Evidence of IL-17 producing innate lymphoid cells in peripheral blood from patients with enteropathic spondyloarthritis

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Abstract

Objective

Both the innate and the adaptive immune responses contribute to the onset of chronic inflammation in spondyloarthritis (SpA). The association between SpA and inflammatory bowel disease (IBD, enteropathic SpA-ESpA) has been largely established and suggests a shared pathophysiology. There is evidence that innate lymphoid cells (ILC) are involved in the pathogenesis of both SpA and IBD while no evidence has been reported to date on ESpA. We aimed to analyse for the first time the frequency and cytokine expression of ILC in peripheral blood from ESpA patients compared with both IBD and healthy subjects. Correlations between immunophenotyping and disease activity were also explored.

Methods

ESpA patients (n=20) were prospectively enrolled. Healthy controls (HC, n=10) and IBD patients (n=10) served as control groups. Peripheral blood Interferon (IFN)-γ and interleukin (IL)-17 expressing T and non-T cells as well as ILC subsets (ILC-1: IFN- γ +; ILC-3: IL-17+; natural killer-NK) were characterised by flowcytometry. Correlations between IL-17+ cells and SpA disease activity were analysed.

Results

ESpA patients showed higher levels of ROR-γ expressing non T-cells with the respect to the controls. IL-17 producing non-T cells were higher than the HC and positively correlated with IFN-γ expressing cells levels as well as with SpA disease activity. ESpA showed higher levels of ILC-1 and ILC-3 than both IBD and HC. IFN-γ expressing NK cells were higher in ESpA than HC.

Conclusion

Our preliminary findings indicate that peripheral blood of ESpA patients is enriched for IL-17 expressing ILC which distinguishes the blood compartment from both IBD and HC. The increased IL-17 production by ILC indicates a novel role for ILC in ESpA.

Key words

inflammatory bowel disease, innate lymphoid cells, interleukin-17, spondyloarthritis

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Introduction

Spondyloarthritis (SpA) represents a distinct group of inflammatory disorders that primarily affect entheses, small and large joints, and axial skeleton joints sharing similar clinical features and genetic background (1). The ASAS recommendations include the inflammatory bowel disease (IBD) among the parameters for a diagnosis of SpA (2). The joint involvement observed in IBD is usually classified as axial and peripheral (3). Cumulative evidence from both experimental and human models strongly supports the involvement of the interleukin (IL)-17 in the pathogenesis of SpA and, instead of IBD, anti-IL-17A specific antibody rapidly reduces clinical and biological signs of active spondylitis and is well tolerated (4, 5). However, an emerging family of innate immune cells has been recently characterised, termed innate lymphoid cells (ILC), that has revealed to play an essential role in the initiation, regulation and resolution of inflammation (6). IL-17 expressing ILC have been found in gut mucosal tissues, skin, and lungs, and are considered as important sentinels for the immune system for their role in preserving barrier function and epithelial integrity as well as by cooperating with T cells in cytokine secretion (6). Several data are reported in the literature on the role of RORγ-driven inflammatory type 17 immune responses T cell-mediated in the immune-pathogenesis of SpA, but to date no evidence is documented on the contribution of IL-17 ILC-mediated in SpA patients with concomitant IBD (enteropathic SpA [ESpA]) (7, 8). That issue might be useful to improve the management of patients with ESpA since ILC could represent a new potential source of more detailed disease markers as well as more tailored targets of therapy. We thus aimed to analyse for the first time the frequency and cytokine expression of peripheral blood T and non-T cells with particular focus on ILC from patients with ESpA compared with both healthy controls (HC) and IBD patients.

Methods

The study was designed as a prospective cohort study performed at the Department of Medicina dei Sistemi, University of Rome Tor Vergata, (Italy), during the period November 2012 to July 2015. Patients were enrolled from the combined gastrointestinal-rheumatologic outpatient clinic (3). ESpA patients were included in the study if they met the following inclusion criteria: they had a diagnosis of both IBD and SpA (1-3), they were ≥18 years old, and were naive for biologic treatment. IBD patients included in the study met the same inclusion criteria excepted for the joint involvement. Matched HC were enrolled at the same clinic. Demographic and clinical data were recorded in a database during the combined visit by experienced gastroenterologists and rheumatologists (3). Rheumatologic assessment included: physical examination with 68 tender and 66 swollen joint count, presence of dactylitis, enthesitis, inflammatory spinal pain and buttock pain. Joint imaging was requested (musculoskeletal ultrasound, joint traditional radiography and magnetic resonance imaging of the sacroiliac joints with STIR sequences, where appropriate). ASAS criteria were used in order to classify patients as affected by axial or/and peripheral SpA (3, 9). Disease activity in SpA patients was assessed using Disease Activity Score [DAS, on 44 joints, C-reactive protein (CRP)-based], Ankylosing Spondylitis Disease Activity Score (ASDAS, CRP-based), Bath Ankilosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI), and Health Assessment Questionnaire for SpA (HAQ-S). A high SpA disease activity was defined with DAS ≥1.6, ASDAS ≥2.1, or BASDAI ≥4 (10) .The clinical activity of the intestinal disease was evaluated by using the Mayo Clinic Score in ulcerative colitis (UC) and the Crohn's Disease (CD) Activity Index (CDAI), in both ESpA and IBD subjects: a Mayo score <3 and a CDAI score <150 indicated remission (3). All the patients enrolled in the study were naïve for treatment with biologic agents and were steroid-free at the time of the laboratory assays.

