a NSAID-related ileitis although no stricture were observed in all the patients enrolled. PsA patients enrolled in our study had

high PASI score and a relative short duration of disease (~ 40 months in our study compared to 40 years in the Belgian study). The proportion of PsA patients with axial involvement displaying ileal subclinical inflammation was comparable with AS. Differently from 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 CD.

An increased numbers of PC was also observed in PsA patients. PC 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 the maintenance of crypt sterility and the regulation of balance with colonizing microbiota and entheric pathogens [28]. PC origin from the stem cell zone located in the lower portion of the crypt of Lieberkuhn and their differentiation/hyperplasia seem to be dependent by pro-inflammatory cytokines such as CSF-1[29], IL-9 and IL-13[30] highlighting the role of inflammation in modulating the PC differentiation. On the other hand, PC have been also demonstrated to participate in the regulation of several pathways of innate and adaptive immunity including that of IL-23/IL-17 axis[26, 31]. The presence of a significant intestinal dysbiosis, different from AS dysbiosis [32], has been recently demonstrated in PsA patients[33]. In this context, the increased number of Paneth cells and the increased levels of anti-microbial peptides such as α-defensin 5, we demonstrated in the present study, may suggest the activation of innate defensive responses in PsA gut in response to intestinal dysbiosis.

The immunological characterization of the inflamed gut of PsA patients we performed, demonstrated a singular complexity of immune responses. Similarly to AS[26], we could not demonstrate a significant up-regulation of Th1-related cytokines. On the other hand,

differently from AS and similarly to CD a fully developed Th17 response seems to be present in the gut of PsA characterized by the strong up-regulation of both IL-17 and IL-22.

Beyond the IL-23/Th17 axis, however, a new subset of effector T cells has been suggested to play a role in the pathogenesis of human inflammatory diseases and in particular of psoriasis, the so-called Th9 cells[34]. Th9 cells have been demonstrated to be involved in the pathogenesis of psoriasis but their developmental origin remains elusive[6, 22]. In this study we demonstrate for the first time that IL-9 is strongly over-expressed in the gut of PsA patients, being essentially produced by infiltrating inflammatory cells. Among inflammatory cells, Th9 lymphocytes were the major source of IL-9 in PsA, highlighting the role of adaptive branch of immunity in the production of IL-9. Of note, IL-9 was also produced by PC and these cells expressing also the specific IL-9 receptor. These data, together with the evidence that stimulation of epithelial cells with IL-9 up-regulates the expression of anti-microbial peptides and of cytokines, such as IL-23, may also suggest the possibility of a functional autocrine loop involving IL-9/IL-9R. In PsA patients both peripheral arthritis and spine inflammation occur. In this regard, PsA synovial samples were evaluated for IL-9 and IL-9R expression demonstrating a significant over-expression of both IL-9 and IL-9R. In PsA synovial tissues synovial fibroblasts and synovial vessels were demonstrated to be the main source of IL-9 together with Th9 cells. A constitutive production of IL-9 was also observed in OA synovial samples, where endothelial cells and synovial fibroblasts were demonstrated to produce IL-9.

Th9 cells were also expanded in the peripheral blood and synovial fluids of PsA patients and were correlated with the disease activity. The relative low percentage of circulating Th9 cells (although significantly increased compared to controls) was similar to that described in other autoimmune diseases[35] indicating that probably these cells specifically re-circulate in the sites of inflammation. Notably, both circulating and synovial

Th9 cells expressed in large part the intestinal homing receptor $\alpha4\beta7$ suggesting a predominant intestinal origin for these cells. Although a few number of PsA patients were studied before and after biologic treatments, in both the anti-TNF and ustekinumab-treated patients we, together with a clinical improvement, observed a significant reduction of circulating Th9 cells.

In conclusion, specific histologic and immunologic features seem to characterize the subclinical gut inflammation in PsA patients. We also demonstrated that Th1 is predominant in CD but not in SpA (AS and PsA), that TH17/22 is increased in all 3 diseases, and mainly that Th9 is the only that is specific for PsA. In this perspective IL-9 and Th9 cells may play a role in driving local and systemic inflammation in these patients and might be considered as a potential future therapeutic target.

Funding

This study was in part supported by a grant of Ministero dell'Istruzione, dell'Università e della ricerca scientifica from Italy

Competing interests

The authors declare no competing interests

Acknowledgements: we would like to deeply thank Dr Francesca Raiata for her technical support in immunohistochemical experiments.

