Combined manual and automated immunophenotypisation identified disease-specific peripheral blood immune subpopulations in rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis

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Abstract

Objective

Rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA) are associated with abnormal immune cell functions. We combined manual and automated profiling in subpopulations of T-cells, B-cells and monocytes, in parallel to functional testing and clinical correlation.

Methods

Using flow cytometry, we analysed the expression of CCR4, CCR6 and CXCR5 on helper and cyotoxic T-cells, CD32B and CD86 on naïve and memory B-cells, and CCR1, CCR2, CCR4 and CXCR4 on monocytes in chronic high-disease activity patients to identify peripheral blood subpopulations. Cell activation, proliferative capability and osteoclastogenic effects were tested in vitro. Comparison with synovial compartment, clinical data and anti-TNF treatment were added to peripheral blood analysis.

Results

PsA had lower double-negative T-cell frequency, while RA had lower double-positive T-cell frequency and expanded Th1-like and cytotoxic T-cell subsets. CD32B expression was increased on naïve and memory B-cells in AS and associated with disease activity. $CCR6^+$ and $CXCR5^+$ cytotoxic T-cells and $CD32B^+$ naïve and memory B-cells were highly enriched within the synovial compartment. T-cells and B-cells from AS exhibited enhanced activation and proliferation in vitro, whereas T-cell conditioned medium from RA produced an increased osteoclastogenic effect. CCR1 and CXCR4 were upregulated on osteoclastogenic monocyte subsets of RA, AS and PsA patients. Bioinformatic Citrus analysis identified additional T-cell, B-cell and monocyte clusters specifically associated with each disease.

Conclusion

By combining manual and automated data analysis, our study revealed several disease-specific immune cell subpopulations, particularly cytotoxic T-cell subsets in RA and memory B-cell subsets in AS, which may serve as an indicator of active disease or possible therapeutic target.

Key words

lymphocyte, monocyte, rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis

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Introduction

In rheumatic diseases, aberrancies in inflammatory and immune cell populations, precipitated by genetic and environmental factors, promote autoimmunity and persistent inflammation, resulting in both systemic and local pathology (1-3). Chronic systemic immune inflammation represents a central hallmark of rheumatic diseases and accounts for substantially increased cardiovascular risk and associated comorbidities in these patients. On the local level, inflammation and associated cytokine milieu alters bone cell activity, resulting in bone and joint pathology (4, 5).

While there has been substantial improvement in clinical outcomes of inflammatory joint diseases since the introduction of novel therapeutical approaches, the crucial component(s) responsible for the pathogenesis of these diseases have yet to be elucidated and, consequently, current treatment remains more or less non-specific. Moreover, some patients exhibit poorly controlled disease, having high disease activity and progression despite prolonged treatment (6). Rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS) are the most common inflammatory rheumatic diseases (7). They differ substantially in immune mechanisms as well as in the patterns of bone and cartilage damage (8). RA commonly manifests as a symmetric and erosive arthritis typically affecting small and medium-sized joints, with erosion resulting from a continuous inflammatory attack of the synovial membrane on bone (1). In RA, T-cells, B-cells, and the concerted interaction of pro-inflammatory cytokines play key roles in disease pathophysiology. T-cells detected in established disease have features of both Th1 and Th17, with most T-cells specific for citrullinated tetramers being of Th1 phenotype. RA mostly presents as a seropositive arthritis due to autoantibody production, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (aCPA), which are closely related to the chronicity of the disease (9, 10). PsA is a more heterogeneous inflammatory disease, varying from mild monoarthritis to severe polyarthritis, and may also involve the axial skeleton and the entheses (2). Chronic inflammation mediated by activated T-cells and macrophages is thought to play important roles in the induction of articular inflammation and destruction (11). AS is a systemic inflammatory disorder that primarily affects the sacroiliac joints and the spine, but can also affect peripheral joints and entheses (3). Although the disease-associated HLA-B27 would be predicted to generate autoreactive CD8 T-cells, T-cell targeting therapies have shown unpromising results. Moreover, effectiveness of rituximab suggests the role of B-cells and immune complexes (12). Despite significant clinical improvements being achieved in the outcome of inflammatory rheumatic diseases, systemic disturbances in immune cell populations seem to persist in chronically active forms, with these cells permanently homing to affected joints and triggering pathology (8). The primary aim of our study was to associate specific aberrancies of circulatory lymphocyte and monocyte subsets with a distinct form of rheumatic disease, by evaluating manual and bioinformatic phenotypisation extended by functional characterisation and correlations with clinical parameters. Since peripheral blood samples are readily obtainable as a part of routine assessment, it would be beneficial to elucidate one or more key disease specific immune cell populations, which may indicate highly active disease and serve as possible therapeutic target. As secondary objectives, we set out to evaluate, firstly, whether these specific aberrancies carry over into the synovial compartment and, secondly, if their frequency changes in response to anti-TNF therapy.

Materials and methods

Patients

Arthritic patients (RA, n=30; AS, n=22; and PsA, n=22), admitted to the Department of Rheumatology, Physical Medicine and Rehabilitation at Clinical Hospital Center "Sestre Milosrdnice" or to the Department for Clinical Immunology, Rheumatology and Pulmology at Clinical Hospital "Sveti Duh", were included in the study after obtaining approval from the Ethics Commit-

Table I. Demographic and clinical characteristics of enrolled patients with rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis.

	Ankylosing spondylitis	Rheumatoid arthritis	Psoriatic arthritis	Controls
Age (years)	60 [52-66]	66 [52-73]	55 [51-64]	55 [43-68]
Gender (male/female)	9/13	3/27	9/13	6/18
Disease duration (years)	22 [14-29]	15 [7-21]	15 [10-21]	
BASDAI	6.07 [4.90-7.21]	-	7.31 [5.71-8.30]	
ASDAS	2.91 [2.41-3.46]	-	3.51 [2.94-4.12]	
DAS28	4.73 [3.1-6.1]	6.2 [5.1-6.5]	5.6 [4.6-6.3]	
DAPSA	-	-	46.5 [36.5-54.6]	
CDAI	30.6 [20.0-66.6]	40.9 [24.8-44.2]	35.4 [17.5-45.1]	
ESR (mm/h)	10 [4.0-16.0]	24.0 [18.0-31.0]	15.0 [8.5-29.0]	
CRP (mg/L)	3.7 [0.65-15.6]	9.4 [3.1-19.6]	7.5 [2.5-12.7]	
RF (IU/L)	-	38.6 [10.7-75.9]	-	
aCPA (EU/L)	-	3.7 [1.1-284.5]	-	
Tender joint count (28; 66/68)	26 [10-45]	17 [12-22]	15 [6-26]	
Swollen joint count (28 score)	1 [0-2]	7 [1-15]	5 [1-12]	
Disease activity (physician VAS)	7.3 [5.9-8.6]	6.7 [4.8-8.7]	6.5 [6.0-7.0]	
Disease activity (patient VAS)	7.0 [6.0-8.5]	7.2 [5.7-8.9]	6.7 [6.2-7.7]	
Disease activity (high/moderate)	18/4	22/8	12/10	

*Values are presented as median with interquartile range. ASDAS – Ankylosing Spondylitis Disease Activity Score; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein, CDAI: clinical disease activity index; DAS28: disease activity score including a 28-joint count (calculated with ESR); DAPSA: Disease Activity index for PSoriatic Arthritis; VAS: visual analogue scale (cm); RF: rheumatoid factor (determined by turbidimetry), aCPA: anti-citrullinated protein antibodies (determined by ELISA), Tender joint count - 28 for RA and PsA, 66/68 for AS. Therapy consisted of non-steroidal anti-inflammatory drugs (NSAID) for AS (mostly ibuprofen 600 mg twice a day), a combination of NSAID and disease-modifying anti-rheumatic drugs (DMARD) for PsA (mostly ibuprofen 600 mg twice a day and methotrexate 15 mg once a week), and a combination of NSAID, DMARD and/or glucocorticoid for RA (mostly ibuprofen 600 mg twice a day and methotrexate 15 mg once a week or methylprednisolone 4 mg once a day). Moderate disease activity was defined as 3.2<DAS28≤5.1 in RA, 2.1<ASDAS≤3.5 in AS, and 14<DAPSA≤28 in PsA. High disease activity was defined as DAS28>5.1 in RA, ASDAS>3.5 in AS, and DAPSA>28 in PsA. There were no remitting or low disease activity patients included in the study.

tee and informed consent from patients (Table I). A rheumatology specialist established the diagnosis of RA based on the American College of Rheumatology (ACR) 2010 criteria, AS based on 1984 Modified New York classification criteria, while PsA according to the Moll and Wright criteria (13-15). Patients were enrolled based on: age of 40 or more years; disease duration of at least 5 years after diagnosis was established by a specialist; being on therapy consisting of non-steroidal anti-inflammatory drugs (NSAID), disease-modifying anti-rheumatic drugs (DMARD) and/or glucocorticoids (with no history of biological therapy); and having Disease activity score including a 28-joint count (DAS28)>3.2 for RA patients, Ankylosing spondylitis disease activity score (ASDAS)>2.1 for AS patients, or Disease activity index for psoriatic arthritis (DAPSA)>14 for PsA patients (16-18). In patients with significant accumulation of synovial fluid in the knee (n=8 RA, n=3 PsA), the effusion was aspirated and analysed for immune cell subpopulations as later described. Control subjects (CTRL, n=24), aged 40 years or above, were admitted to same institutions due to non-inflammatory aetiology, with no previous history of arthritic or autoimmune diseases. An additional, smaller, group of patients (RA n=3, AS=5, PsA=8) of similar age and disease duration scheduled for anti-TNF treatment (etanercept 50 mg weekly or adalimumab 40 mg every two weeks) was followed up by analysing their samples before, 1 month and 3 months after introduction of anti-TNF therapy (19, 20).