Routine laboratory examinations and genetic testing (HLA-B27) were performed in all ESpA and IBD patients

including CRP that was tested by nephelometric measurement (normal range, 0–3 mg/l). For immunophenotyping, peripheral blood mononuclear cells (PBMCs) were isolated from all the subjects using fresh EDTA-K3 anti-coagulated blood samples and according to the standard Ficoll gradient procedure. Freshly isolated PBMCs were phenotypically characterised by flow cytometry using the following antibodies (1:50, final dilution): anti-CD3- peridinin chlorophyll protein (PerCP), anti-CD45- allophycocyanin (APC) H7, anti-T-bet- R-Phycoerythrin (Pe) Cy7, anti-Foxp3- Fluorescein isothiocyanat (FITC), anti-RORγ-APC. All of the antibodies were from BD Biosciences (San Jose, CA). In order to further investigate the CD45+CD3- cells, the Lineage negative cells (Lin-) were counted, and in this group the ILC (Lin-/CD127+) phenotype, the NK (Lin-/CD127-/CD56+) phenotype, and the CD56+ILC (Lin-/CD127+/CD56+) phenotype, in the peripheral blood of the cases (EspA) and controls (boh IBD and HC). For this analysis, freshly isolated PBMCs were characterised with anti-human lineage cocktail 3 (Lin 3, CD3/CD14/CD19/CD20)-FITC, anti-CD56-PECy7, anti-CD45-APCH7, and anti-CD127-PerCPCy5.5 (anti-Lin3, -CD56, -CD45 from BD Biosciences, San Jose, CA; anti-CD127 from eBioscience, San Diego, CA). For detection of intracellular cytokine production in CD45+CD3+ and CD45+CD3- cells, PBMCs were resuspended in RPMI 1640 medium, supplemented with 10% inactivated FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml), seeded in 96-well U-bottom culture dishes, and stimulated with phorbol myristate acetate (4 μg/mL) and ionomycin (1 μg/mL) and brefeldin A (1 μg/mL; eBioscience, San Diego, CA). After 4 h, cells were stained for IL-4, IFN-γ, and IL-17A with the following antibodies (1:50, final dilution): anti-IL-4-FITC, IFN-γ–APC and IL-17A–V450 (BD Biosciences, San Jose, CA). In order to further investigate the ILC, both IL-17 and IFN-γ producing Lin negative cells were counted, and among them, IFN-γ and IL-17 expressing ILC (ILC-1 and ILC-3 respectively), IFN-γ and IL-17 expressing NK cells, and IFN-γ and IL-17 expressing CD56+ILC were characterised. Appropriate isotype-matched controls from BD Biosciences were included in all the experiments. Flow cytometric analysis was performed using a FACSCVerse (Becton Dickinson) and analysed by FACSsuite software. At least 100000 cells (events) were acquired for each sample. PBMCs were expressed as percentage of cells within the lymphocyte gate. The acquired data were analysed using the CellQuest and FlowJo software programmes.

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was consistent with the guidelines for good clinical practice. Informed consent was obtained from each participant of the study.

Statistical analysis

To test the normality of data sets, the D'Agostino and Pearson omnibus test was used. Normally distributed variables were expressed as mean ± standard error of the mean (SEM). Continuous variables were compared using the unpaired *t*-test. Contingency analyses were performed by Chi-square test or Fisher's exact test where appropriate. A measure of the correlation (linear dependence) between two variables was obtained using Pearson's r. Statistical analysis was considered significant when two-tailed p=values were <0.05. All data were stored on a server and statistical analyses were performed using GraphPad Prism Software v. 6.0.

Table I. Demographic and clinical data of subjects in the study population.

	ESpA (n=20)	IBD (n=10)	HC (n=15)
Age (years)	42 ± 15	50 ± 8	37 ± 8
Female sex (n/%)	17/85	8/80	13/86.7
UC/CD	6/14	7/2*	NA
IBD disease duration (years)	13 ± 9	10.5 ± 14	NA
SpA disease duration (years)	4.6 ± 7	N.A.	NA
Mayo / CDAI remission (n/%)	20/100	10/100	NA
CRP levels (mg/dl)	2.2 ± 2	1 ± 0.9	NA
HLA-B27 (yes/no)	2/18	1/9	NA
Joint involvement: - axial (n/%)	3/15		
- peripheral (n/%)	12/60	NA	NA
- both (n/%)	5/25		
DAS44-CRP	2 ± 0.9	NA	NA
ASDAS-CRP	2.9 ± 1	NA	NA
BASDAI	5.7 ± 2.2	NA	NA
BASFI	5 ± 8	NA	NA
HAQ-S	0.8 ± 0.5	NA	NA
DMARDs (yes/no)	14/6**	2/8**	NA
Corticosteroids (yes/no)	0/20	0/10	NA

ESpA: enteropathic spondyloarthritis; IBD: inflammatory bowel diseases, HC: healthy controls; UC: ulcerative colitis; CD: Crohn's disease; CDAI: Crohn's disease activity index; CRP: C-reactive protein; DAS: Disease Activity Score (on 44 joint count); ASDAS: Ankylosing Spondylitis Disease Activity Score; BASDAI: Bath Ankilosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; Health Assessment Questionnaire for SpA (HAQ-S); DMARD: disease-modifying anti-rheumatic drugs; NA: not applicable. *One IBD patient has a diagnosis of indeterminate colitis. **Fisher's exact test p<0.05.

