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IL-10 producing Bregs are impaired in psoriatic arthritis and psoriasis and inversely correlate with IL-17- and IFNγ-producing T cells☆

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ABSTRACT

Our aim was to study CD19(+)CD27(+)CD24(high) memory and CD19(+)CD24(high)CD38(high) transitional and IL-10+Breg cells, known to inhibit Th1 and Th17 cells in experimental arthritis, in psoriatic arthritis (PsA) and psoriasis (Ps). Peripheral blood Breg cells from 60 patients with PsA, 50 patients with Ps and 23 healthy controls were analyzed by flow cytometry. IL-17A-producing CD3(+) T cells and IFNγ-producing CD3(+) T cells and activation of p38 MAPK and STAT3 were also studied. CD19(+)CD27(+)CD24(high) and CD19(+)CD24(high)CD38(high) Breg cells were decreased in PsA and Ps. In Ps patients, CD19(+)CD27(+)CD24(high) Breg cells inversely correlated with PASI score. IL-10+Bcells were also decreased and inversely correlated with IL-17A+CD3+ and IFN-γ+CD3+ T cells. B cells from patients exhibited impaired activation of p38 MAPK and STAT3. In conclusion, IL-10+Breg cells are decreased PsA and Ps and inversely correlated with the severity of psoriasis and IL-17A+ and IFNγ+ T cells.

1. Introduction

The pathogenesis of psoriatic arthritis (PsA) and psoriasis (Ps) are incompletely understood. Both genetic factors, such as HLA-class I alleles, and environmental factors, such as infections and traumas, have been implicated [1]. In Ps skin lesions and PsA synovial tissues there is increase in innate immune cells and adaptiveTh1 and Th17 cells, and increased levels of TNFα, IL-6, IFNγ, IL-23, and IL-17A cytokines [2,3]. In PsA, the oligoclonal expansion of T cells shared between psoriatic skin lesions and arthritic synovial tissues [4,5], suggests that a common antigen drives the T cell response in both skin and joints of PsA.

B cells may also be implicated. Ectopic lymphoid tissue, with microanatomical features for germinal center formation, was found frequently in PsA synovial membranes [6]. These ectopic lymphoid structures allow interaction among T cells, B cells and dendritic cells and antigen recognition that drives B cell differentiation to antibody-secreting plasma cells. Analysis of immunoglobulin heavy chain variable region (IgVH) genes of synovial membrane from 5 patients with PsA showed identical amino acid replacements, indicative of antigen-driven activation of B cells [7]. Furthermore, a recent study reported that antibodies against a peptide, that shares sequence homology with autoantigens from psoriatic skin lesion and entheses, were frequently detected in patients with PsA [8]. More interestingly, these autoantibodies were reactive to toll-like receptor 2 (TLR2). TLRs activate innate immune cells, and TLR2 agonists promote Th17 differentiation [9,10].

We have recently shown that TLR9-induced IL-10 expressing regulatory B cells (Bregs), a subset of B cells mainly enriched within CD19(+)CD27(+)CD24(high) and CD19(+)CD38(high)CD24(high) cell compartments, are numerically decreased and functionally impaired in systemic sclerosis, a disease with B cell activation [11]. Bregs are major negative regulators of the immune response. They inhibit Th1 and Th17 cell differentiation [12–14], considered to be pathogenic

Abbreviations: BSA, body surface area; Bregs, regulatory B cells; DMARDs, disease-modifying anti-rheumatic drugs; MAPK, mitogen-activated protein kinase; MTX, methotrexate; PASI, psoriasis area severity index; PBMC, peripheral blood mononuclear cells; PsA, psoriatic arthritis; Ps, psoriasis; STAT3, signal transducer and activator of transcription 3; PMA, Phorbol 12-myristate 13-acetate.

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in both PsA and Ps. Although the phenotypic classification of Bregs is still evolving and the IL-10-independent capacity of Breg populations is also under investigation [15], Bregs exert their inhibitory function mainly through IL-10 production [13,16]. Therefore, we explored the role of these Bregs in PsA and Ps.

