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ORIGINAL RESEARCH

Type 17-specific immune pathways are active in early spondyloarthritis

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ABSTRACT

Objective Undifferentiated, early inflammatory arthritis (EIA) can differentiate into seropositive or seronegative rheumatoid arthritis (RA), peripheral spondyloarthritis (SpA) or remain as seronegative undifferentiated inflammatory arthritis (UIA). Little is known about immune pathways active in the early stages of SpA and seronegative UIA, in contrast to detailed knowledge of seropositive RA. The aim of this study was to examine if specific immune pathways were active in synovial CD4+ and CD8+ T cells in EIA.

Methods Synovial fluid (SF) samples from 30 patients with EIA were analysed for expression of IL-17A, IFN   and TNF   in CD8+ or CD4+ T cells. Final clinical diagnoses were made at least 12 months after sample collection, by two independent clinicians blind to the study data.

Results Flow cytometry analysis of all EIA samples indicated considerable variation in synovial IL-17A+CD8+ T cells (Tc17) cell frequencies between patients. The group with a final diagnosis of SpA (psoriatic arthritis or peripheral SpA, n=14) showed a significant enrichment in the percentage of synovial Tc17 cells compared with the group later diagnosed with seronegative UIA (n=10). The small number of patients later diagnosed with seropositive RA (n=6) patients had few Tc17 cells, similar to our previous findings in established disease. In contrast, RA SF contained a significantly higher percentage of CD8+IFN  + T cells compared with SpA or seronegative UIA.

Conclusion These results suggest that adaptive T cell cytokine pathways differ not only between RA and SpA but also seronegative UIA early in the disease process, with a particular activation of Tc17 pathways in early SpA.

INTRODUCTION

Early inflammatory arthritis (EIA) can be described as a duration of inflammatory features of greater than 6 weeks but less than 1–2 years.^{1–3} Patients in this category are classically defined as having seronegative undifferentiated arthritis if they do not satisfy the criteria for diagnosis of rheumatoid arthritis (RA), psoriatic arthritis (PsA) or axial and peripheral spondyloarthritis (SpA).

It is increasingly recognised that there are distinct differences in the clinical manifestations, genetics, serological and cellular features between established RA and SpA/

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Many studies have reported on the presence of pro-inflammatory cytokine-expressing T cell subsets in the joints of patients with established rheumatoid arthritis (RA) and spondyloarthritis (SpA). Much less is known about the immune pathways that are active in early inflammatory arthritis (EIA), particularly in early SpA and seronegative undifferentiated inflammatory arthritis (UIA). We aimed to examine if specific immune pathways are active in synovial CD4+ and CD8+ T cells from patients with EIA.

WHAT THIS STUDY ADDS

⇒ Our results suggest that adaptive T cell cytokine pathways differ already early in the disease process between patients who go on to be diagnosed with seropositive RA versus those later diagnosed with seronegative SpA or UIA. We report evidence for a particular activation of type 17 pathways in CD8+T cells in early SpA.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These data add to the increasing evidence that the immunopathology of seropositive RA and seronegative SpA and UIA is different.

PsA,^{4–6} with much less research into undifferentiated arthritis or seronegative RA. In contrast to RA, patients with SpA/PsA can have significant extra-articular disease associations (psoriasis, inflammatory bowel disease, uveitis).⁷ Furthermore, PsA is associated with increased body mass index in many patients with concomitant metabolic syndrome and cardiovascular morbidity.⁸ There are strong associations between seropositive (ACPA+) RA and cigarette smoking and *HLADRB1* shared epitope alleles.^{3 9–12} These associations are not observed in seronegative RA. Instead, it has been suggested that *HLADRB3* and interferon regulatory factor 5 (*IRF5*) may predispose to seronegative RA.^{13–15} It is also unclear as to whether seronegative RA is one or several disease entities.³ Finally, we recently

reported quantitative differences in the composition of synovial tissue-resident memory T cells between patients with established RA versus PsA.⁵