Table II. Phenotype of analysed circulating immune cell subpopulations.

Subpopulation	Phenotype
Helper T-cells	
Th1-like	$CD3^+CD4^+CCR4^-CCR6^-$
Th2	$CD3^+CD4^+CCR4^+CCR6^-$
Th9	$CD3^+CD4^+CCR4^-CCR6^+$
Th17	$CD3^+CD4^+CCR4^+CCR6^+$
Cytotoxic T-cells	
immature effector memory	$CD3^+CD8^+CCR4^+$
early effector memory	$CD3^+CD8^+CCR6^+$
follicular	$CD4^+CD8^+CXCR5^+$
B-cells	
naïve	$CD19^+IgD^+CD27^-$
unswitched memory	$CD19^+IgD^+CD27^+$
class-switched memory	$CD19^+IgD^-CD27^+$
double-negative memory	$CD19^+IgD^-CD27^-$
plasmablasts	$CD19^+IgD^-CD27^+CD38^+$
Monocytes	
mature (classical) monocytes	$CD3^-CD19^-CD56^-CD11b^+CD14^+$
osteoclastogenic monocytes	$CD3^-CD19^-CD56^-CD11b^+CD14^+CD115^+$

Isolation of mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation (20 min at 600 g) with Histopaque (Sigma-Aldrich, Saint Louis, MO, USA) and subjected for flow cytometry phenotyping, lymphocytic

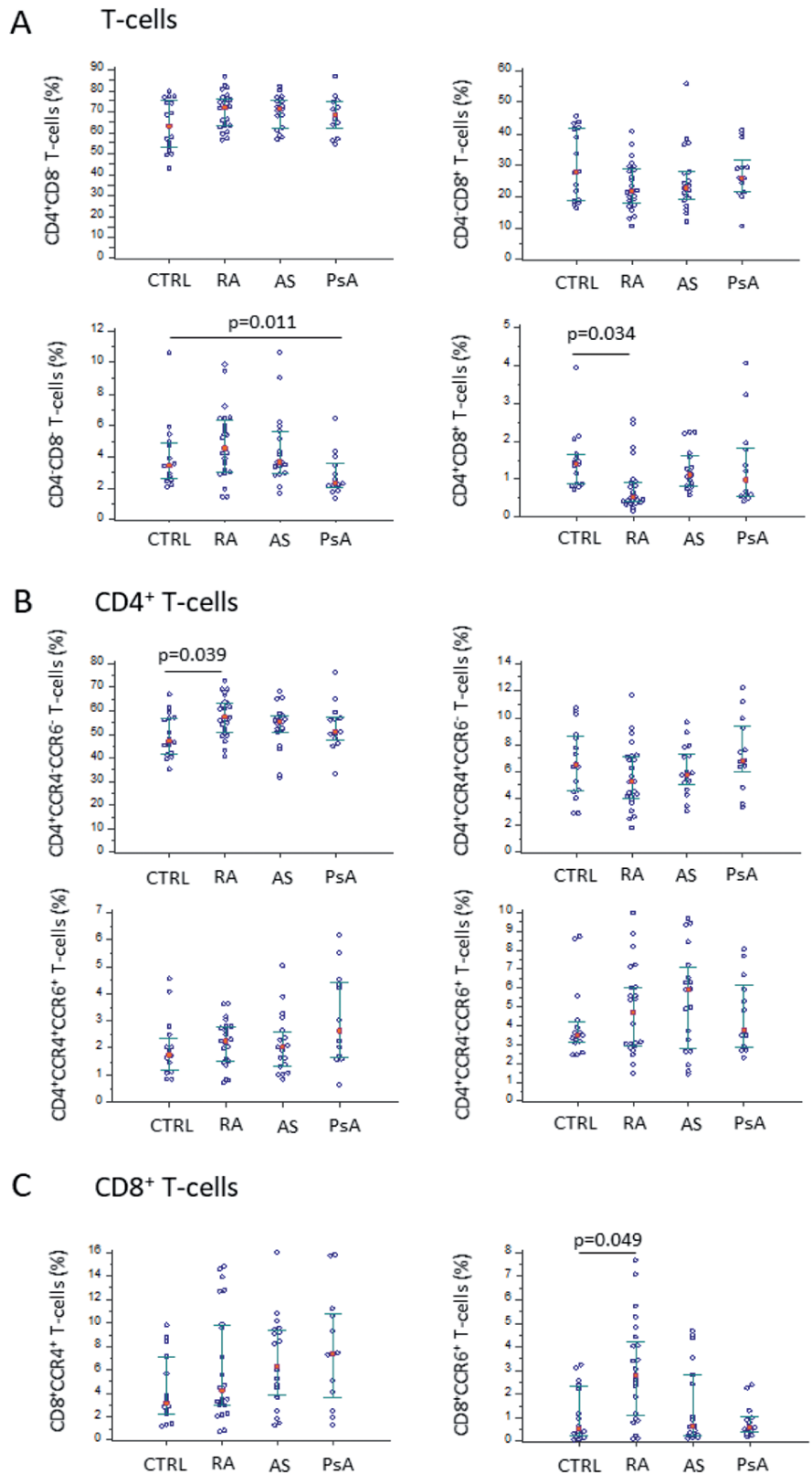

Fig. 1. T-cell subpopulations in peripheral blood of arthritic patients.
A: Frequencies of helper ($CD4^+CD8^-$), cytotoxic ($CD4^-CD8^+$), double-positive ($CD4^+CD8^+$) and double negative ($CD4^-CD8^-$) T-cells of control (CTRL) subjects and rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA).
B: Frequencies of Th1-like ($CCR4^-CCR6^-$), Th2 ($CCR4^+CCR6^-$), Th9 ($CCR4^-CCR6^+$), Th17 ($CCR4^+CCR6^+$) and Tfh ($CXCR5^+$) cells.
C: Frequencies of immature effector memory ($CCR4^+$), early effector memory ($CCR6^+$) and follicular ($CXCR5^+$) cytotoxic T-cell subpopulations.
Values are presented as medians (square), with boundaries (horizontal lines) representing interquartile range (IQR), and circles representing individual values. Disease-to-control comparisons were performed using non-parametric Mann-Whitney test, p-values <0.05 are shown.

proliferation assay and osteoclastogenic cocultures. Synovial fluid-derived cells were isolated by sequential centrifugation (5 min at 100 g) of aspirated effusion diluted in increasing amounts of phosphate-buffered saline and phenoytped using flow cytometry. Cell viability, checked by Trypan Blue exclusion, was determined to always be greater than 95%.

Flow cytometry

Phenotype characterisation was performed on the Attune flow cytometer (Life Technologies, ABI, Carlsbad, CA, USA) and analysed by FlowJo software

(TreeStar, Ashland, OR, USA FlowJo LLC, Ashland, OR USA). Cells were labelled using commercially available monoclonal antibodies (eBiosciences; San Diego, CA, USA). Flow cytometry gating was performed according to suggested strategies (8, 21-23) for T-cell helper (22, 23), T-cell cytotoxic, B-cell (21) (with additional analysis of CD32B and CD86 expression (24, 25)) and osteoclast progenitor-enriched monocyte (with additional analysis of CCR1, CCR2, CCR4 and CXCR4) subpopulations (26) (phenotype noted in Table II, gating strategy in Supplementary Figure 1, antibody-producing clones in Suppl. Table S1).