Figure legends

Figure 1. High level of tissue inflammation and Paneth cell hyperplasia characterize subclinical gut inflammation in PsA. A-D: representative images of H&E stained ileal sections of PsA. Arrows in **D** indicate Paneth cells. **E-F:** representative images of a-defensin 5 staining in healthy controls **(E)** and PsA **(F). G:** semiquantitative evaluation of PC in PsA. **H-J:** inflammatory infiltrating cells, CD3 **(H),** CD19 **(I)** and CD68 **(J)** positive cells in PsA gut. **K:** semiquantitative evaluation of CD3, CD19, CD68 in PSA. **L-M:** Relative m-RNA levels of a-defensin 5 **(L)** and SOX9 **(M)** were assessed by quantitative RT-PCR in ileal samples obtained from 25 PsA, 15 AS, 10 CD patients and 20 control subjects. Data are expressed as mean fold of induction (SEM). **A,C, E-F, H-J:** original magnification x 250. **D:** original magnification x 400**.**

Figure 2. Th1 and Th17 cytokines in the gut of PsA patients. A-F: Relative m-RNA levels of IFN-g **(A),** IL-12 **(B),** IL-6 **(C),** IL-22 **(D),** IL-17 **(E)** were assessed by quantitative RT-PCR in ileal samples obtained from PsA, AS, CD patients and control subjects (HCs). **F-I:** Representative microphotographs showing IL-17 immunostainings in PsA **(F),** AS **(G)** patients and normal controls **(H). J:** IL-17 semiquantitative score in the ileum of patients and controls. **K-M:** Representative microphotographs showing IL-22 immunostainings in PsA **(K),** AS **(L)** patients and HCs **(M). O:** IL-22 semiquantitative score in the ileum of patients and controls. Data are expressed as mean (SEM). *p<0.05 compared to control subjects. **I, N:** representative images showing isotype control staining for IL-17 **(I)** and IL-22 **(N)** in the gut of PsA patients. **F-G, J-K:** original magnification x 400. **H, L:** original magnification x 100; **I-N:** original magnification x250.

Figure 3. IL-9 in the gut of PsA patients. A-C: Relative m-RNA quantification of IL-9 **(A),** PU.1 **(B)** and IRF4 **(C)** was assessed by quantitative RT-PCR in ileal samples obtained from 25 PsA, 15 AS, 10 CD patients and 20 control subjects. **D-I:** Representative

microphotographs showing IL-9 immunostainings in PsA **(D-F),** AS **(G)** and CD **(H)** patients and normal controls **(I). J:** representative image showing isotype control staining in PsA patients. **K:** IL-9 semiquantitative score in the ileum of patients and controls. Data are expressed as individual data points (mean). *p<0.05 compared to control subjects. **D-J:** original magnification x 250.

Figure 4. Th9 cells but not Th17/Th9 cells produce IL-9 in PsA gut. A-D: Th9 cells in PsA gut. **A**: single staining for IL-9. **B**: single staining for CD4; **C**: single staining for PU.1. D: merged triple staining for IL-9/CD4/PU.1. **E-G**:Th17 in PsA gut. **E**: single staining for IL-17. **F**: single staining for IL-9. **G**: merged double staining for IL-17 and IL-9. **H-J**: Th2 cells in PsA gut. **H**: single staining for IL-4. **I**: single staining for IL-9. **J**: merged double staining for IL-4 and IL-9. **K-M**: Paneth cells in PsA gut. K: single staining for IL-9; **L**: single staining for α-sefensin5. **M**: merge double staining for IL-9 and α-defensin 5. **A-D, K-M**: original magnification x630; **E-J**: original magnification x 250.

Figure 5. IL-9R in the gut of PsA. A: Relative m-RNA levels quantification of IL-9R **(A)** were assessed by quantitative RT-PCR in ileal samples obtained from 25 PsA, 15 AS, 10 CD patients and 20 control subjects. **B-H:** Representative microphotographs showing IL-9 immunostainings in healthy subject **(B), AS (C), CD (D)** and PsA patients **(E-H). I:** representative picture showing isotype control staining in PsA gut. **J:** IL-9 semiquantitative score in the ileum of patients and controls. Data are expressed as individual data points (mean). *p<0.05 compared to control subjects. **D-F:** original magnification x 250; **G:** original magnification x 400; H: original magnification x 630. I: original magnification x100