Key cytokine pathways with broad unified activities have been identified, with the type 1 pathway indicated by interferon-gamma (IFN γ), and the type 17 pathway indicated by IL-17A.¹⁶ These two pathways are known to be expressed in the joints of patients with inflammatory arthritis. IFN γ is a potent activator of myeloid cells and of MHC class I/II expression, highlighting its proinflammatory role, although it should be noted that certain aspects of its pathogenic action in inflammatory arthritis remain incompletely elucidated.^{17 18} IL-17A is a proinflammatory cytokine with broad-ranging effects on angiogenesis, neutrophil infiltration and stromal cell activation.^{19–21} In the context of inflammatory arthritis, IL-17A can synergise with TNF α to promote osteoclast activation²² and induce proinflammatory cytokine and chemokine production from fibroblasts and synovocytes.^{23–27} Paradoxically, and relevant to SpA, IL-17A has also been shown to promote new bone formation in HLA-B27/h β (2) m-transgenic rats.²⁸ Our previous work demonstrated that IL-17A+CD8+ T cells with a tissue-resident profile are enriched in the synovial fluid (SF) of patients with established PsA/SpA, but not in patients with RA suggesting that IL-17 expression is an important differentiating pathway between these two diseases.^{5 29 30}

To enhance our understanding of the lymphoid cytokine pathways that may operate early on in the disease process of inflammatory arthritis, we investigated T cell expression of IL-17, IFN γ and TNF α in the inflamed joints of patients with EIA.

MATERIALS AND METHODS

Clinical diagnosis

We enrolled patients with early inflammatory arthritis which we defined as symptoms of arthritis of no more than 12 months duration, with an initial clinical diagnosis of an inflammatory arthritis including RA, SpA—which included PsA, enteric-related IA and reactive arthritis—and seronegative undifferentiated inflammatory arthritis (UIA). Patient samples were collected between Feb 2014 and May 2021 (online supplemental table 1). Final clinical diagnoses were independently applied by two investigators (BWK and BM) who were blind to each other's diagnosis and research laboratory data, using clinical, imaging and laboratory data including rheumatoid factor, ACPA and ANA autoantibodies, from patient electronic records. Diagnoses were compared and two patients with slightly different diagnoses were discussed to complete a final consensus diagnosis. The changes in diagnosis from the time SF samples were taken to final diagnoses were: four patients initially diagnosed as reactive arthritis, with a weak history of a preceding infection arthritis, developing into an undifferentiated inflammatory arthritis (UIA) pattern of disease, one patient diagnosed with UIA who subsequently had a preceding infection confirmed

with a final diagnosis of reactive arthritis, one patient diagnosed as UIA and another with reactive arthritis who subsequently developed a PsA pattern of disease and one patient initially diagnosed with PsA who did not have psoriasis with a final diagnosis of UIA.

Samples and cell isolation

SF samples from patients with EIA were collected following written informed consent by the Rheumatology Department at Guy's Hospital (REC reference 06/Q0705/20 and 17/LO/1940). Patient demographic and clinical information is shown in table 1. SF mononuclear cells (SFMCs) were isolated by density gradient centrifugation using Lymphoprep (Alere Technologies) and cryopreserved in liquid nitrogen until use.

Flow cytometric analysis

For intracellular cytokine staining, SFMC were thawed, rested for 1 hour and then stimulated in culture medium (RPMI 1640 with 1% Pen/Strep/Glutamine and 10% fetal calf serum (FCS)) with phorbol myristate acetate (PMA) (50 ng/mL) and ionomycin (750 ng/mL, both Sigma-Aldrich) for 3 hours at 37°C in the presence of GolgiStop (monensin, according to manufacturer's recommendation, BD Biosciences). Cells were washed and labelled with fixable viability dye (LIVE/DEAD eFluor780, eBioscience), alongside extracellular staining with mouse-anti-human antibodies (see table 2 for details), for 20 min at 4°C. Cells were fixed with 2% paraformaldehyde for 15 min at 4°C, then permeabilised using 0.5% saponin (Sigma-Aldrich) and stained for 30 min at 4°C with the mouse-anti-human antibodies. For fluorescence minus (FM) control staining, small sample aliquots were combined and stained as above but without addition of anti-cytokine mAbs. Samples were acquired using either a FACS CantoII or LSRFortessa (BD Biosciences). Flow cytometry data were analysed using FlowJo software (V.10.8.1, TreeStar). The vast majority of samples were stained and acquired between January 2018 and December 2019. Once all samples were acquired, an analysis pipeline was set up and applied to ensure that all samples were gated and analysed in an identical manner (analysis performed between July 2020 and July 2021). For this, CD8+ and CD4+ T cells were gated followed by FM control gating, which was set separately for each individual sample and cytokine to aid determination of cytokine-expressing cell populations (online supplemental figure 1).