Lymphocyte proliferation and activation assay

PBMCs of CTRL, RA, AS and PsA samples were first labelled with Cell Proliferation Dye eFluor 670 (eBiosciences), according to the manufacturer instruction, then with anti-CD3 for T-cells and anti-CD19 for B-cells, and sorted using BD FACSAria II (BD Biosciences, Franklin Lakes, NJ, USA). The mitogenic pulse was carried out by adding 50 ng/mL Phorbol 12-myristate 13-acetate (PMA) and 0.5 ng/mL calcium ionophore A23187 (both from Sigma-Aldrich) and incubating for 3 hours at 37 °C. The cells were washed and plated into 96-well plates at a density of 250,000 T-cells per well and 70,000 B-cells per well in RPMI with 10% fetal bovine serum (FBS) and cultured for 72h. Corresponding non-pulsed lymphocytes were used as non-stimulated controls. Upon harvesting, lymphocytes were labelled with anti-CD69 as a marker of activation (27) and analysed on Attune flow cytometer, while proliferation was evaluated as the percent of divided cells by assessing Proliferation Dye eFluor 670 fluorescence intensity and using the FlowJo Proliferation platform (setting the number of peaks to 2).

Osteoclastogenic culture treated by lymphocyte-conditioned medium

Osteoclast progenitor-enriched monocytes ($CD3^-CD19^-CD56^-CD11b^+CD14^+$) were sorted from peripheral blood and plated at a density of 4×10^4 cells per well in 96-well plate in α-MEM/10%

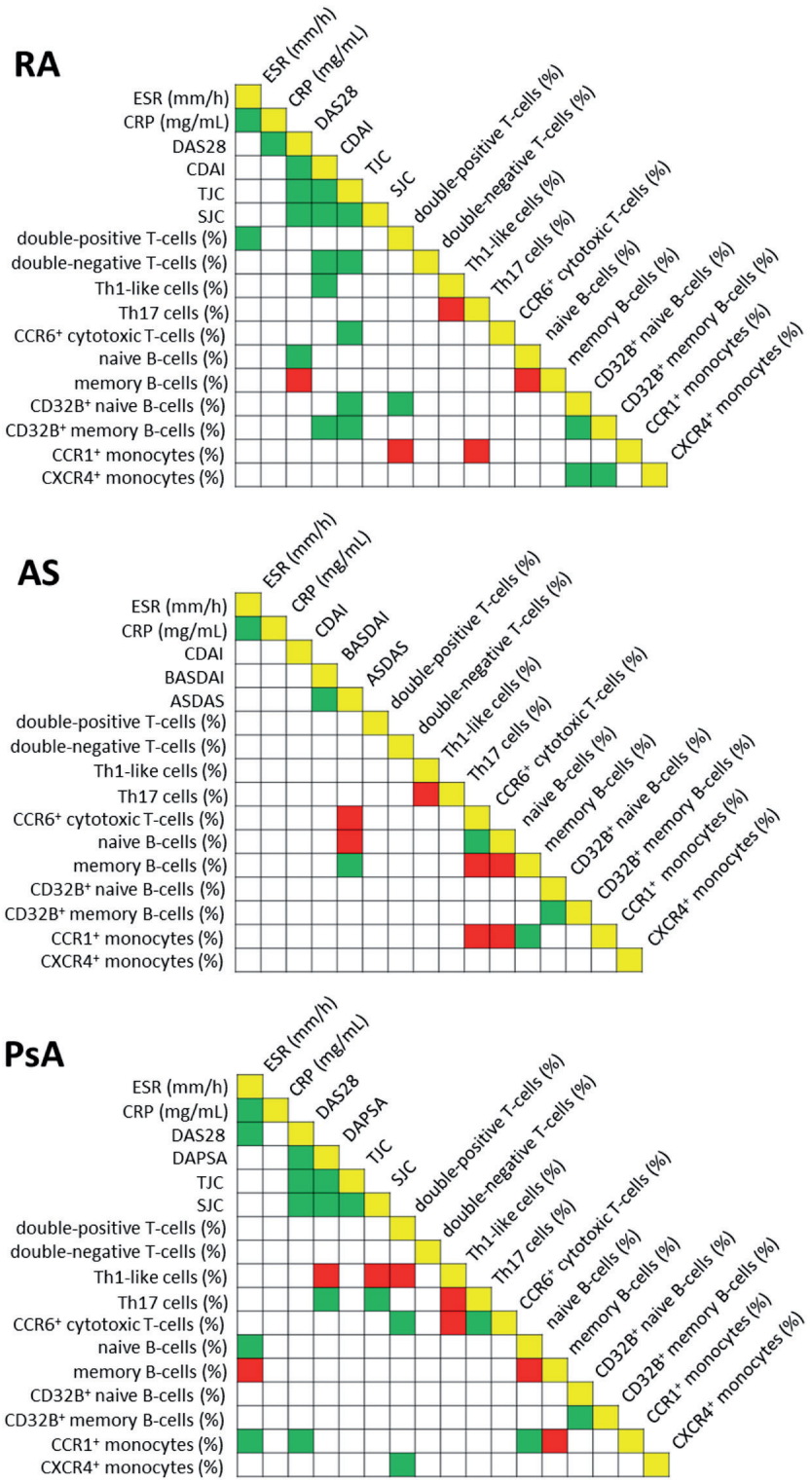

Fig. 2. Correlation of clinical parameters and significantly changed lymphocyte subpopulations in peripheral blood of rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis. Association was established using rank correlation. Correlations were calculated only for significantly changed cell populations, of which those with $p<0.05$ and Spearman´s coefficient $\varrho>0.5$ or $\varrho<-0.5$ are shown. Significant strongly positive ($\varrho>0.5$ and $p<0.05$) correlations are marked with green, significant strongly negative ($\varrho<-0.5$ and $p<0.05$) correlations are marked with red, variable self-correlation is marked yellow and unsignificant correlations are unmarked.

ASDAS: Ankylosing Spondylitis Disease Activity Score; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; ESR: erythrocyte sedimentation rate; CDAI: clinical disease activity index; DAS28: Disease Activity Score out of 28 joint count; SJC: swollen joint count (out of 28); TJC: tender joint count (out of 28); AS: ankylosing spondylitis; PsA: psoriatic arthritis; RA: rheumatoid arthritis.