Results

Twenty ESpA patients were enrolled in the study. Age/sex-matched IBD patients and HC were used as controls. Clinical and laboratory data of the study cohort were described in Table I. In ESpA group, 16 patients (80%) showed ASDAS ≥2.1 and BASDAI ≥4 and 13 patients (65%) had DAS ≥1.6. All ESpA and IBD patients were on Mayo / CDAI remission. No correlations occurred between SpA disease activity scores and respective CDAI or Mayo scores. Demographic and clinical features of ESpA patients were compared with those of IBD non-SpA patients and no differences in IBD diagnosis/disease duration/ disease activity (CDAI/Mayo), and CRP levels were found (Table I). The use of disease-modifying

Supplementary Table I. Immunophenoyping of peripheral blood mononuclear cells in the study population.

	ESpA (n=20)	IBD (n=10)	HC (n=15)
CD45+ CD3+ cells	73.7 ± 7.8	71.6 ± 6	70 ± 6
T-bet	38 ± 4.5	20 ± 3	29.5 ± 4
ROR-γ	11.8 ± 5.8	3.6 ± 1.7	3.8 ± 1.2
Foxp-3	3.7 ± 1.2	1.5 ± 0.5	1.7 ± 0.5
IFN-γ	7.3 ± 1.8	4.5 ± 2.3	5.4 ± 2.3
IL-4	2.7 ± 0.8	1.2 ± 0.6	1 ± 0.2
IL-17	5.3 ± 1.3	1.7 ± 0.8	2.9 ± 0.4
CD45+ CD3- cells	24.2 ± 4.9	24 ± 3	23 ± 5
T-bet	39 ± 5	49 ± 24	31.3 ± 3.3
ROR-γ	38.7 ± 9.6*	18 ± 10	13.8 ± 3.4
Foxp-3	15 ± 4.2	4.5 ± 3	5.4 ± 1.8
IFN-γ	10.4 ± 2.5*	4.6 ± 2.3	4 ± 1.2
IL-4	0	0	0
IL-17	15.4 ± 3*	7 ± 3.5	5 ± 1.2

Frequencies of CD45+CD3+ and CD45+CD3- cells in peripheral blood mononuclear cells from subjects in the study groups. Data are expressed as mean ± standard error of the mean. ESpA: enteropathic spondyloarthritis; IBD: inflammatory bowel disease; HC: healthy controls; IFN: interferon; IL: interleukin. Differences between mean values were analysed with unpaired *t*-test. *$p<0.05$ compared with both IBD and HC.

anti-rheumatic drugs (DMARDs) were more prevalent in ESpA than in IBD patients ($p=0.02$, Table I).

Elevated levels of IL-17A expressing non-T cells in ESpA patients

Frequencies of CD45+CD3+ cells and CD45+CD3- in PBMCs from subjects in the study groups were reported in Supplementary Table I. ESpA, IBD, and HC showed comparable frequencies of T-bet/ROR-γ/Foxp3 expressing CD45+CD3+ cells (Fig. 1A). The same was registered analysing IFN-γ/IL-4/IL-17 expressing CD45+CD3+ cells (Fig. 1B). The analysis of CD45+CD3- cells reported significantly higher levels of ROR-γ expressing cells in ESpA than in HC ($p=0.03$) (Fig. 1C). The T-bet expression was similar among the three groups while a not significant increase of Foxp-3 CD45+CD3- expressing cells occurred in ESpA than in HC and IBD (Fig. 1C). ESpA patients had higher IL-17 and IFN-γ expressing CD45+CD3- cells when compared with HC ($p=0.04$ and $p=0.02$, respectively) while the differences between ESpA and IBD did not reach the statistical significance (Fig. 1D). In ESpA patients, IL-17 expressing CD45+CD3- cells were positively correlated with IFN-γ+CD45+CD3- cells (r=0.6, $p=0.009$) (Fig. 2A). Moreover, the frequency of IL-17+CD45+CD3- cells were positively related to DAS (r=0.7, $p=0.02$) and BASFI (r=0.8, $p=0.002$) while no correlations were found with ASDAS and BASDAI in ESpA group (Fig. 2B-C). No correlations were found between the frequencies of IFN-γ+CD45+CD3- cells and the SpA disease activity scores (data not shown). CRP levels did not correlate with IL-17+ and IFN-γ + expressing CD3- cells in both ESpA and IBD patients (data not shown).

Higher ILC-1 and ILC-3 levels in peripheral blood from ESpA patients compared to controls