Figure 6. Th9 and IL-9/IL-9R in PsA synovial compart. A-C: representative images showing IL-9 immunostaining in synovial tissues obtained from patients with PsA **(A-B)** and osteoarthritis **(OA) (C-D). E:** numbers of IL-9+ cells in synovial tissues. **F:** merged double staining of IL-9 and PU.1 in synovial tissues of PsA patients demonstrating the

presence of Th9 cells. **G-I:** representative images showing IL-9R immunostaining in PsA synovial tissues **(G-H)** and OA **(I). J:** numbers of IL-9R+ cells in PsA and OA synovial tissues. **K-L:** representative immunostainings with isotype antibodies in synovial tissues from PsA patients. **M:** representative dot plots showing Th9 cells in synovial fluid obtained from 5 PsA patients. A large percentage of Th9 cells also express $\alpha4\beta7$. **N:** representative dot plots showing Th9 cells in synovial fluid obtained from 5 OA patients. **O:** percentages of Th9 cells and **(P)** of $\alpha4\beta7$+ Th9 cells in the synovial fluid obtained from PsA and OA patients. **A-D, G-I, K-L:** original magnification x250. **E, J. O-P:** data are expressed as mean (SEM).

Supplemental Figure 1. Numbers of IL-9+ cells are correlated with the degree of intestinal inflammation and IL-9 modulates the expression of PC associated proteins. A: numbers of IL-9+ cells were correlated with the severity of intestinal inflammation. **B-D**: Epithelial cells isolated from the ileal biopsies of 5 patients with PsA were treated with recombinant IL-9 and the modulation of the expression of Paneth cells related genes was assessed by RT-PCR. After stimulation with IL-9 a significan increased expression of α-defensin 5 (**B**), IL-23p19 (**C**) and SOX9 (**D**) was observed. Data are expressed as mean (SEM).

Supplemental Figure 2. Th9 cells among peripheral blood mononuclear cells in PsA patients. A: representative dot plots showing gating strategy for Th9 cells and Th9 cells in the peripheral blood of PsA patients. CD4+ IL-9+ cells were almost exclusively represented by PU.1+ cells also expressing $\alpha4\beta7$. **B:** percentages of Th9 cells among PBMC in PsA, AS, CD patients and controls. **C:** percentages of $\alpha4\beta7$+Th9 cell in PsA, AS, CD patients and controls. **D:** correlation between the percentage of circulating Th9 cells and BASDAI and DAS28. *$p<0.05$

Supplemental figure 3. Effects of Golimumab and Ustekinumab on peripheral Th9 cells in PsA patients. A-B. representative dot plots showing the percentages of Th9 cells in Golimumab (N=5) (**A**) and Ustekinumab ((N=4) (B) treated PsA patients. C-D. Th9 cells before and after Golimumab (C) and Ustekinumab (D) treatment. Data are shown as individual data points.

Reference list

[1] 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; 72:1200-1205.

[2] 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.

[3] Noordenbos T, Yeremenko N, Gofita I, van de Sande M, Tak PP, Caňete JD et al. Interleukin-17-positive mast cells contribute to synovial inflammation in spondylarthritis. *Arthritis Rheum* 2012; 64: 99-109.

[4] 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: 691-693.

[5] Schmitt E, Klein M, Bopp T. Th9 cells, new players in adaptive immunity. *Trends Immunol,* 2014; 35: 61-68.

[6] Schlapbach C, Gehad A, Yang C, Watanabe R, Guenova E, Teague JE*,* et al*.* Human TH9 cells are skin-tropic and have autocrine and paracrine proinflammatory capacity. *Sci Transl Med* 2014; 6: 219ra8.

[7] 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: 901-907.

[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: 743-755.

[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),* Apr 2015.

[10] Taylor W, Gladman D, Helliwell P, Marchesoni A, Mease P, Mielants H et al*.* Classification criteria for psoriatic arthritis: development of new criteria from a large international study. *Arthritis Rheum* 2006; 54: 2665-2673.

[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: 361-368.

[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: 434-441.

[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: 2286-2291.

[14] van der Heijde DM, van 't Hof MA, van Riel PL, Theunisse LA, Lubberts EW, van Leeuwen MA et al*.* Judging disease activity in clinical practice in rheumatoid arthritis: first step in the development of a disease activity score. *Ann Rheum Dis* 1990; 49: 916-920.