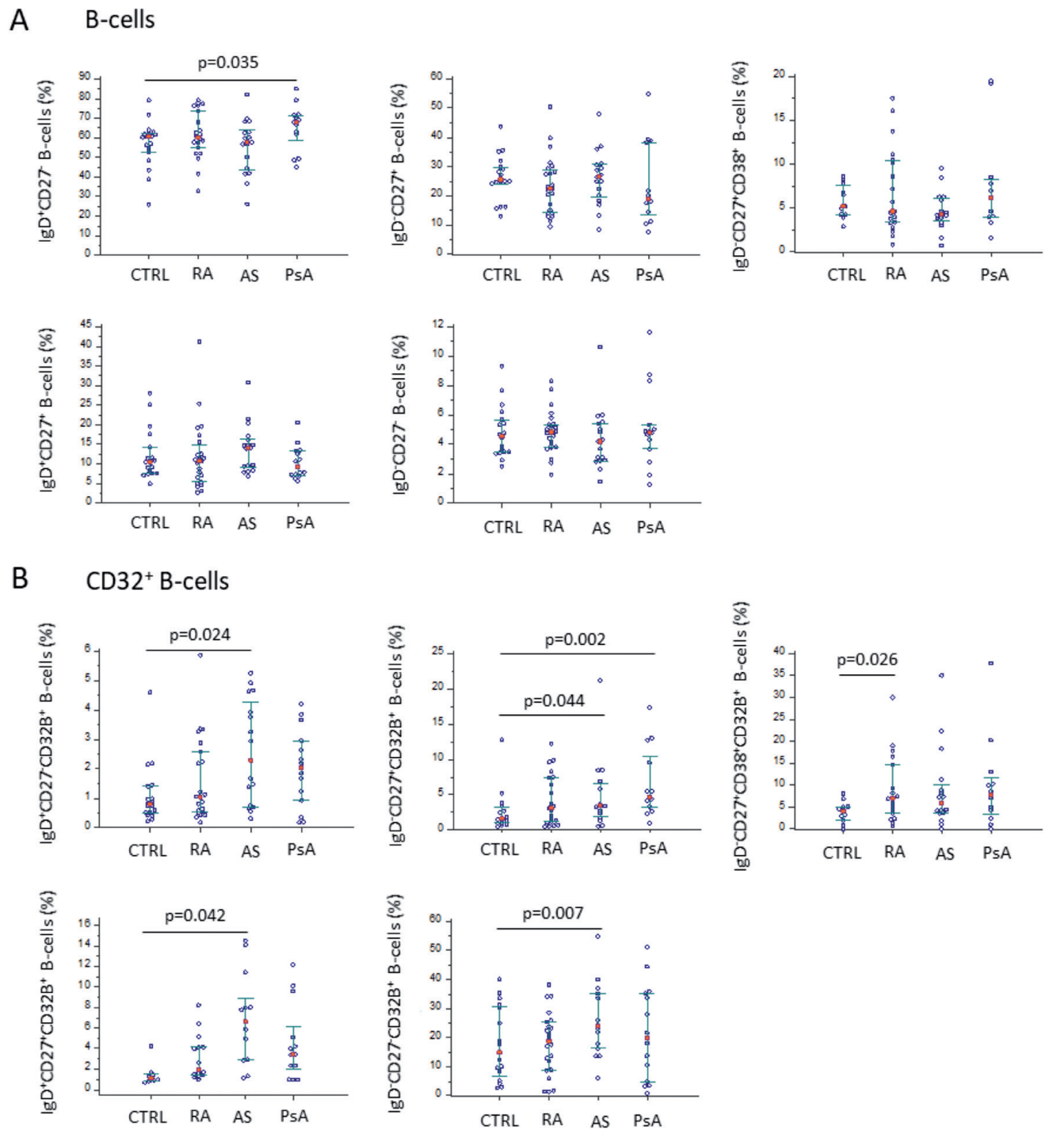

Fig. 3. B-cell subpopulations in peripheral blood of arthritic patients.
A: Frequencies of naïve (IgD^+CD27^-), unswitched memory (IgD^+CD27^+), class-switched memory (IgD^-CD27^+), double-negative memory (IgD^-CD27^-) and plasmablast ($IgD^-CD27^+CD38^{hi}$) B-cells of control (CTRL) subjects and rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA). **B**: Expression of inhibitory Fc-receptor CD32B on B-cell subpopulations. Values are presented as medians (square), with boundaries (horizontal lines) representing interquartile range (IQR), and circles representing individual values. Disease-to-control comparisons were performed using non-parametric Mann-Whitney test, *p*-values <0.05 are shown.

FBS, supplemented with osteoclastogenic growth factors, 30 ng/mL of recombinant human (rh) macrophage colony-stimulating factor (M-CSF) and 100 ng/mL rh receptor activator of nuclear factor-kappaB ligand (RANKL) (R&D Systems, NE Minneapolis, MN, USA) and additionally treated with lymphocyte-conditioned medium in the dilution 1:3. At day 11–13 of culture, cells were stained for tartrate-resistant acid phosphatase (TRAP) using a commercially available set of chemicals according to the manufacturer's instructions (Leukocyte acid phosphatase kit; Sigma-Aldrich Corp.). Using light microscopy on Axiovert 200 (Carl Zeiss, AG, Oberkochen, Germany), osteo-

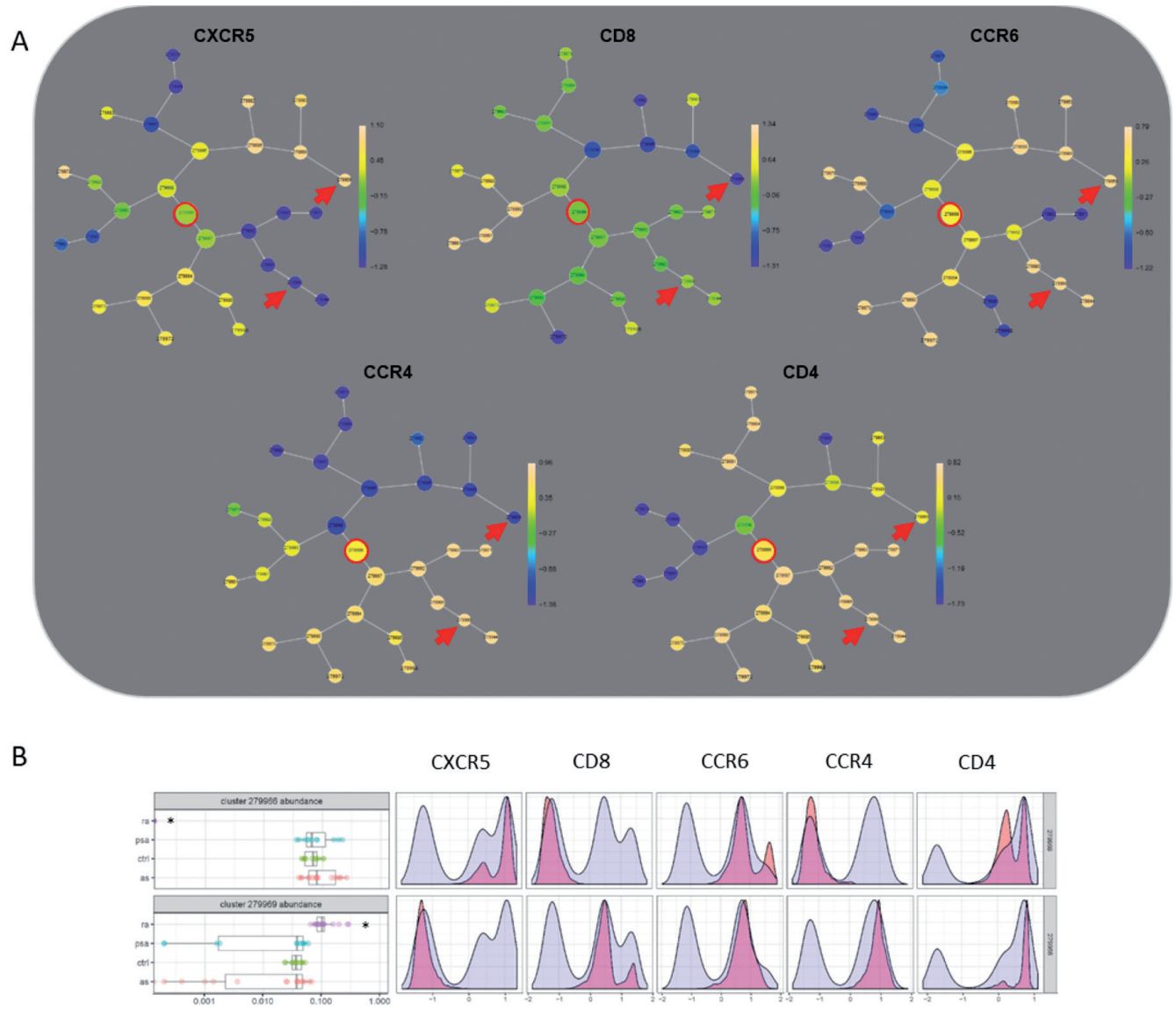

Fig. 4. Citrus automated flow cytometry analysis and disease associated subset selection for T-cell panel in arthritic patients.
A: Visual representation of unsupervised hierarchical clustering by Citrus. The analysis was done on CD3⁺ cells, while CD4, CD8, CCR4, CCR6 and CXCR5 were used as markers for clustering. The colour scale indicates median intensity of respective marker expression, while node size is relative to the frequency of cells in the cluster. Red circle indicates the source cluster at the start of automated hierarchical gating which includes all cells, while red arrows indicate significantly different clusters.
B: Histograms of marker expression and relative abundance of disease-associated cell clusters. Analysis of clusters was done on account of abundance in the cluster between groups using "significance analysis of microarrays" (sam) model. Histograms show the marker expression profile for the cluster (red) *vs.* background (purple) expression in all clusters. Results are mean fold change ± SEM. Disease-to-control comparisons were performed using non-parametric Mann-Whitney test, clusters for *p*-values <0.05 are shown.

clasts were identified as TRAP⁺ cells with two or more nuclei per cell.