In order to further characterise IFN-γ and IL-17 expressing CD45+CD3- cells, we firstly counted Lin- cells in CD45+ cells. Representative dot-plots of gating strategies and flow cytometry analyses of Lin- cells in CD45+ cells from an ESpA patient were reported (Fig. 3A). Representative dot-plots of IFN-γ and IL-17 expressing Lin- cells from ESpA, IBD, HC, and isotype controls are shown in Figure 3B-E. IFN-γ expressing Lin- cells were more abundant in ESpA than both IBD and HC ($p=0.04$ for both the comparisons, Fig. 3F). Likewise, IL-17 expressing Lin- cells were higher in ESpA than in HC ($p=0.01$) but even they were higher in ESpA compared with IBD that difference did not reach the statistical significance (Fig. 3F). No differences occurred between IBD and HC (Fig. 3F). We further analysed peripheral blood ILC (CD127+Lin- cells), CD56+ILC (CD56+CD127+Lin- cells) and NK cells (CD56+Lin- cells) in the three groups. Representative dot-plots of gating strategies and flow cytometry analyses of ILC, CD56+ILC, and NK cells from peripheral blood CD45+ Lin- cells from a patient with ESpA were reported in Fig. 4A. We analysed ILC-1 cells, represented by IFN-γ producing ILC, and ILC-3, represented by IL-17 producing ILC. ESpA showed higher levels of ILC-1 than both IBD and HC ($p=0.04$ for both comparisons) (Fig. 4B). ILC-3 were higher in ESpA than both IBD ($p=0.03$) and HC ($p=0.01$, Fig. 4C). We also measured both IL-17 and IFN-γ producing CD56+ILC and NK cells from the peripheral blood Lin- cells of the cases (ESpA) and controls (IBD and HC). IFN-γ expressing CD56+ILC were not different among the groups while IL-17 expressing CD56+ILC were higher in ESpA compared to HC ($p=0.04$) but not to IBD (Fig. 4B-C). IFN-γ+ NK cells levels were higher in ESpA than in IBD while there was no difference between ESpA and HC (Fig. 4B). IL-17 expressing NK cells were registered in ESpA patients while they were not detectable in IBD and HC (Fig. 4C). Representative dot-plots of IFN-γ+ and IL-17+ ILC, CD56+ILC, and NK cells from peripheral blood CD45+ Lin- cells from a patient with ESpA, IBD, HC, and an isotype control were reported in Figure 5.

Discussion

Current evidence highlights the emerging role of innate immune cells and their mechanisms in the pathogenesis of several autoimmune/auto-inflammatory conditions, such as SpA, suggesting a potential functional equivalent of adaptive cells in these conditions (4, 7, 11). Cumulative data strongly support the involvement of the IL-23/IL-17 axis in the pathogenesis of SpA and there is an increasing genetic and functional evidence that ILC contribute to the RORγ-driven inflammatory type 17 immune responses in SpA (4, 7, 8, 12, 13). ESpA represents a complex condition that supports a possible relationship be-

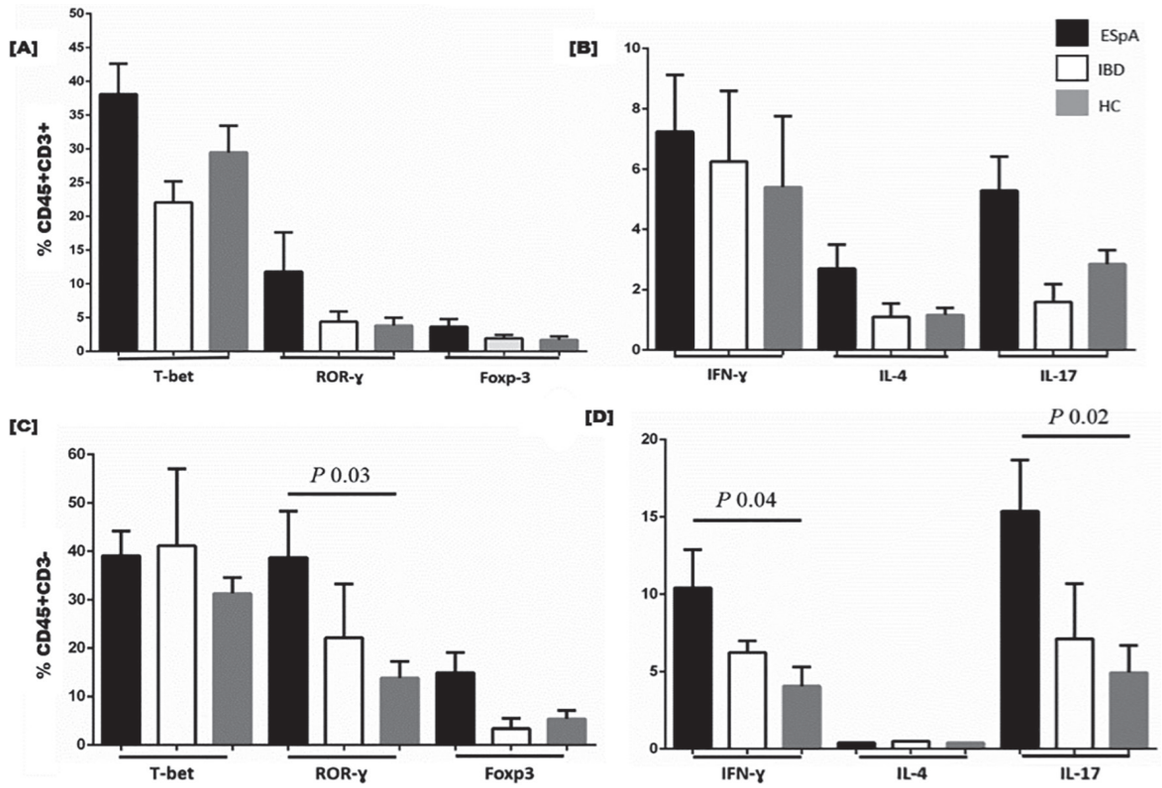

Fig. 1. Frequencies of CD45+CD3+ and CD45+CD3- in peripheral blood from subjects in the study population. ESpA: enteropathic spondyloarthritis; IBD: inflammatory bowel disease; HC: healthy controls. T-bet, ROR-γ, and Foxp-3 expressing CD3+ (panel **A**) and CD3- (panel **C**) were evaluated in freshly isolated peripheral blood mononuclear cells. The expression interferon (IFN)-γ, interleukin (IL)-4, and IL-17 was recorded in CD3+ (panel **B**) and CD3- (panel **D**) cells after 4-hours *in vitro* stimulation with PMA and ionomicin. Data are expressed as percentage (%) of cells (mean ± SEM). Unpaired t-test registered a significant difference between mean values with $p<0.05$.