Autoantibody analysis

Almost all patients (90%) had routine ACPA testing at our centre at the time of SF sampling, using the Elia test (Thermo-Fisher, with a Phadia250 instrument). To extend our characterisation of seronegative undifferentiated arthritis patients, we also determined ACPA antibodies and anti-CarP antibodies, in patients with saved serum. These ACPA IgG antibody levels were determined using an in-house ELISA, essentially as described before.³¹

Table 1 Patient demographic and clinical information

	SpA	Seronegative UIA	Seropositive RA
n	14	10	6
Disease duration months, mean (range)	5.13 (0.5–12)	3.75 (2–7)	5.33 (1–11)
Age, mean (range)	30 (23–52)	48 (21–79)	52 (25–84)
Female, n (%)	4 (29)	6 (60)	5 (83)
No treatment, n (%)	4 (29)	2 (20)	5 (83)
NSAID*, n (%)	8 (57)	1 (10)	1 (17)
DMARD†, n (%)	1 (7)	6 (60)	0
Biologic‡, n (%)	1 (7)	0	0
Steroid§, n (%)	1 (7)	5 (50)	1 (17)
ACPA+, n (%)¶	0	0	3/4 (75%)
Anti-CarP+, n (%)¶	0	1/5 (20%)	2/4 (50%)

Demographic and clinical information for patients included in this study.

*Naproxen, etoricoxib and ibuprofen.

†Methotrexate and sulfasalazine.

‡Guselkumab.

§Prednisolone.

¶Serum available for ACPA/anti-CarP testing SpA n=7, seronegative UIA n=5, seropositive RA n=4.

ACPA+, anti-citrullinated peptide antibody positive; Anti-CarP, anti-carbamylated peptide antibody positive; DMARD, disease-modifying antirheumatic drug; NSAID, non-steroidal anti-inflammatory drug; SpA, spondyloarthritis; UIA, undifferentiated inflammatory arthritis.

In short, streptavidin coated plates were incubated with biotinylated cyclic citrullinated peptide 4 or the arginine control peptide. In between each sequential step, plates were washed five times with PBS/0.05% Tween. Serum samples, diluted 1/50 in PBS/1% BSA/0.05% Tween, were added to the plate and incubated for 1 hour at 37°C. Next, ACPA IgG were detected as described above. ACPA IgG binding was quantified relative to a standard curve and expressed in arbitrary units per mL. A cut-off based on healthy controls was determined as described

above. Patient samples were considered positive if the ACPA level was higher than the cut-off, as well as >2 times higher than the control peptide.

Anti-CarP IgG antibody levels were detected using an in-house ELISA as described previously.³² In brief, a Nunc Maxisorp plate (ThermoFisher) was coated with carbamylated FCS or non-modified FCS. In between each sequential step, plates were washed three times using PBS/0.05% Tween (Sigma). Plates were blocked for 6 hours at 4°C and were then incubated with 1/50 diluted serum overnight at 4°C. After incubation, IgG levels were detected using Rabbit-anti-Human IgG-HRP (Dako). Plates were developed using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)/0.015% H₂O₂ (both from Merck) and absorbance was measured at 415 nm. Antibody binding was quantified relative to a standard line and expressed in arbitrary units per mL. Next, antibody binding to the control proteins was subtracted from the antibody binding to carbamylated protein. The cut-off for positivity was set as the mean arbitrary units plus two times the SD of 100 healthy controls.

Statistical analysis

Graphs were constructed with GraphPad Prism V.9.5.1. Statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. P values were considered significant if p<0.05.

RESULTS

We recruited 45 patients with early inflammatory arthritis with symptoms of no more than 12 months, whose clinical management required SF aspiration from a knee joint.

Table 2 Antibodies used for sample staining

Epitope	Fluorochrome	Clone	Company
CD3	PE-Cy7	UCHT1	Biolegend
CD3	BUV737	UCHT1	Biolegend
CD4*	PerCP-Cy5.5	SK3	Biolegend
CD4*	BV785	RPA.T4	Biolegend
CD8	Pacific Blue	RPA-T8	Biolegend
CD8	BUV395	RPA-T8	Biolegend
CD14	APC-Cy7	REA559	Miltenyi
CD19	APC-Cy7	HIB19	Biolegend
IFN γ *	FITC	B27	Biolegend
IFN γ *	PerCP-Cy5.5	4S.B3	Biolegend
IL-17A*	PE	BL168	Biolegend
TNF α *	APC	Mab11	Biolegend
TNF α *	FITC	Mab11	Biolegend

Antibodies used for sample staining.

*Indicates these antibodies were added intracellularly.