[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: 439-444.

[16] Langley RG, Ellis CN. Evaluating psoriasis with Psoriasis Area and Severity Index, Psoriasis Global Assessment, and Lattice System Physician's Global Assessment. *J Am Acad Dermatol* 2004; 51: 563-569.

[17] Schatteman L, Mielants H, Veys EM, Cuvelier C, De Vos M, Gyselbrecht L et al*.* Gut inflammation in psoriatic arthritis: a prospective ileocolonoscopic study. *J Rheumatol* 1995; 22: 680-683.

[18] Ciccia F, Bombardieri M, Rizzo A, Principato A, Giardina AR, Raiata F et al*.* Over-expression of paneth cell-derived anti-microbial peptides in the gut of patients with ankylosing spondylitis and subclinical intestinal inflammation. *Rheumatology (Oxford)* 2010; 49: 2076-2083.

[19] Ciccia F, Accardo-Palumbo A, Alessandro R, Rizzo A, Principe S, Peralta S et al. "Interleukin-22 and IL-22-producing NKp44(+) NK cells in the subclinical gut inflammation of patients with ankylosing spondylitis.," *Arthritis Rheum,* Dec 2012; 64:1869-1878.

[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 spondyloarthropathy. *Ann Rheum Dis* 2001; 60: 495-499.

[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: 635-648.

[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: 1031-1037.

[24] 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; 27:1241-1246.

[25] Scarpa R, Ayala F, Caporaso N, Olivieri I. Psoriasis, psoriatic arthritis, or psoriatic disease? *J Rheumatol* 2006; 33: 210-212.

[26] 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: 955-965.

[27] 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

[28] Keshav S. Paneth cells: leukocyte-like mediators of innate immunity in the intestine," *J Leukoc Biol* 2006; 80: 500-508.

[29] 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: 136-144.

[30] 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: 4737-4743.

[31] Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* 2010; 10: 479-489.

[32] Costello ME, Ciccia F, Willner D, Warrington N, Robinson PC, Gardiner B et al. Intestinal dysbiosis in ankylosing spondylitis. *Arthritis Rheumatol* 2014 Nov 21. doi: 10.1002/art.38967..

[33] Scher J, 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.

[34] Kaplan MH, Hufford MM, Olson MR. The development and in vivo function of T helper 9 cells. *Nat Rev Immunol* 2015; 15:295-307.

[35] 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.

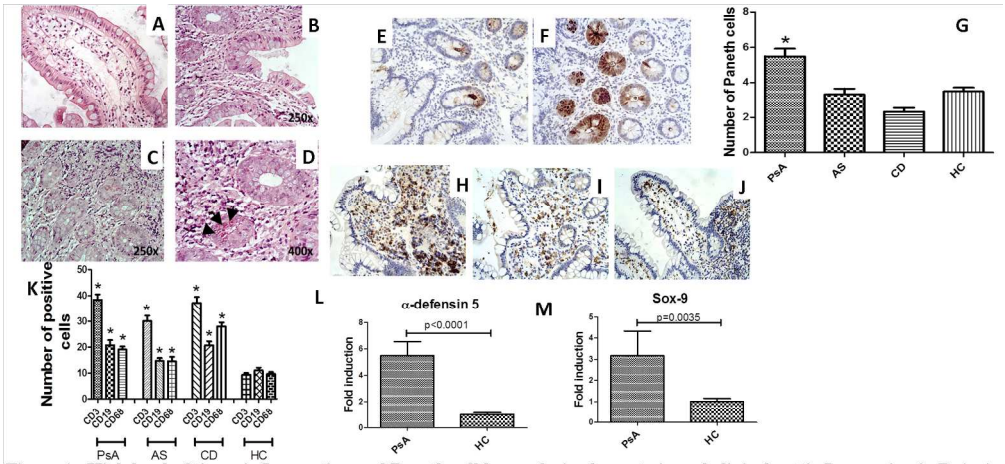

Figure 1. High level of tissue inflammation and Paneth cell hyperplasia characterize subclinical gut inflammation in PsA. A-D: representative images of H&E stained ileal sections of PsA. Arrows in **D** indicate Paneth cells. **E-F**: representative images of α-defensin 5 staining in healthy controls (**E**) and PsA (**F**). **G**: semiquantitative evaluation of PC in PsA. **H-J**: inflammatory infiltrating cells, CD3 (**H**), CD19 (**I**) and CD68 (**J**) positive cells in PsA gut. **K**: semiquantitative evaluation of CD3, CD19, CD68 in PSA. **L-M**: Relative m-RNA levels of α-defensin 5 (**L**) and SOX9 (**M**) were assessed by quantitative RT-PCR in ileal samples obtained from 25 PsA, 15 AS, 10 CD patients and 20 control subjects. Data are expressed as mean fold of induction (SEM). **A,C, E-F, H-J**: original magnification x 250. **D**: original magnification x 400.