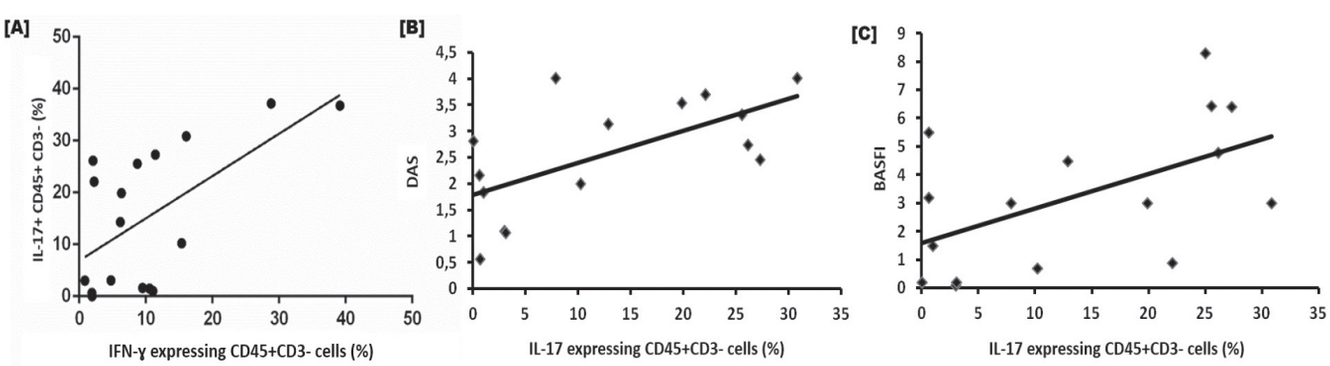

Fig. 2. Interleukin-17+ CD3- cells correlated with interferon-γ+ CD3- cells and disease activity in patients with enteropathic spondyloarthritis. Panel **A**: a positive correlation occurred between interleukin (IL)-17 expressing CD45+CD3- cells and interferon (IFN)-γ expressing CD45+CD3- cells (Pearson's r=0.6, p=0.009) in patients with enteropathic spondyloarthritis (EspA). Panel **B**-**C**: positive correlations were recorded between interleukin IL-17 expressing CD45+CD3- cells and Disease Activity Score (DAS) on 44 joints, C-reactive protein (CRP)-based (Pearson's r=0.7, p=0.02, panel B) and Bath Ankylosing Spondylitis Functional Index (BASFI) (Pearson's r=0.7, p=0.02, panel **C**) in patients with EspA. Pearson's correlation analyses were considered significant with r values related to $p<0.05$.

tween inflammation of the gut mucosa and arthritis and where genetic factors (such as HLA-B27) may play a predisposing role while environmental factors, such as bacterial gut infections, may play a causative role (14). ESpA could be thus considered *per se* a defined disease that shares with IBD and SpA common genetic predisposition and immunologic pathomechanisms. No studies have been performed to date on peripheral blood immunophenotype from patients with ESpA. With this purpose, we investigated peripheral blood T and non-T cells in ESpA patients who were steroid-free and naïve for biologic treatment. Firstly, we observed that the frequencies of Tbet/ROR-γ/Foxp-3 expressing T cells were similar in ESpA patients compared to both the control groups (IBD and HC). We registered the same analysing IL-17 and IFN-γ expressing T cells. These results were consistent with some data from the literature reporting a similar frequency of IL-17 and IFN-γ expressing T cells in PBMCs from SpA patients and healthy