2. Material & methods

2.1. Patients

A total of 60 consecutive patients with psoriatic arthritis (PsA) (34 females; mean age [±SD], 52.6 ± 13.2 years) and 50 consecutive patients with psoriasis (Ps) (17 females; mean age, 51.2 ± 14.2 years) were included in the study. Twenty-three healthy volunteers were included as healthy controls (HCs) (13 females; mean age 47.3 ± 10.6 years). Patients with PsA and patients with Ps attended the Rheumatology and Dermatology out-patient clinic, respectively, of the University General Hospital of Larissa, in Larissa, central Greece. PsA patients were diagnosed according to the CASPAR (ClASsification criteria for Psoriatic Arthritis) criteria [17]. Psoriasis was graded according to the psoriasis area severity index (PASI). Patients underwent testing for anti-nuclear antibodies by indirect immunofluorescence, rheumatoid factor, and anti-CCP3 antibodies (Generic Assays, Germany). Demographics, clinical and laboratory features of PsA and Ps patients are shown in Table 1.

A written informed consent was obtained by all patients and healthy blood donors according to the Declaration of Helsinki. The protocol was approved by the Local Ethical Committee of the University General Hospital of Larissa.

2.2. Preparation of mononuclear cells

Heparinized peripheral blood samples (~20 mL) from patients and controls were drawn by venipuncture and peripheral blood mononuclear cells (PBMCs) were collected by centrifugation on LymphoPrep gradient (Axis-Shield, Oslo, Norway) [18]. PBMCs were washed twice with serum free RPMI-1640 medium (Invitrogen Life Technologies, Paisley, UK) and cell viability was determined bytrypan blue dye exclusion. Relative lymphocyte percentages among viable cells (based on scatter properties) were also assessed by flow cytometry and routinely exceeded 95–97%.

Table 1
Demographics and main clinical features of patients with psoriatic arthritis (PSA, $n = 60$) and psoriasis (Ps, $n = 50$).

	PsA ($n = 60$)	Ps ($n = 50$)
Female/male (n)	34/26	17/33
Age (mean ± SD years)	52.6 ± 13.2	51.2 ± 14.2
Polyarthritis (n, %)	27 (45%)	NA
PASI score (mean ± SD)	–	5.44 ± 11.94
BSA (mean ± SD)	–	10.87 ± 12.57
Current treatment	53 (88.3%)	46 (92%)
On DMARDs	40	23
MTX/other	27[a]/12[b]	19[c]/4[d]
TNF inhibitor/anti-IL-12-IL-23/CTLA-4/human IgG1 Fc	23/4/1	20/10/0

DMARDs, disease-modifying anti-rheumatic drugs; TNF, tumor necrosis factor; CTLA, cytotoxic T lymphocyte antigen A; PASI, psoriasis area and severity index; BSA, body surface area.

[a] 24 on MTX alone, 1 on MTX plus cyclosporine, 1 on MTX plus sulfasalazine and 1 on MTX plus leflunomide.

[b] 5 on leflunomide, 3 on sulfasalazine, 2 on cyclosposporine, and 2 on hydrochloroquine.

[c] 18 on MTX alone, 1 on MTX plus cyclosporine.

[d] 4 on cyclosporine.

2.3. Bregs phenotypic analysis by flow cytometry

The following anti-human MoAbs were used for phenotypic detection of Breg subsets: FITC-conjugated anti-CD27 (clone MT271), phycoerythrin (PE)-conjugated anti-CD19 (clone HIB19), PC5-conjugated anti-CD24 (clone ML5), and APC- conjugated anti-CD38 (clone HIT2) MoAb (BD Biosciences). Appropriate titration of antibodies was performed for optimal detection of surface epitopes prior to flow cytometry staining. PBMCs ($0.5–1 \times 10^6$ cells) were incubated with MoAbs for 30 mins on ice, washed twice in PBS containing fetal calf serum (FCS) (2%) and fixed with paraformaldehyde (2%). Four-color isotypic non-specific mouse IgMoAbs were used for background autofluorescence. Insignificant background staining was observed using appropriate control FITC-conjugated, PE-, PC5- APC-conjugated antibodies.