170x103mm (300 x 300 DPI)

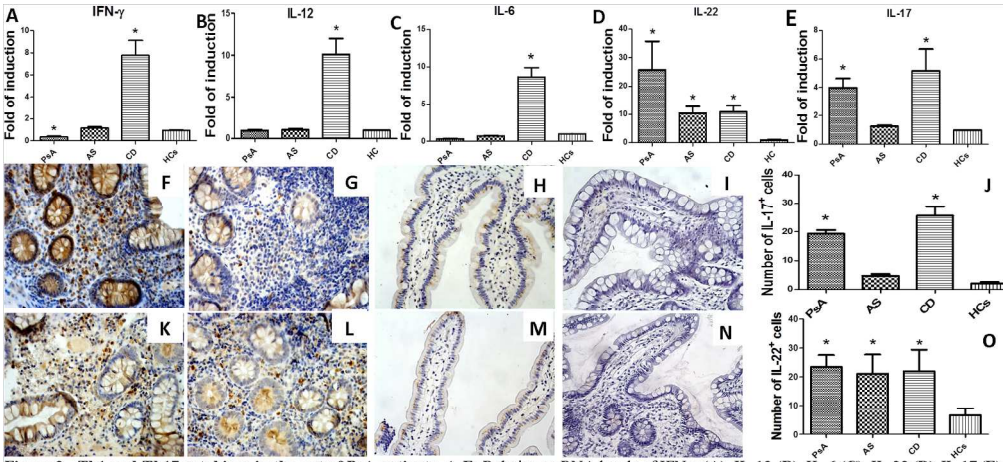

Figure 2. Th1 and Th17 cytokines in the gut of PsA patients. **A-F:** Relative m-RNA levels of IFN-γ (**A**), IL-12 (**B**), IL-6 (**C**), IL-22 (**D**), IL-17 (**E**) were assessed by quantitative RT-PCR in ileal samples obtained from PsA, AS, CD patients and control subjects (HCs). **F-I**: Representative micro-photographs showing IL-17 immunostainings in PsA (**F**), AS (**G**) patients and normal controls (**H**). **J**: IL-17 semiquantitative score in the ileum of patients and controls. **K-M**: Representative microphotographs showing IL-22 immunostainings in PsA (**K**), AS (**L**) patients and HCs (**M**). **O**: IL-22 semiquantitative score in the ileum of patients and controls. Data are expressed as mean (SEM). *p<0.05 compared to control subjects. **I, N**: represen-tative images showing isotype control staining for IL-17 (**I**) and IL-22 (**N**) in the gut of PsA patients. **F-G, J-K**: original magnification x 400. **H, L**: original magnification x 100; **I-N**: original magnification x250.

170x103mm (300 x 300 DPI)