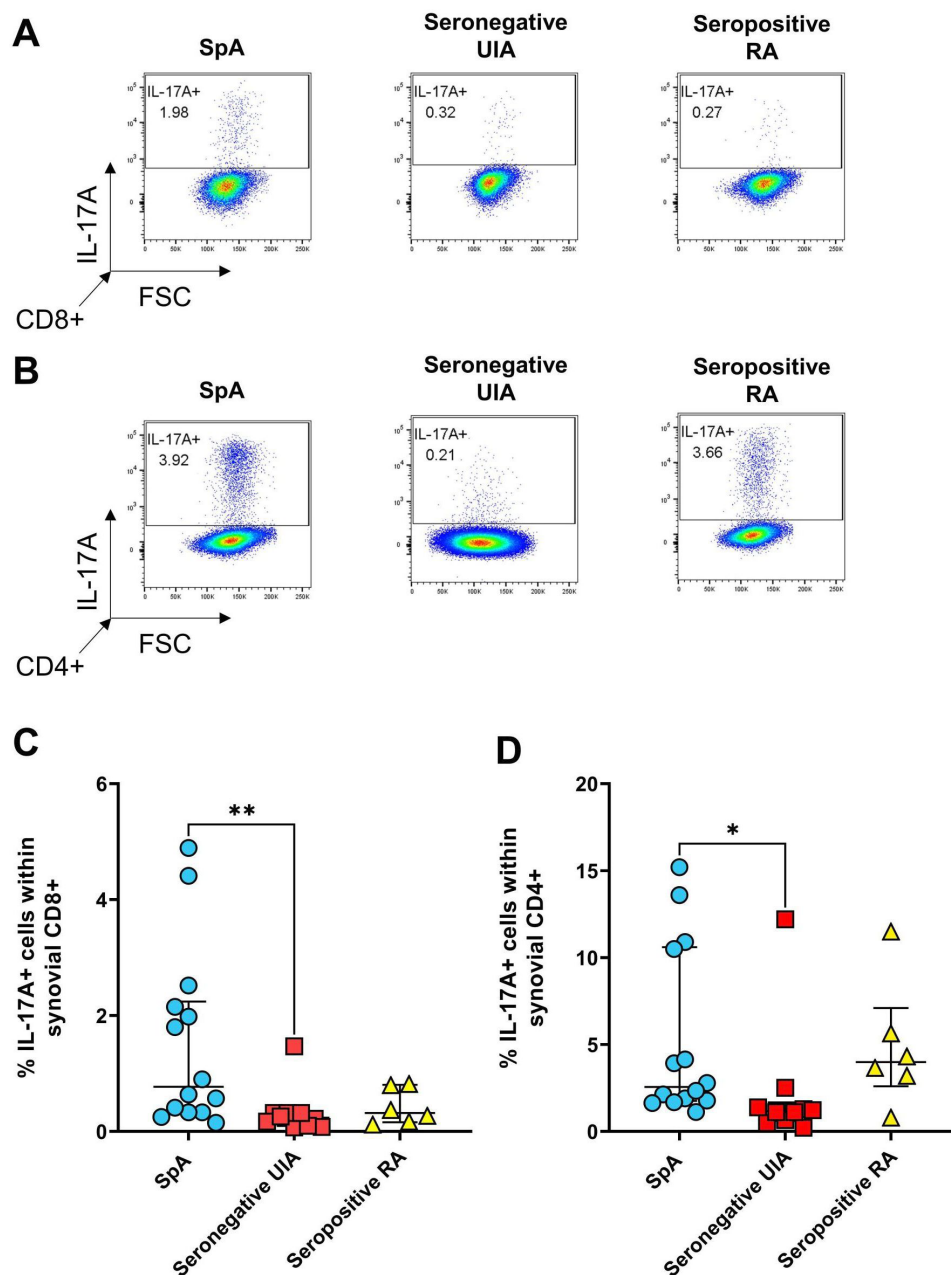


Figure 1 Presence of IL-17A+ CD8+ or CD4+ T-cells in the synovial fluid of patients with EIA. SFMC was stimulated for 3 hours with PMA and ionomycin in the presence of GolgiStop followed by staining for the presence of IL-17A producing T cells. Representative dot plots (A, B) and cumulative data (C, D) showing the presence of IL-17A+ cells within CD8+ (A, C) and CD4+ (B, D) T cells from the synovial fluid of patients with EIA who were subsequently diagnosed as having SpA (n=14, blue circles), seronegative UIA (n=10, red squares) or seropositive RA (n=6, yellow triangles). Data analysed using the Kruskal-Wallis test. EIA, early inflammatory arthritis; PMA, phorbol myristate acetate; RA, rheumatoid arthritis; SFMC, synovial fluid mononuclear cell; SpA, spondyloarthritis; UIA, undifferentiated inflammatory arthritis. * $p < 0.05$; ** $p < 0.01$.