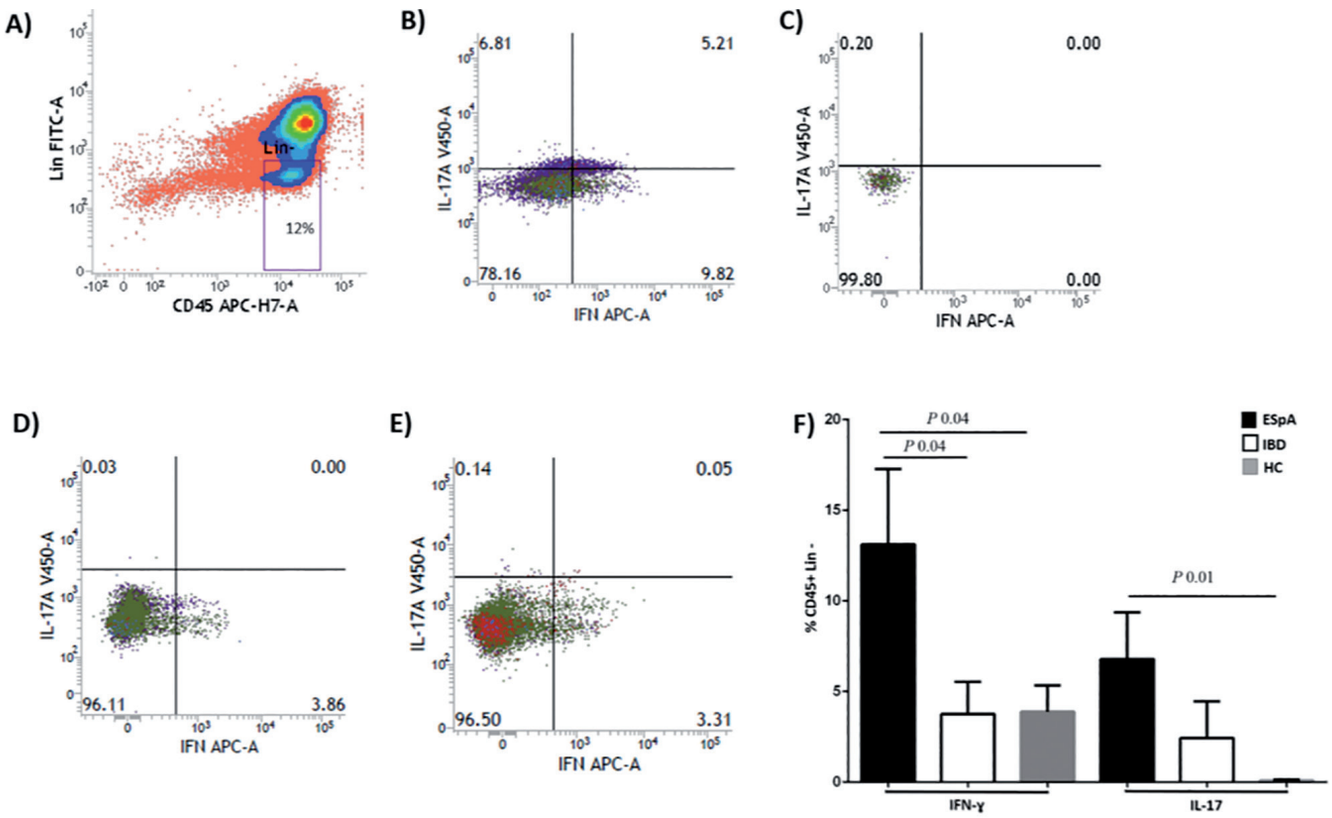

Fig. 3. Flow cytometry analysis of lineage negative cells in the study population.
ESpA: enteropathic spondyloarthritis; IBD: inflammatory bowel disease; HC: healthy controls. Lineage negative (Lin-) cells were characterised in peripheral blood CD45+ cells as CD3/CD14/CD19/CD20 negative cells (representative plots from an ESpA patient were reported in panel **A**). Representative plots of interferon (IFN)-γ and interleukin (IL)-17 expressing cells in Lin- cells from an ESpA patient (panel **B**), an isotype control (panel **C**), an IBD patient (panel **D**), a HC (panel **E**). Frequencies of IFN-γ and IL-17 expressing Lin- cells from the study population (panel **F**). Data are expressed as percentage (%) of cells (mean ± SEM). Unpaired *t*-test registered a significant difference between mean values with $p<0.05$.

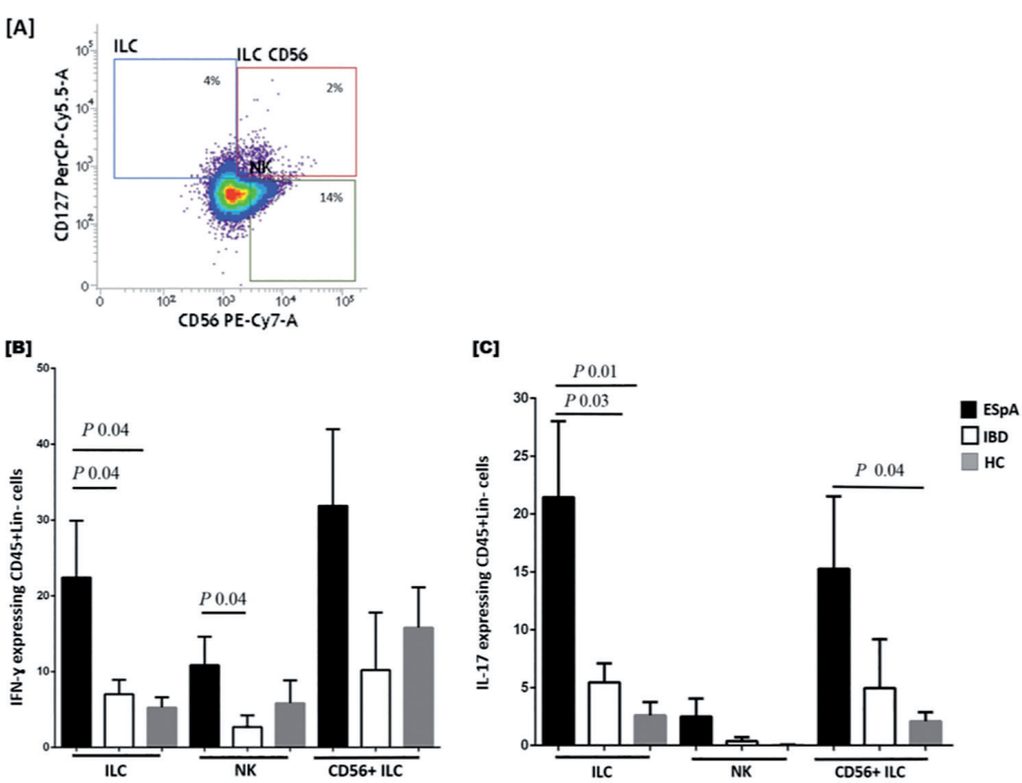

Fig. 4. Flow cytometry analysis of innate lymphoid cells in the study population.
ESpA: enteropathic spondyloarthritis; IBD: inflammatory bowel disease; HC: healthy controls. Innate lymphoid cells [ILC = CD127+; CD56+ILC = CD56+CD127+; natural killer (NK) = CD56+] were characterised in peripheral blood CD45+/lineage negative cells (representative plots and gating strategies from an ESpA patient were reported in panel **A**). A single group analysis of the prevalence of interferon (IFN)-γ expressing cells (panel **B**) and interleukin (IL)-17 expressing cells (panel **C**) in ILC (ILC-1 and ILC-3, respectively), CD56+ILC, and NK cells from subjects in the study population was reported. Data are expressed as percentage (%) of cells (mean±SEM). Unpaired *t*-test registered a significant difference between mean values with $p<0.05$.