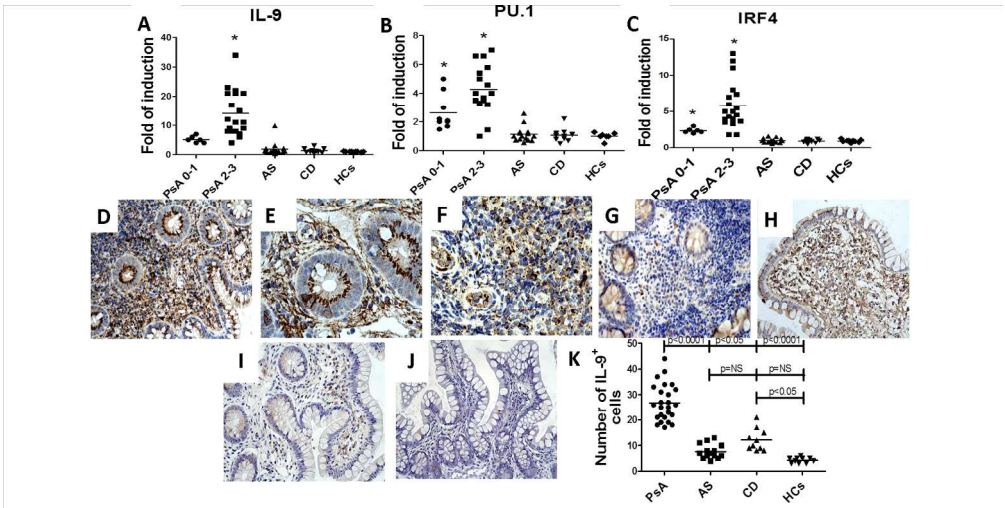

Figure 3. IL-9 in the gut of PsA patients. A-C: Relative m-RNA quantification of IL-9 (**A**), PU.1 (**B**) and IRF4 (**C**) was assessed by quantitative RT-PCR in ileal samples obtained from 25 PsA, 15 AS, 10 CD patients and 20 control subjects. **D-I**: Representative microphotographs showing IL-9 immunostainings in PsA (**D-F**), AS (**G**) and CD (**H**) patients and normal controls (**I**). **J**: representative image showing isotype control staining in PsA patients. **K**: IL-9 semiquantitative score in the ileum of patients and controls. Data are expressed as individual data points (mean). *p<0.05 compared to control subjects. **D-J**: original magnification x 250.

170x103mm (300 x 300 DPI)

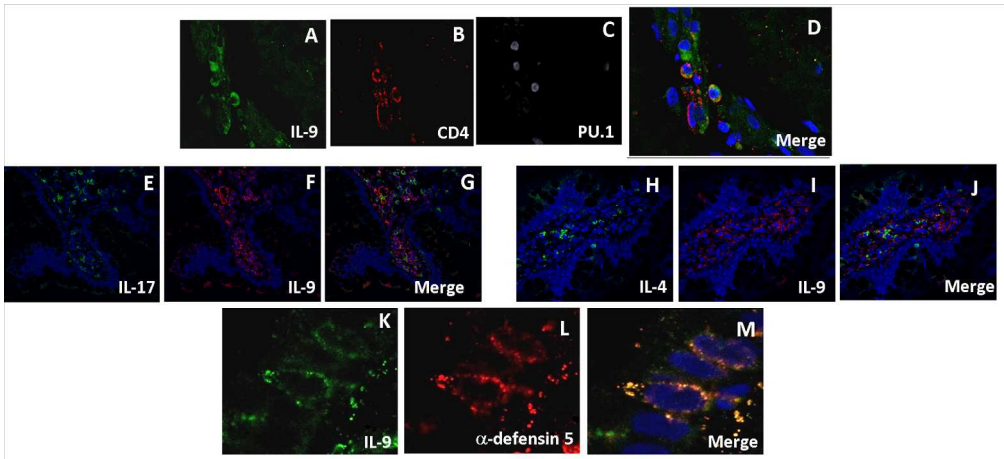

Figure 4. Th9 cells but not Th17/Th9 cells produce IL-9 in PsA gut. A-D: Th9 cells in PsA gut. A: single staining for IL-9. B: single staining for CD4; C: single staining for PU.1. D: merged triple staining for IL-9/CD4/PU.1. **E-G**:Th17 in PsA gut. **E**: single staining for IL-17. **F**: single staining for IL-9. **G**: merged double staining for IL-17 and IL-9. **H-J**: Th2 cells in PsA gut. **H**: single staining for IL-4. **I**: single staining for IL-9. **J**: merged double staining for IL-4 and IL-9. **K-M**: Paneth cells in PsA gut. **K**: single staining for IL-9; **L**: single staining for α-defensin5. M: merge double staining for IL-9 and α-defensin 5. **A-D, K-M**: original magnification x630; **E-J**: original magnification x 250.