Bioinformatic analysis

Flow cytometry data were analysed in an automated hierarchical clustering algorithm and analysis software Citrus v. 0.08 using R v. 3.5.2, as described by Bruggner *et al*. (28), which organises cells into clusters depending on the expression of various markers. The autogating hierarchy of nodes is then depicted in cluster plots, with node size being relative to the number of cells, and colour marking the expression level of a noted marker, followed by group comparison for defined populations. Analyses were performed by using data from all groups to build a tree in Citrus for each staining panel, with randomised sampling of 5000 events, transformed with a cofactor of 150, from individual sample and defining smallest cluster or subpopulation size as 5% of cells in total data. Analysis between groups was done on account of cell abundance in a cluster using "significance analysis of microarrays" (sam) model which identified values associated with a particular group. For T-cell panel, CD3⁺ cells were exported from FlowJo with applied compensation and then clustered and analysed in Citrus on the basis of expression of CD4, CD8, CCR4, CCR6, CXCR5. For B-cell panel, CD19⁺ cells were export-

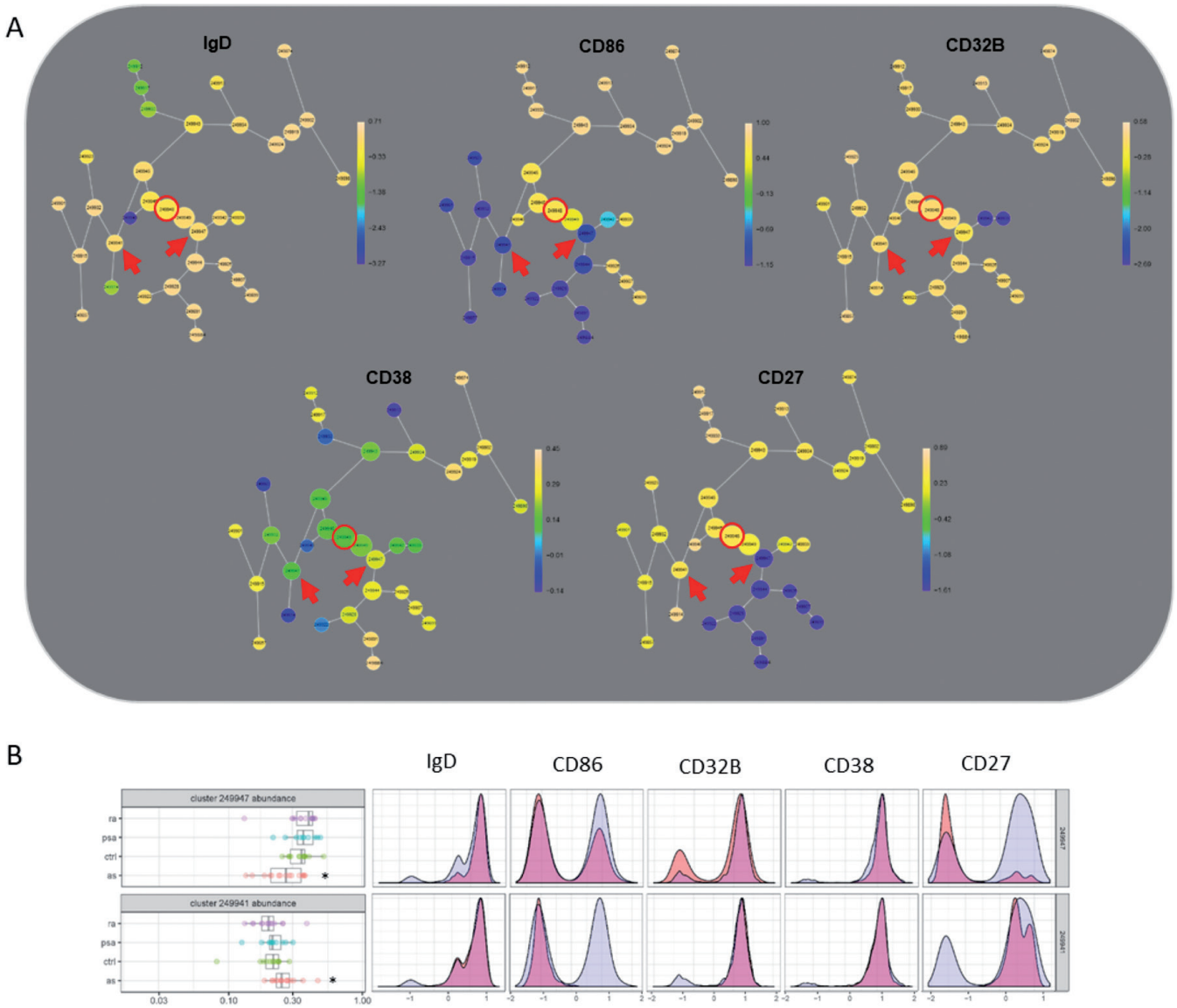

Fig. 5. Citrus automated flow cytometry analysis and disease associated subset selection for B-cell panel in arthritic patients.
A: Visual representation of unsupervised hierarchical clustering by Citrus. The analysis was done on CD19⁺ cells, while IgD, CD27, CD32B, CD38 and CD86 were used as markers for clustering. The colour scale indicates median intensity of respective marker expression, while node size is relative to the frequency of cells in the cluster. Red circle indicates the source cluster at the start of automated hierarchical gating which includes all cells, while red arrows indicate significantly different clusters.
B: Histograms of marker expression and relative abundance of disease-associated cell clusters. Analysis of clusters was done on account of abundance in the cluster between groups using "significance analysis of microarrays" (sam) model. Histograms show the marker expression profile for the cluster (red) *vs.* background (purple) expression in all clusters. Results are mean fold change ± SEM. Disease-to-control comparisons were performed using non-parametric Mann-Whitney test, clusters for *p*-values <0.05 are shown.

ed from FlowJo and then analysed on account of IgD, CD27, CD38, CD86, CD32B. For monocytes, $CD3^{-}CD19^{-}CD56^{-}CD11b^{+}$ cells were exported and then analysed on account of CD14, CD115 and either CCR2 and CCR4 or CCR1 and CXCR4.

Statistical analysis

Data were presented as median ± interquartile range (IQR) for continuous variables or categorised for categorical variables. Statistical analysis was performed using non-parametric Kruskal-Wallis test; disease-to-control differences were tested by Mann-Whitney test. Correlations were made using rank correlation and Spearman's coefficient rho (ϱ) with its 95% confidence interval (CI). Statistical analyses were performed using MedCalc for Windows, v. 9.4.2.0 (MedCalc Software, Ostend, Belgium). For all experiments, α-level was set at 0.05.

Results

Decreased double-negative T-cells in psoriatic arthritis and double positive T-cells in rheumatoid arthritis

General T-cell populations (total $CD3^{+}$, helper $CD3^{+}CD4^{+}$, cytotoxic $CD3^{+}CD8^{+}$) did not differ in frequency across the groups (Suppl. Fig. 1D, Fig. 1A). However, double-positive ($CD4^{+}CD8^{+}$) T-cells, were of lower frequency in RA. On the other hand,

PsA had lower frequency of circulating double-negative ($CD4^-CD8^-$) T-cells (Fig. 1A). In our study, this population was enriched among synovial-fluid derived cells in RA patients (Suppl. Table S2). In adittion, we followed-up a small group of arthritic patients under anti-TNF therapy and observed a tendency of recovery in initially decreased circulatory double-positive and double-negative T-cells by anti-TNF, that was statistically significant for double-negative T-cells in PsA paired samples (Suppl. Fig. 2).

Of helper T-cell subpopulations, only Th1-like ($CD4^+CCR4^-CCR6^-$) cells had significantly increased frequency in RA (Fig. 1B). Furthermore, the Th1-like cells strongly positively correlated with Clinical Disease Activity Index (CDAI) (rho=0.821, p=0.021; Fig. 2).

Cytotoxic ($CD3^+CD8^+$) T-cell subpopulations were altered in RA (Fig. 1C), with a specific increase in $CCR6^+$ (early effector memory) and $CXCR5^+$ (follicular) cytotoxic lymphocytes. Cytotoxic lymphocytes expressing CCR6 were also significantly increased in synovial fluid of RA patients (Suppl. Table S2) and strongly positively correlated with the number of tender joints (rho=0.811, p=0.027; Fig. 2), suggesting possible intra-articular accumulation.