Five patients declined to have SF samples tested, nine samples did not yield sufficient SFMC to analyse and one patient remained with an inconclusive diagnosis. Of the 30 patients analysed, 14 had a final diagnosis of SpA (7 PsA, 2 enteropathic SpA, 5 reactive arthritis), 10 of seronegative undifferentiated inflammatory arthritis (UIA) and 6 of seropositive RA. Serum for detailed autoantibody profiling was available for 16 out of 30 patients. Patient demographic, serology and clinical parameters at the time of SF collection in the 30 tested samples are

shown in [table 1](#). The final diagnosis was made at least 12 months (range 12–48 months) after symptom onset independently by two clinicians blinded to the research laboratory data. Final clinical diagnoses were linked to the laboratory data after completion of the flow cytometry acquisition and analysis.

Recent findings suggest some seronegative undifferentiated inflammatory arthritis patients are positive for antibodies such as anti-carbamylated proteins (anti-CarP), which are not yet routinely tested in the clinic.³³

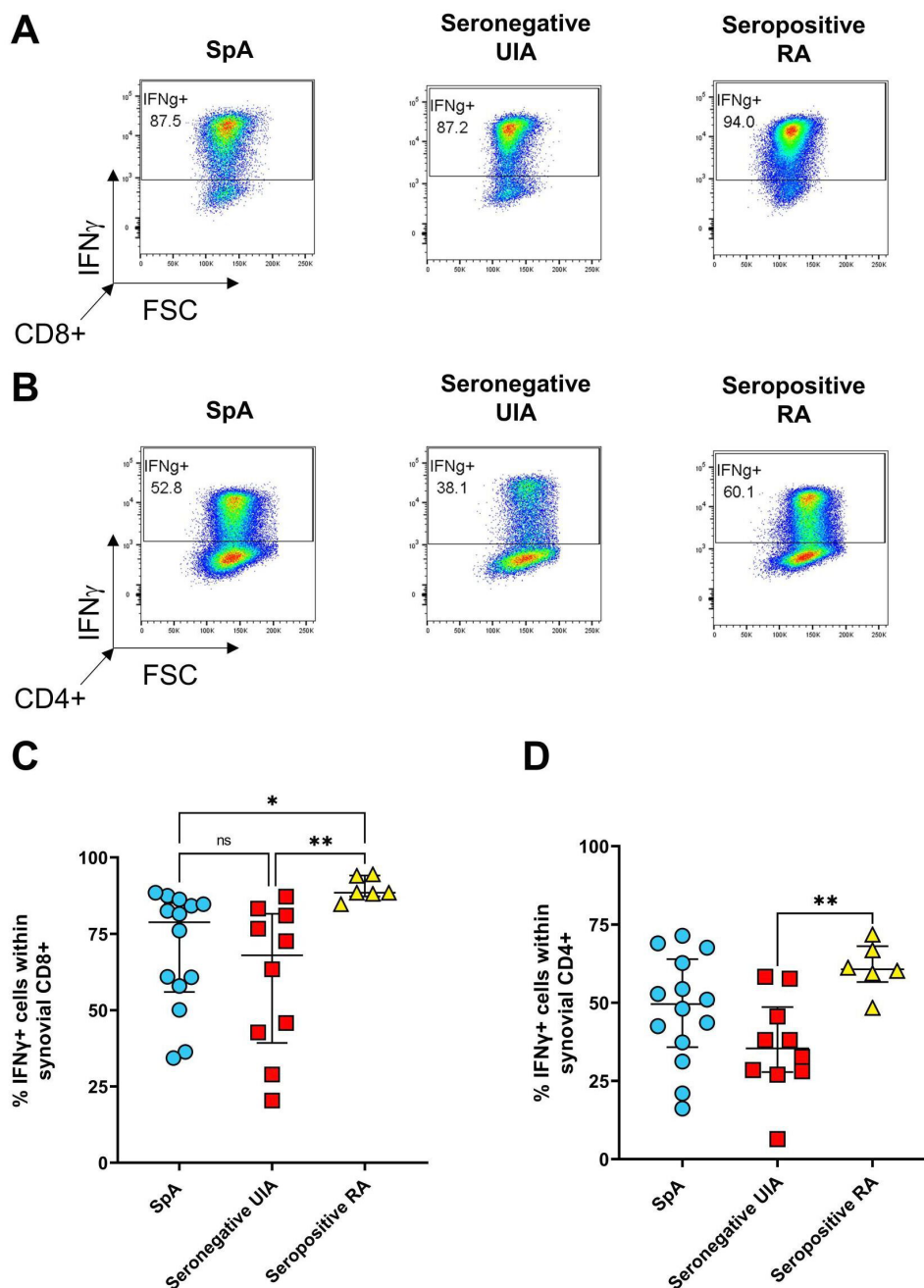


Figure 2 Presence of IFN γ + CD8+ or CD4+ T-cells in the synovial fluid of patients with EIA. SFMC was stimulated for 3 hours with PMA and ionomycin in the presence of GolgiStop followed by staining for the presence of IFN γ producing T cells. Representative dot plots (A, B) and cumulative data (C, D) showing the presence of IFN γ + cells within CD8+ (A, C) and CD4+ (B, D) T cells from the synovial fluid of patients with EIA who were subsequently diagnosed as having SpA (n=14, blue circles), seronegative UIA (n=10, red squares) or seropositive RA (n=6, yellow triangles). Data analysed using the Kruskal-Wallis test. EIA, early inflammatory arthritis; PMA, phorbol myristate acetate; RA, rheumatoid arthritis; SFMC, synovial fluid mononuclear cell; SpA, spondyloarthritis. * p < 0.05; ** p < 0.01.

In addition to our routine laboratory ACPA testing, we assessed serum levels of ACPA and anti-CarP antibodies in 16 of the 30 patients, using in-house assays. Of the tested samples, the sera from patients later diagnosed with SpA were all ACPA and anti-CarP negative (n=7) and those from patients later diagnosed with seronegative UIA (n=5) were all negative for ACPA with one patient testing positive for anti-CarP only. Of the patients later diagnosed with seropositive RA, three and two out of four

serum samples tested positive for ACPA and anti-CarP, respectively.

To investigate the presence of specific cytokine-expressing CD8+ or CD4+ T lymphocytes, SFMC were thawed, rested and stimulated for 3 hours with PMA and ionomycin in the presence of GolgiStop before staining for the appropriate markers.

Overall, the percentage of IL-17A+CD8+ T cells was significantly enriched (p=0.0054) in the SpA group