Flow cytometric analysis was carried out in a FACS Calibur® (Becton Dickinson Mountain View, CA) cytometer using logarithmic amplification and a forward and side scatter-based gate for total lymphocyte populations. CaliBRITE™ beads (BD Biosciences) were used to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity. At least 3×10^5 cells within the lymphocyte gate were collected for each sample. Experimental testing was performed at least in duplicates in most of the significant analyses and the results were reproducible. BD CellQuest software (BD Bioscience) was used for data acquisition and off-line analysis. For each of the gated populations the percentage and geometric mean fluorescence intensity (MFI) was analyzed.

2.4. Detection of intracellular B cell IL-10 production, CD3(+) T cell IL-17A and IFN-γ cytokine production

For the assessment of the function of Bregs, intracellular IL-10 production was measured. PBMCs were left untreated, or cultured with 10 μg/mL ODN2006 (TLR9 stimulator) (InvivoGen, San Diego, CA, USA) in fresh RPMI-1640 medium supplemented with 10% fetal calf serum for 24 h. During the last 6 h of culture, cells were treated with Brefeldin A (GolgiPlug, BD Biosciences) to block cytokine secretion and re-stimulated with 20 ng/mL Phorbol 12-myristate 13-acetate (PMA) plus 1 μg/mL Ionomycin (Sigma-Aldrich). Activated cells were harvested, washed, stained for appropriate surface markers and then permeabilized with Perm Buffer (BioLegend, San Diego, CA, USA) according to the manufacturer instructions. IL-10 was detected using PE-conjugated or unconjugated anti–IL-10 (JES3–19F1, JES3–9D7) MoAbs (BioLegend).

Since Bregs inhibit Th1 and Th17 cell differentiation, we assessed intracellular IL-17A and IFN-γ production by T cells. For this testing, PBMCs were left untreated, or cultured with 50 ng/mL PMA plus 1 μg/mL Ionomycin for 6 h. IL-17A and IFN-γ were detected using PE- and APC-conjugated MoAbs (BL-168 and 4SB3, respectively) (BD Biosciences).

2.5. Activation of p38 MAPK and STAT3 by phosphoflow cytometric analysis

To further assess the functional properties of B cell populations, we measured the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (STAT-3) by phosphoflow, as previously described in detail [18,19]. Briefly, cells were first allowed to rest for 2 h in low serum (1% FCS) and then activated non-specifically with 20 ng/mL PMA plus 1 μg/mL Ionomycin or sodium arsenite (500μM), or specifically with stimuli engaging the BCR (10 μg/mL polyclonal IgM) or TLR9 (10 μg/mL ODN2006). The stimulation lasted for the indicated times to allow proper signal transduction and to determine kinase activity. A parallel culture with unstimulated cells was used to determine basal levels of phosphorylation. Another culture was set up using the same stimuli in the presence of 3 mM H2O2, in order to deactivate phosphatases and enhance the strength of B cell signaling [11]. Pre-warmed (at 37 °C) paraformaldehyde at a final concentration of 2% was added to the culture medium, and cells