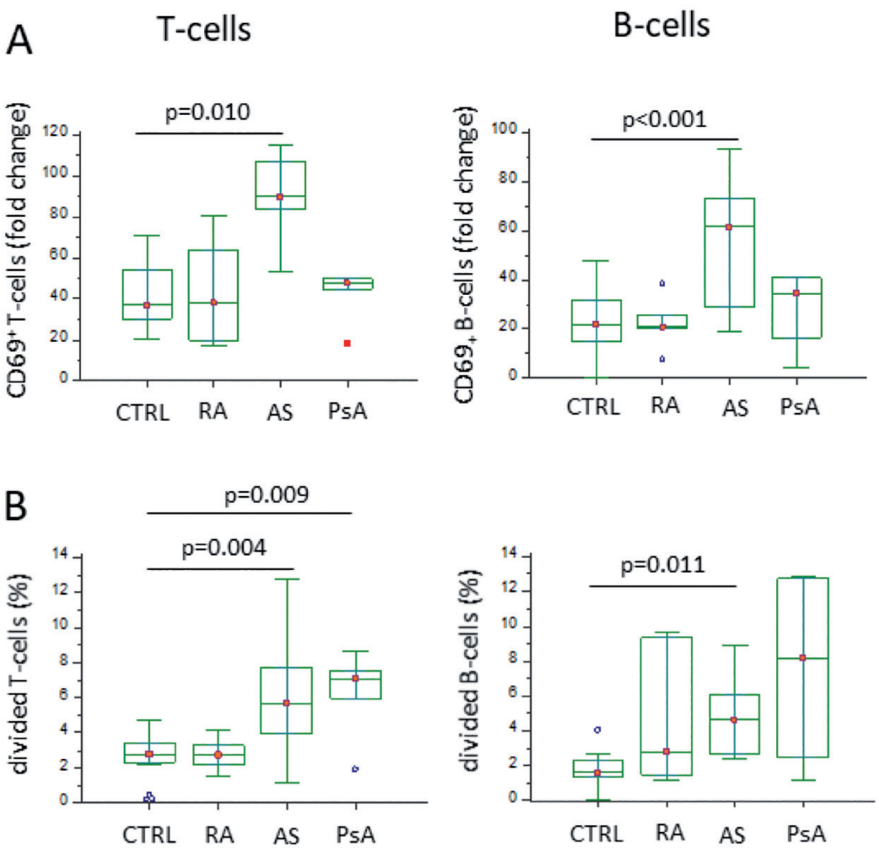

Fig. 6. *In vitro* lymphocyte proliferation and activation assay.
A: CD69 (activation marker) relative expression on T-cells and B-cells of control (CTRL), rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA) samples relative to mitogen-untreated sample.
B: Percentage of divided T-cells and B-cells from peripheral blood of CTRL, RA, AS and PsA samples. Values are presented as medians (middle line), with boxes representing interquartile range (IQR), whiskers representing 1.5 times the IQR and squares or circles representing outliers. Disease-to-control comparisons were performed using non-parametric Mann-Whitney test, p-values <0.05 are shown.

Increased $CD32B^+$ B-cell subpopulations in ankylosing spondylitis associated with disease activity indices

Total B-cells ($CD19^+$) were significantly increased in our group of patients with long disease duration and high disease activity, specifically in RA and, more pronounced, in AS (Suppl. Fig. 1D). Other than a slight increase in frequency of naïve B-cells (IgD^+CD27^-) in PsA, there were no significant changes among main B-cell subpopulations across groups (Fig. 3A). Thus, we proceded to analyse expression of inhibitory receptor CD32B (antibody Fc-receptor II B, FcγRIIB) and costimulatory molecule CD86 (B7-2), both of which have been implied in development of autoimmune diseases, including inflammatory arthritis (29, 30).

There was a notable increase in CD32B expression on naïve (IgD^+CD27^-) and memory (unswitched IgD^+CD27^+, class-switched IgD^-CD27^+ and double-negative IgD^-CD27^-) B-cells in AS, on plasmablasts ($IgD^-CD27^+CD38^+$) in RA and class-switched memory B-cells in PsA (Fig. 3B). Furthermore, CD32B expression on double-negative memory B-cells was strongly positively associated with both Bath ankylosing spondylitis disease activity index (rho=0.721, p=0.019) and, especially, ankylosing spondylitis disease activity score (rho=0.794, p=0.006). In addition, $CD32B^+$ subsets of both naïve and memory B-cells accumulated in RA and PsA synovial fluid (Suppl. Table S3). In contrast to CD32B, changes in CD86 expression were unremarkable, with only significant finding of reduced $CD86^+$ class-switched memory B-cell subset in AS (4.8% [3.9–6.1] in AS *vs.* 7.0% [5.1–8.5] in CTRL, p<0.001).

Bioinformatics analysis links T-cell clusters with rheumatoid arthritis and B-cell clusters with ankylosing spondylitis

Using Citrus algorithm to automatically cluster cells in specific nodes depending on surface marker expression and unbiasely assess the generated hierarchical trees of each panel, several specific cell subpopulations were detected for each disease.

In the T-cell panel, Citrus detected two clusters of cells which had significantly altered frequency in RA (Fig. 4). First cluster (279966) which contained a subpopulation of helper T-cells ($CD4^+CD8^-$), positive for both CCR6 and CXCR5, but negative for CCR4, was virtually absent in RA samples; second cluster (279969) consisted of double-positive ($CD4^+CD8^+$) T-cells positive for both CCR4 and CCR6, but

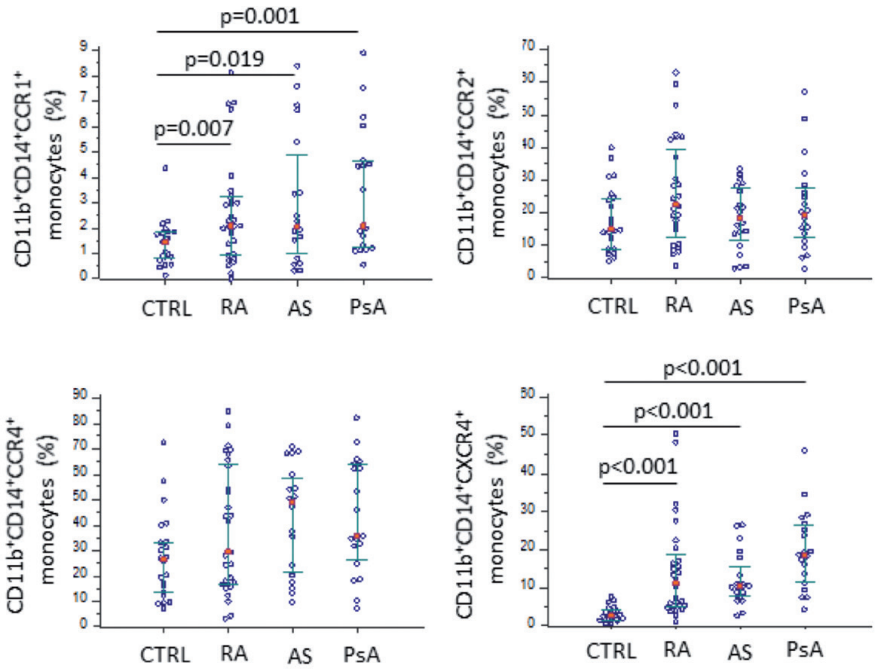

Fig. 7. Expression of chemokine receptors on peripheral osteoclast progenitor-enriched monocytes in arthritic patients. Expression of CCR1, CCR2, CCR4 and CXCR4 on $CD3^-CD19^-CD56^-CD11b^+CD14^+CD115^+$ monocytes of control (CTRL), rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA) patients. Values are presented as medians (square), with boundaries (horizontal lines) representing interquartile range (IQR), and circles representing individual values. Disease-to-control comparisons were performed using non-parametric Mann-Whitney test, *p*-values <0.05 are shown.

negative for CXCR5, was of increased frequency in RA.

Citrus analysis of the B-cell panel revealed two distinct subpopulations associated with AS (Fig. 5). One cluster (249947) had a decreased subpopulation of naïve B-cells (IgD^+CD27^-) which was positive for CD38, while the other cluster (249941) had an increased subpopulation of unswitched memory B-cells (IgD^+CD27^+) positive for both CD32B and CD38, but negative for CD86.

Enhanced activation and proliferation of both T- and B-cells from ankylosing spondylitis

In addition to phenotype, we assessed the activation and proliferation potential of peripheral T- and B-cells in a small group of RA, AS and PsA samples. Sorted T- and B-cells were treated by mitogenic pulse and assessed for the activation and proliferation profile compared to corresponding unstimulated cells.

Both T- and B-cells were found to significantly upregulate CD69, as a marker of activation, and contained more divided cells in AS samples (Fig. 6). There was a similar tendency in PsA samples, though it was only significant for the number of divided T-cells, whereas there were no significant differences between RA samples and controls.

Increased $CCR1^+$ and $CXCR4^+$ osteoclastogenic monocyte subsets in rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis

Based on our previous work, we selected a double-positive, $CD11b^+CD14^+$, monocytic subpopulation expressing CD115, noted to contain peripheral osteoclast progenitors, and profiled it for chemokine receptors - CCR1, CCR2, CCR4 and CXCR4 (26). Even though the general subpopulation did not differ in frequency across the groups (Suppl. Fig. 1D), its expression of CCR1 and CXCR4 was significantly increased in RA, AS and PsA (Fig. 7).