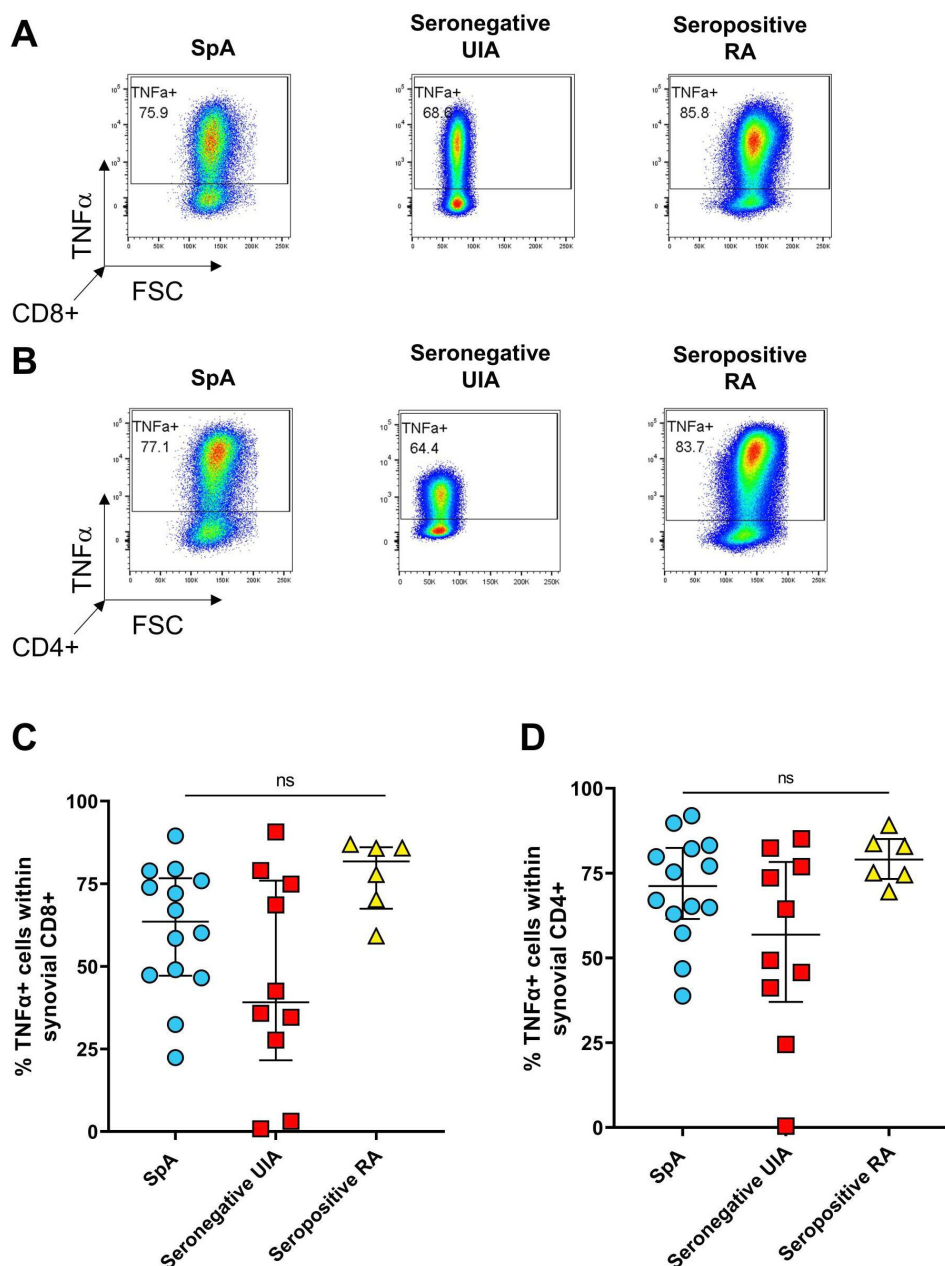


Figure 3 Presence of TNF α + CD8+ or CD4+ T-cells in the synovial fluid of patients with EIA. SFMC was stimulated for 3 hours with PMA and ionomycin in the presence of GolgiStop followed by staining for the presence of TNF α producing T cells. Representative dot plots (A, B) and cumulative data (C, D) showing the presence of TNF α + cells within CD8+ (A, C) and CD4+ (B, D) T cells from the synovial fluid of patients with EIA who were subsequently diagnosed as having SpA (n=14, blue circles), seronegative UIA (n=10, red squares) or seropositive RA (n=6, yellow triangles). Data are analysed using the Kruskal-Wallis test. EIA, early inflammatory arthritis; PMA, phorbol myristate acetate; RA, rheumatoid arthritis; SFMC, synovial fluid mononuclear cell; SpA, spondyloarthritis; UIA, undifferentiated inflammatory arthritis.

compared with the group later diagnosed with seronegative UIA (figure 1A and C). A similar pattern was observed for synovial IL-17A+CD4+ T cells ($p=0.0253$) (figure 1B and D). The small group of patients later diagnosed with seropositive RA (n=6) all had low levels of IL-17A+CD8+ T cells, while the percentage of IL-17A+CD4+ T cells was similar to that in SpA.

Online supplemental figure 2 shows the individual datapoints of the synovial Tc17 or Th17 cell

frequencies in the three patients subgroups in relation to age, medication, gender or SpA subgroup. Given the heterogeneity and relatively small sample size in each group, statistical analysis was not possible, but no immediate trends were discernible. We also analysed whether there was any relation between %Tc17 cells and time since first symptom onset or C reactive protein level, but no statistical significance was found (online supplemental figure 3). A negative