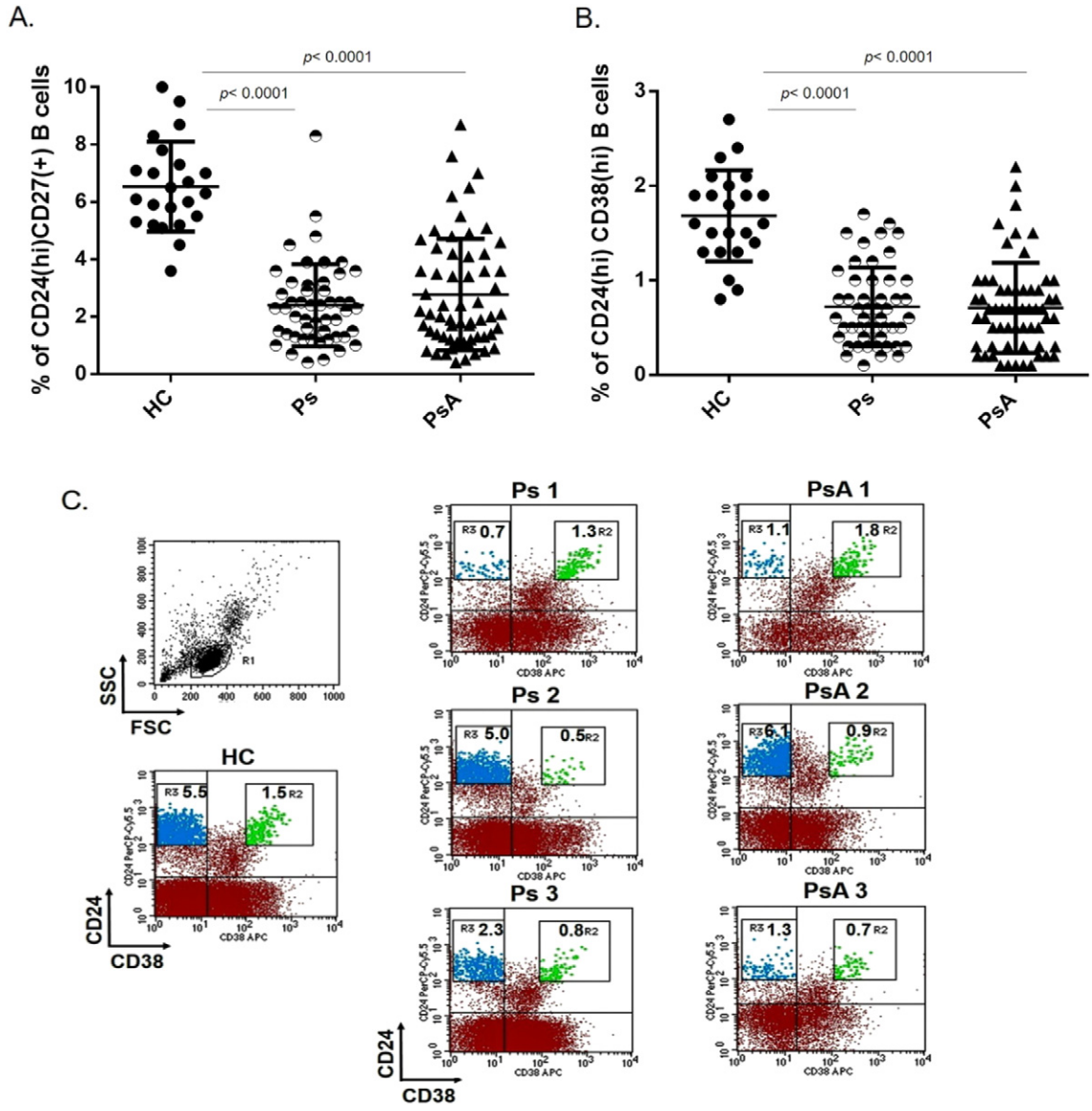

Fig. 1. A. Distribution of memory Bregs in PsA and Ps. Scatter dot-plot illustrations showing decreased percentages of memory Bregs calculated as mean ± SD within total lymphocytes in patients with PsA ($n = 60$) and Ps ($n = 50$) compared to healthy controls (HCs) ($n = 23$). B. Distribution of transitional Bregs in PsA and Ps. Transitional Bregs are significantly decreased in PsA or Ps compared to HC. C. Typical flow cytometric analysis of memory and transitional Bregs in PsA and Ps. PBMCs from patients with PsA or Ps and HCs were stained with CD19, CD24, CD27 and CD38 MoAbs and analyzed by flow cytometry. Viable total lymphocytes were gated based on forward-side scatter characteristic excluding dead cells and debris (gate R1). Transitional Bregs are identified based on positivity for CD19 and high expression of CD38 and CD24 markers (green color-gate R2). Memory Bregs are identified based on positivity for CD19, CD27 markers, high expression of CD24 and lack of CD38 expression (blue color-gate R3). The phenotype and percentages of each cell subset are indicated in three representative cases of Ps and PsA.

were fixed at 37 °C for 15 min. Cells were harvested, washed and permeabilized by slowly adding 1 mL of 75% (v/v) methanol (Fisher Scientific, Pittsburgh, PA) in TBS, while thoroughly vortexing at medium speed. Paraformaldehyde-fixed, methanol-permeabilized cells were rehydrated for 30–60 min by adding 1 mL of TBS-based wash buffer, followed by gentle re-suspension, and centrifugation. To allow blocking of Fc receptors, cells were washed and treated with an FcR blocking reagent (Miltenyi Biotech, Bergisch Gladbach, Germany), followed by 15 min incubation at room temperature (RT). The cell pellet was washed twice, re-suspended in 50 μL 