Since double-positive monocytes exhibit osteoclastogenic potential and can contribute to joint and bone destruction in inflammatory arthritis (26), we also performed a functional osteoclastogenic assay by co-culture of a sorted subset of peripheral monocytes and T-cell-conditioned medium, which is known to contain osteoclastogenic factors (8) (Suppl. Fig. 3). Supernatant obtained from unstimulated lymphocytes did not alter the osteoclastogenic potential of sorted progenitors. However, supernatant from mitogenically-pulsed control T-cells significantly enhanced osteoclastogenesis and the effect was further stimulated by supernatants from mitogenically-pulsed RA T-cells, indicating a change in their secretory profile, even though the activation and proliferation profile was similar to that of control.

Bioinformatics analysis reveals monocyte clusters associated with psoriatic arthritis, ankylosing spondylitis and rheumatoid arthritis

Monocytes were analysed by two panels using Citrus algorithm. The first panel (Fig. 8A) detected a cluster (261174) of monocytes ($CD11b^+CD14^+$) expressing CD115 and double positive for CCR1 and CXCR4 which was of significantly higher frequency in PsA and AS samples (Fig. 8A). Two detected clusters in the second panel were indifferent in regards to CD115 expression (Fig. 8B). The first cluster (296636) contained a monocyte subpopulation of $CD11b^+CD14^+$ cells that express CCR2 and was of increased frequency in RA, AS and PsA. The second cluster (296627) contained $CD11b^+CD14^+$cells positive for CCR4 and negative for CCR2, and was of lower frequency in RA and AS.

Discussion

Phenotyping of peripheral immune cell populations has been widely appreciated as the method to assess autoimmune diseases. However, by following predefined gating strategies based on the preceding research, it is not possible to fully utilise the advances in flow cytometry interpretation and to profile novel disease-specific cell subsets. Moreover, rapid technical improvements of cytometry platforms, while increasing the effectiveness and reducing time for acquiring, have been generating high-dimensional datasets inappropriate for meaningful and uniformed analysis

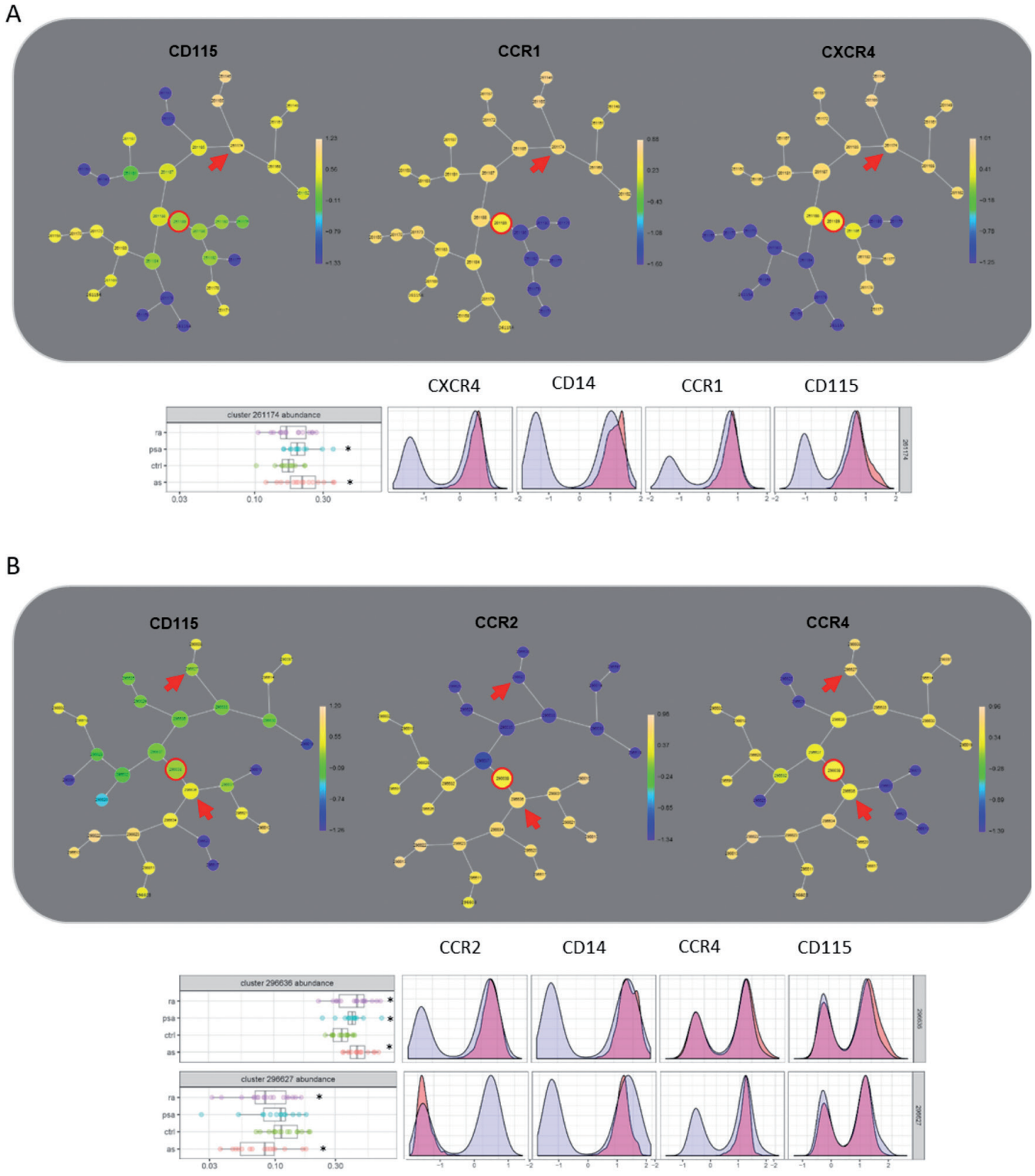

Fig. 8. Citrus automated flow cytometry analysis and disease associated subset selection for monocyte panels in arthritic patients. Visual representation of unsupervised hierarchical clustering by Citrus and histograms of marker expression and relative abundance of disease-associated cell clusters.
A: Analysis done on $CD3^{-}CD19^{-}CD56^{-}CD11b^{+}$ cells, with CD14, CD115, CCR1 and CXCR4 as markers for clustering.
B: Analysis done on $CD3^{-}CD19^{-}CD56^{-}CD11b^{+}$ cells, with CD14, CD115, CCR2 and CCR4 as markers for clustering.
The colour scale indicates median intensity of respective marker expression, while node size is relative to the frequency of cells in the cluster. Red circle indicates the source cluster at the start of automated hierarchical gating which includes all cells, while red arrows indicate significantly different clusters. Histograms indicate marker expression and relative abundance of disease-associated cell clusters. Analysis of clusters was done on account of abundance in the cluster between groups using "significance analysis of microarrays" (sam) model. Histograms show the marker expression profile for the cluster (red) *vs.* background (purple) expression in all clusters. Results are mean fold change ± SEM. Disease-to-control comparisons were performed using non-parametric Mann-Whitney test, clusters for p-values <0.05 are shown.

using standard bivariate dot-plots (26, 31, 32). To overcome these limitations, automated cytometry analysis was introduced to enable comprehensive and unbiased approach in fraction of time and labor. Among new algorithms and software, Citrus is, so far, the only one enabling completely automated identification of unique cell subpopulations associated with a particular disease in an objective and reproducible way. However, the proposed algorithms still need to be applied in different settings and experimentally verified to become widely accepted as method for flow cytometry data interpretation (28, 33). By combining data of manual/automated immunophenotyping, functional analysis and clinical assessment, we identified several pathognomonic immune cell subpopulations in different forms of arthritis (Fig. 9).