correlation was found for %Th17 cells and time since symptom onset. Finally, we analysed whether there was a correlation between Tc17 and Th17 cells in the EIA SF samples and found a significant correlation when we compared all samples ($n=30$), but not when we only included the SpA subset ($n=14$) which may be due to the lower n -number (online supplemental figure 4).

EIA patients later diagnosed with seropositive RA showed a significantly higher percentage of synovial IFN γ +CD8+ T cells compared with both the SpA ($p=0.0138$) and the seronegative UIA ($p=0.0016$) groups, and in IFN γ +CD4+ T cells compared with seronegative UIA ($p=0.0089$) (figure 2).

The percentages of synovial TNF α +CD8+ or CD4+ T cells were not different between EIA patients later diagnosed with SpA, seronegative UIA or seropositive RA (figure 3).

DISCUSSION

At present, our understanding of the immunological mechanisms driving the early phases of SpA and seronegative UIA is incomplete. Recent evidence indicates that more patients are now presenting with seronegative UIA patterns, which could relate to reduced smoking and increased population adiposity.³⁴ A deeper understanding of the underlying immunopathology of diverse forms of inflammatory arthritis is therefore important. In this report, we focused on two key functional immune pathways, namely type 1, characterised by IFN γ expression and type 17 where IL-17A is the signature cytokine, and investigated if these pathways were present early in the disease process of different types of EIA.