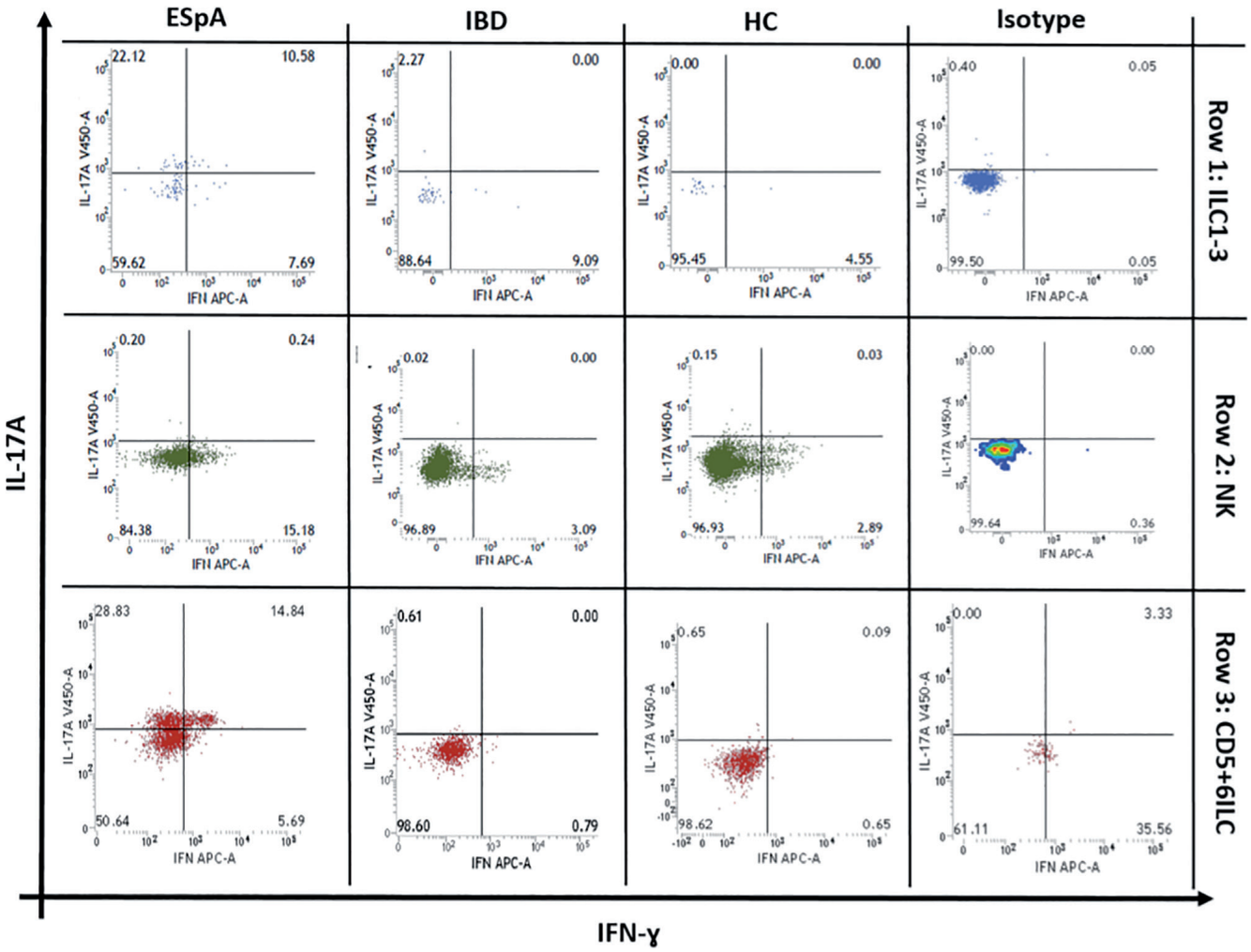

Fig. 5. Flow cytometry analysis of innate lymphoid cells subsets: representative dot plots. ESpA: enteropathic spondyloarthritis; IBD: inflammatory bowel disease; HC: healthy controls. Innate lymphoid cells subsets [ILC = CD127+; CD56+ILC = CD56+CD127+; natural killer (NK) = CD56+] were characterised in peripheral blood CD45+/lineage negative cells. Row 1 reported representative plots of ILC-1 [interferon (IFN)-γ expressing ILC] and ILC-3 [interleukin (IL)-17 expressing ILC] from an ESpA patient, an IBD patient, a HC, and an isotype control. Row 2 and row 3 reported IFN-γ and IL-17 NK and CD56+ILC, respectively, from an ESpA patient, an IBD patient, a HC, and an isotype control.