RA patients mostly presented with a disturbed peripheral T-cell compartment, including increased proportion of Th1-like ($CD4^+CCR4^-CCR6^-$) cells. RA is traditionally considered as a Th1-predominant disease with the expansion of citrulline-specific autoreactive Th1 cells that contribute to aCPA production (34). In addition, Citrus identified a cluster of Th9-like cells ($CD4^+CCR6^+CCR4^-$) expressing the marker of Tfh, CXCR5, as absent from peripheral blood of RA patients. Th9 cells accumulate in RA synovium, whereas CXCR5 serves to attract T-cells to germinal centres, thus this subpopulation might stimulate B-cell differentiation inside the synovial germinal centre-like structures (35). Moreover, expansion of peripheral $CD8^+CCR6^+$ early effector memory cytotoxic T-cells was found in RA, which accumulated in synovial fluid and associated with number of tender joints. Recent human studies indicate that CD8 T-cell may be important for establishment of germinal centre-like structures in the inflamed synovium, secreting cytokines that positively correlate with disease activity (36, 37). In particular, early effector memory cells mainly secrete proinflammatory Th1 cytokines, whereas immature effector memory T-cells are predominantly of secretory Th2 nature and are associated with immune-mediated skin diseases (38). We

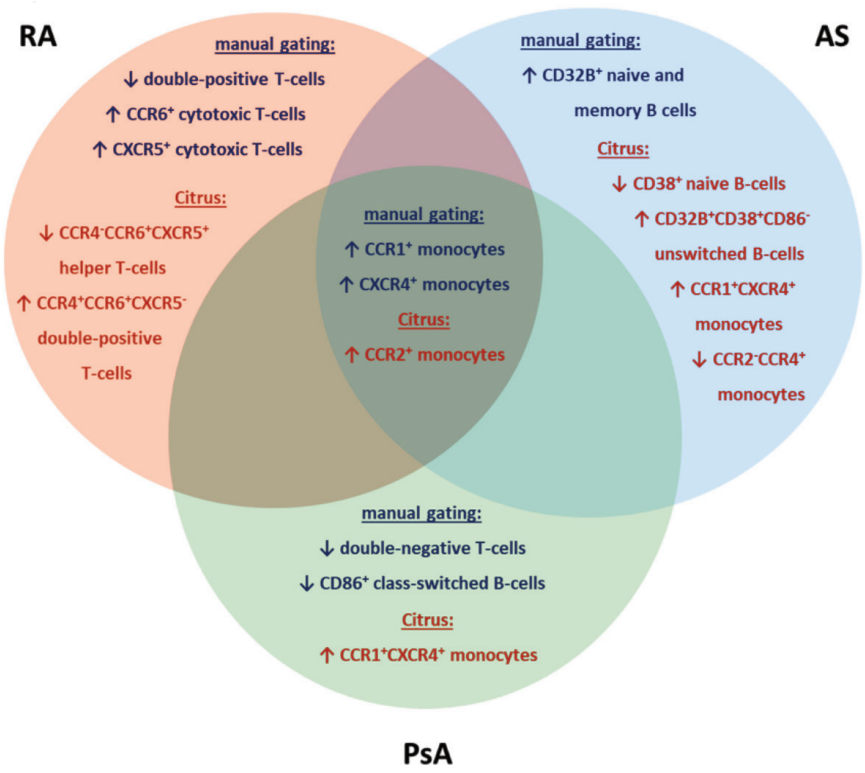

Fig. 9. Disease-specific immune subpopulations in rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis. The diagram summarises pathognomonic circulating immune cell subpopulations in three rheumatic diseases. Our results suggest significant phenotypic and functional abnormalities in B-cell subsets in ankylosing spondylitis, cytotoxic T-cell subsets in rheumatoid arthritis, and chemokine receptor expressing monocyte subsets in all three diseases. Changes in immune subsets were less polarised in PsA patients. AS: ankylosing spondylitis; PS: psoriatic arthritis; RA: rheumatoid arthritis.

also observed a significant decrease of double-negative ($CD4^-CD8^-$) T-cells in PsA and double-positive ($CD4^+CD8^+$) T-cells in RA, subsets associated with autoimmune, malignant and graft-versus-host diseases (39, 40). Interestingly, Citrus revealed enlarged $CCR4^+CCR6^+$ subset among double-positive T-cells in RA that has not been previously studied. Double-negative T-cells have been detected inside entheses of AS and skin lesions of PsA patients (41, 42), whereas we found this population expanded within synovial compartment in RA and recovered among PBMCs upon anti-TNF therapy in PsA patients. However, the roles of double-positive and double-negative populations remain largely unclear since studies are giving controversial results (43, 44).

The most striking findings within B-cell compartment were, unexpectedly, observed in AS. AS is considered a seronegative spondyloarthritis with dominantly abberant autoreactive T-cells and dendritic cells, whereas RA is referred to as a prototype of a seropositive arthritis with B-cell pathology, emphasised by effectiveness of rituximab (45). However, in recent years, it has been shown that AS develops even in total absence of $CD8^+$ T-cells, with several types of newly discovered autoantibodies (including anti-CD47, anti-sclerostin and anti-noggin), thereby shifting focus to B-cell importance (12). Namely, we found increased CD32B expression on naïve and memory B-cell subpopulations in AS, particularly on double-negative (IgD^-CD27^-) memory B-cells that correlated positively with disease activity. Still controversial, double-negative B-cells have been proposed to be exhausted or anergic B-cells, a product of abnormal germinal centre reaction, or to be a unique population formed extra-follicular. This subset has been upregulated in some autoimmune or malignant diseases and, moreover, the expression of CD32B on double-negative B-cells was found to correlate with disease activity in sys-

temic sclerosis (21, 46). Citrus also detected more abundant unswitched memory B-cells with high expression of inhibitory CD32B and virtually absent co-stimulatory CD86 in AS. Unswitched memory B-cells seem to have regulatory characteristics, linked with improved clinical outcomes in inflammatory states (47). Moreover, $CD32B^+$ subsets of naïve and memory B-cells were highly enriched in the synovial compartment of both RA and PsA patients. We also found that plasmablasts in RA increasingly expressed CD32B, shown to be associated with low level of autoantibodies (48, 49). Since CD32B (FcγRIIb) acts as an inhibitory receptor important for peripheral tolerance (50), enhanced expression could represent a compensatory reaction to abnormal activation of B-cells. Citrus also revealed a decrease in $CD38^+$ naïve B-cells that we have not detected manually (since CD38 is used as a plasmablast marker on memory B-cells). However, CD38 deficiency has been noted to lead to autoimmunity (51), while overexpression stimulates regulatory B-cell function (52). In the functional assay, activation marker CD69 was upregulated upon *in vitro* polyclonal B- and T-cell activation in AS, in parallel to enhanced lymphocyte proliferative response. While the T-cell response in AS confirms tradionally mentioned T-cell aberrancies, the enhanced B-cell activation and proliferation reinforces the notion of B-cell abnormalities in AS.

Changes in peripheral osteoclast progenitor-enriched monocytes were more uniform between arthritis subtypes, showing increased CCR1 and, especially, CXCR4 expression. We have previously shown that CXCR4 expression on peripheral monocytes positively correlates with resorptive and immune parameters in RA, whereas anti-TNF therapy has decreased CXCR4 expression (26). CXCR4 has been shown to play an important role in development of psoriasis, while CXCR4 inhibition suppressed osteogenic potential in AS (53-55). CCR1 has been mostly studied in the context of RA, considering its role in monocyte attraction into the affected joints (56). Furthermore, a cluster of monocytes co-expressing CCR1 and CXCR4 was identified by Citrus to be pathognomonic for AS and PsA. In addition, Citrus singled out a subset of $CCR2^+$ monocytes which was enlarged in all three forms of arthritis, while a subset of $CCR4^+$ monocytes was decreased in RA and AS. We have previously shown that ligands for CCR2 and CCR4 enhanced differentiation of osteoclast progenitors defined within $CD11b^+CD14^+CD115^+$ subset of peripheral monocytes in RA (26, 57). In an *in vitro* functional assay, we observed enhanced osteoclastogenic effect of conditioned medium from mitogen-stimulated RA T-cells, indicating they may produce osteoresorptive mediators in a stimulatory environment.

While the study maintains the possibility to distinguish aberrancies across different diseases, the approach did have limitations in the ability to test secondary objectives. Thus, supplementary data pertaining to anti-TNF effect and synovial fluid analysis are primarily indicative due to small sample sizes. Furthermore, inclusion criteria and clinical characteristics of enrolled patients did not allow analysis of possible differences according to disease activity subgroups (in particular remitting/low activity patients). Finally, PsA patients were diagnosed according to the Moll and Wright criteria, instead of widely accepted CASPAR criteria, but for chronic high activity disease both should have similar sensitivity and thus, we believe, should not have had a notable impact on our results. Despite the listed limitations, we believe that the study successfully elucidated changes related to rheumatic diseases and evaluated its disease-specificity.

To conclude, our results revealed significant aberrancies in peripheral lymphocyte and monocyte populations, in particular phenotypic and functional abnormalities in, traditionally overlooked, B-cells in AS (Fig. 9). Both manual gating strategies and automated software emphasised the importance of enriched $CD32B^+$ subsets that are further expanded within synovial compartment. Several populations, not fully defined in existing literature, emerged as possible additional targets, including early effector memory and follicular cytotoxic T-cell subsets in RA, which could accumulate in the affected joints. Identified subpopulations may serve as disease specific markers that could further be used as indicators of therapeutic response or as novel therapeutic targets.

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