Our data indicate that the frequency of SF IL-17A+CD8+ (Tc17) T cells in patients with early SpA, including both PsA and peripheral SpA, is significantly increased compared with the seronegative UIA group and numerically increased compared with the smaller seropositive RA group. We have previously demonstrated significantly elevated synovial Tc17 frequencies in established SpA compared with RA^{5 29 30} but similar enrichment of Th17 cell frequencies,^{29 30} suggesting the patterns we show here in EIA persist over time, despite most patients in the established arthritis group receiving DMARD and biological therapy.

In contrast, we show a significantly higher percentage of type 1 CD8+IFN γ + SF T cells from patients with early seropositive RA compared with both the seronegative UIA and SpA groups, and in CD4+IFN γ + SF T cells in early RA versus seronegative UIA. Our findings of elevated CD8+ and CD4+ IFN γ + SF cells in EIA patients eventually diagnosed with RA support previous reports of an increased type 1/IFN γ signature in seropositive RA.^{35–37} Furthermore, two recent studies reported the presence of clonally expanded cytotoxic CD8+ and CD4+ T cells in the blood and joints of ACPA+RA patients.^{38 39} The cytotoxic CD8+T cells were activated by citrullinated

antigens in an HLA class I-dependent manner leading to expression of pro-inflammatory and cytolytic mediators (IFN γ and/or GZMB)³⁸ while the cytotoxic CD4+T cells were characterised by a GZMB+PRF1+, Hobit+, NKG7^{high} and GPR56+profile.³⁹ Our study focused on type 1 and type 17 cytokine-expressing T cells and did not investigate cytotoxic cell subsets.

Our study is one of few to analyse synovial immunopathology in a broad group of patients with early disease. Previous research in EIA often compared outcomes and pathology in patients with seropositive or seronegative RA, excluding PsA or peripheral SpA.^{40 41}

Importantly, our data show significant differences in early disease between the well-studied group of seropositive RA, in contrast to the less well studied PsA/SpA and the seronegative UIA groups. The latter group has been difficult to study as there are no clear classification criteria but is important as these patients are increasingly common in EIA services.³⁴ We show that the seronegative UIA group is characterised by lower frequencies of IL-17A and IFN γ -expressing CD8+ and CD4+ T cells. Levels of TNF α -expressing cells are not statistically different across all groups but again in the seronegative UIA group appeared at the lower range of the other two groups.

Do these differences in key immune pathways that we and others have shown in early and established IA, have clinical implications? The clearest clinical relationship is the well-demonstrated response of SpA to IL-17A inhibition therapy in contrast to a much less profound improvement in other types of inflammatory arthritis.^{42 43} Our finding that the Tc17 pathway is active in very early SpA may in part help explain these differences in response to IL-17 inhibition.⁴⁴ Conversely, most arthritis types respond to inhibition of TNF,⁴⁵ which was expressed at similar levels in all the arthritis types tested here.

A limitation of our study is that it contained relatively small numbers, particularly in the seropositive RA group, which makes some comparisons difficult. Furthermore, we did not obtain information on the HLA-B27 status of our patients, which could have helped in the diagnosis of SpA patients. Finally, in SpA it is also recognised that many innate lymphoid cells, such as NK, gamma delta, ILC and MAIT cells express features of the type 17 response, whereas we focused on conventional adaptive T cells. This might underestimate the breadth of the type 17 response in SpA.

CONCLUSIONS

In conclusion, our data show that key type 1 and Tc17 immune activation pathways, that were previously demonstrated to be present in established seropositive RA and peripheral SpA/PsA respectively, are present in these types of inflammatory arthritis at an early stage of the disease process. These findings add to the growing evidence that there are distinct differences in the

immunopathology underlying SpA, seronegative UIA and seropositive RA.

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Contributors CDH, SER and KJAS processed the patient samples, performed and analysed the flow cytometry and interpreted the data. CDH and SER wrote sections of the manuscript. MDvdB and KAJvS performed the autoantibody assays under the supervision of LAT and REMT. BM and BWK recruited patients and performed the clinical diagnoses. BWK and LST designed and supervised the study, interpreted the data and wrote the manuscript. LST acts as guarantor of the study. All authors read, edited and approved the final manuscript.

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Competing interests BWK has received consultancy and speaker fees from Abbvie, Eli Lilly, Pfizer, Novartis and UCB. LST previously received research support from Novartis, UCB, Sanofi and GSK outside the work described in this manuscript.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by Bromley REC reference 06/Q0705/20 and Harrow REC reference 17/L0/1940. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information.

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