170x103mm (300 x 300 DPI)

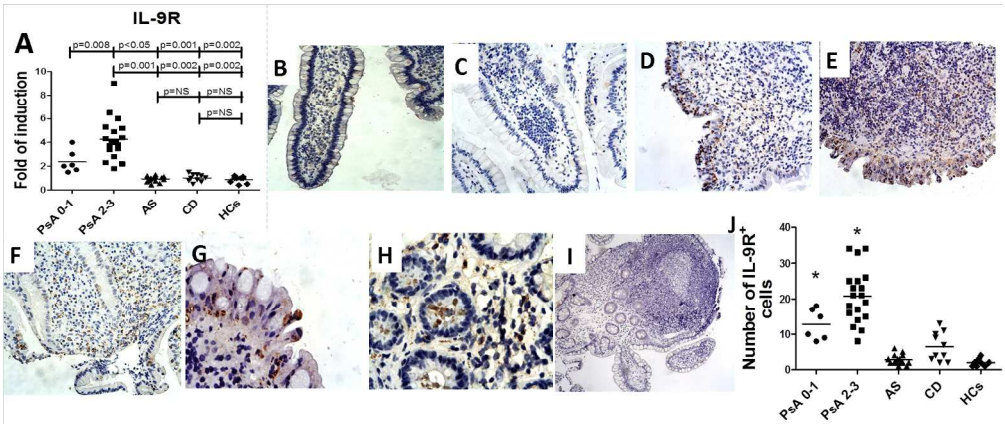

Figure 5. IL-9R in the gut of PsA. A: Relative m-RNA levels quantification of IL-9R (**A**) were assessed by quantitative RT-PCR in ileal samples obtained from 25 PsA, 15 AS, 10 CD patients and 20 control subjects. **B-H**: Representative microphotographs showing IL-9 immunostainings in healthy subject (B), AS (C), CD (D) and PsA patients (**E-H**). **I**: representative picture showing isotype control staining in PsA gut. **J**: IL-9 semiquantitative score in the ileum of patients and controls. Data are expressed as individual data points (mean). *p<0.05 compared to control subjects. **D-F**: original magnification x 250; **G**: original magnification x 400; **H**: original magnification x 630. I: original magnification x100

170x103mm (300 x 300 DPI)

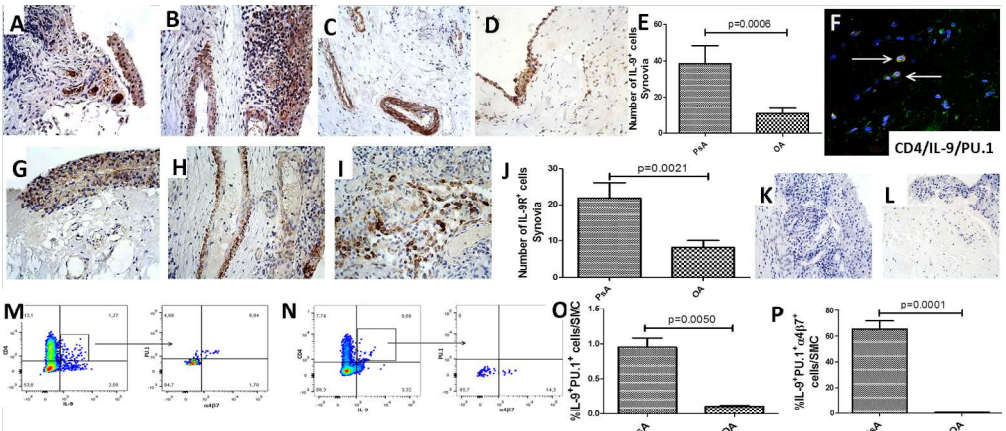

Figure 6. Th9 and IL-9/IL-9R in PsA synovial compart. **A-C**: representative images showing IL-9 immunostaining in synovial tissues obtained from patients with PsA (**A-B**) and osteoarthritis (OA) (**C-D**). **E**: numbers of IL-9$^+$ cells in synovial tissues. **F**: merged double staining of IL-9 and PU.1 in synovial tissues of PsA patients demonstrating the presence of Th9 cells. **G-H**: representative images showing IL-9R immunostaining in PsA synovial tissues (**G-H**) and OA (**I**). **J**: numbers of IL-9R$^+$ cells in PsA and OA synovial tissues. **K-L**: representative immunostainings with isotype antibodies in synovial tissues from PsA patients. **M**: representative dot plots showing Th9 cells in synovial fluid obtained from 5 PsA patients. A large percentage of Th9 cells also express α4β7. **N**: representative dot plots showing Th9 cells in synovial fluid obtained from 5 OA patients. **O**: percentages of Th9 cells and (**P**) of α4β7$^+$ Th9 cells in the synovial fluid obtained from PsA and OA patients. **A-C**, **F-H, JK**: original magnification x250. **D.I. N.O**: data are expressed as mean (SEM).

170x103mm (300 x 300 DPI)