subjects (15, 16). We described for the first time that the frequencies of ROR-γ/IL-17 expressing CD45+CD3- cells were higher in ESpA patients compared with both IBD and HC. These data might support the emerging role of innate immune cells as a source of IL-17 in ESpA. IL-17 is considered a playmaker in SpA pathogenesis and a target for the therapy (4). It is well-known that both IL-17 and IL-22 are produced in excess in CD and UC gut mucosa and, in this context, IL-23-responsive ILC were described as responsible for intestinal inflammation in experimental models of colitis, through secretion of IL-17A or IFN-γ (17, 18). However, differently from IBD, anti-IL-17A specific antibody rapidly reduced clinical or biological signs of active spondylitis and was well tolerated (5). In addition, we observed higher levels of IFN-γ expressing CD3- cells in ESpA compared to HC that can be related to both IL-17/IFN-γ double positive cells and IFN-γ single-producing cells, giving the phenotype and functional plasticity of human innate immune CD45+ cells (6). Accordingly, we reported that IL-17+ CD3- cells positively correlated with IFN-γ+ CD3- cells (6, 19). We demonstrated that in ESpA patients the frequency of IL-17+ CD3- cells were positively related to DAS and BASFI suggesting an association with both the peripheral active involvement and the axial functional impairment. Authors reported that peripheral blood Th17, Th1 and Th17/Th1 cell numbers were related to disease activity indices in HLA-B27(+) early non radiographic SpA patients (20). In our study, no correlations was found with other SpA disease activity scores such as ASDAS or BASDAI. Further investigations on a larger cohort of patients should be considered to better explore the correlation between IL-17 producing non T cells and disease activity in ESpA patients. However, IFN-γ+ CD3- cells did not correlate with SpA disease activity scores supporting a prominent role of IL-17 in this context. We next explored which type of non T-cells could be the potential source of IL-17 in peripheral blood from ESpA patients. We counted the IL-17 and IFN-γ expressing Lin- cells, ILC-1 and ILC-3 cells, NK cells, and CD56+ILC, a transitional cell population that shares surface markers of

innate lymphoid and natural killer cells. We observed that both the IL-17 and IFN-γ expressing Lin- cells, ILC-1 and ILC-3 cells, and IL-17 and IFN-γ expressing CD56+ILC were increased in patients with ESpA while no difference occurred between IBD and HC. Increases in ILC-3 have been observed in peripheral blood and tissue from patients with several chronic inflammatory diseases, including in the skin of patients with psoriasis (21). Studies reported an increased frequency in pro-inflammatory ILC-3 in intestinal tissues from individuals with IBD (22, 23). Indeed, it has been described that IFN-γ producing ILC could contribute to IBD pathogenesis due to elevation of ILC-1 in inflamed intestinal tissues of patients (24). Likewise, many studies have indicated that ILC could contribute to local cytokine-driven immune alterations in SpA and rheumatoid arthritis (15, 25). However, the role of ILC-1 and ILC-3 in promoting inflammation is not well elucidated, given the substantial heterogeneity observed in the cell lineages, the potential lineage plasticity, and the differential surface markers and gating strategies used to identify the cell population (6). For this puspose, we analysed the frequencies and the cytokine expression not only in the classical ILC but also in NK cells and in those cells positive for both CD127 (an ILC phenotype marker) and CD56 (a NK phenotype marker). We registered a high frequency of NK cells producing IFN-γ in ESpA than in IBD while the difference was not significant compared with HC. Studies reported elevated levels of NK cells in peripheral blood of ankylosing spondylitis (AS) patients (26) and these cells were found to be enriched for the KIR3DL1/KIR3DL2 receptors that interact with HLA-B27 (27). The higher levels of IFN-γ expressing NK cells can be related to the elevated SpA disease activity of ESpA patients according with evidence correlating NK cell and disease activity in psoriatic arthritis (19, 28). Previous studies showed differences in NK cell phenotype between SpA and CD patients and also reported gut-derived IL-17+/IL-22+ ILC as expanded in the peripheral blood, synovial fluid and inflamed bone marrow of patients with AS (8, 13). Therefore, in this context, a next challenge could be represented by the characterisation of ILC in SpA patients with concomitant IBD. We described for the first time that cells with ILC-1 phenotype (IFN-γ+ cells) and cells with ILC-3 phenotype (IL-17+ cells) were expanded in the peripheral blood of ESpA compared to both IBD and HC. Although initially linked to Th17, it has been reported that ILC which respond towards IL-23 are present with a specific tissue distribution and could play a vital function in the development or progression of SpA-related pathology (29). A recent evidence from the literaure reported increased serum levels of IL-23 in IBD subjects (especially in CD patients) with concomitant arthritis and sacroiliitis suggesting a potential role for IL-23 as a biomarker of arthritis development in IBD (30). The evidence of peripheral blood IFN-γ and IL-17 expressing ILC in ESpA patients might support the idea of a novel biomarker for ESpA that needs to be addressed in further analysis (31-34). Our research included relatively few subjects that limit the interpretation of the results concerning the causal correlation between specific peripheral blood ILC and disease activity or clinical phenotype. Given the strong gut involvement in ESpA patients, further investigations on the possible relationship between numbers or function of ILC in the blood with those in the gut are awaited. In addition, the heterogeneity of the study population that comprised both CD and UC should be improved given the difference between these diseases. In our research, we described ILC as CD127+CD45+ Lineage negative (CD3/CD14/CD19/CD20) cells but other markers could be used to better characterise these cells in future investigation (*i.e.* NKp44, NKp46, IL-22, CRTH2, *et al.*). However, a universal consensus on gating strategies on human ILC is still under investigation and a more detailed gating strategy could be implemented. Additional studies are needed to carefully define the potential plasticity of ILC populations, and to identify novel functions and regulatory pathways influencing ILC responses. These advances will be critical for our exploring how and whether we can regulate ILC responses to limit the chronic inflammation and to target the therapy of the disease.

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