2% BSA/TBS (w/v) and incubated with fluorochrome-conjugated antibodies for 1 h. The following MoAbs were used for phospho-flow analysis: Alexa 647 dye (Molecular Probes, Invitrogen) phospho-p38 MAPK (T180/Y182) and PE-conjugated STAT3 (pS727) (BD Biosciences). According to the manufacturer specifications STAT3 is phosphorylated at serine 727 (S727) via the MAPK pathway. Activation through the S727 residue leads to initiation of transcription.

2.6. Statistical analysis

Percentages of cells expressing cell surface markers and mean fluorescence intensities (MFI) were described as medians of the individuals in each group. Variation in each patient group was defined by standard deviation (SD) and standard error of the mean (SEM). Differences between healthy controls and patients and between patient groups were tested by two-tailed *t*-test, one-way analysis of variance (ANOVA) and

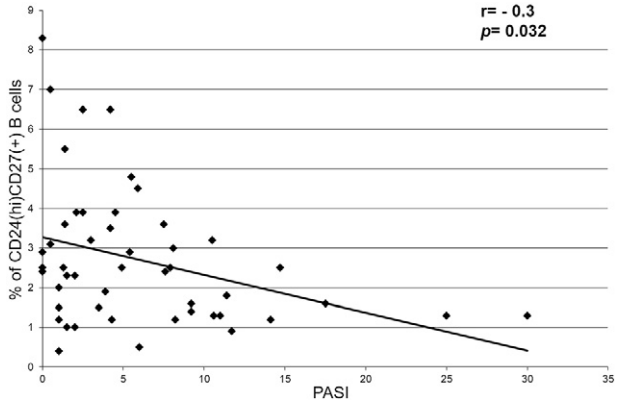

Fig. 2. Inverse correlation of memory Bregs and PASI scores in Ps. PASI scores of patients with Ps ($n = 50$) show a significant negative correlation with the percentages of memory Bregs (Pearson correlation, $r = -0.3$, $p = 0.032$).

the non-parametric Mann-Whitney test. *P*-values smaller than or equal to 0.05 were considered significant. The Pearson correlation (r) method was used in order to measure the correlation between two variables minimally ordinal, with a parametric or normal distribution. The statistical calculations were performed with Graph Pad Prism Software.

3. Results

3.1. CD19(+)CD27(+)CD24(high) memory and CD19(+)CD24(high)CD38(high) transitional Bregs are decreased in psoriatic arthritis and psoriasis

Memory Bregs, identified as CD19(+)CD27(+)CD24(high) and transitional Bregs identified as CD19(+)CD24(high)CD38(high), were quantified in 60 patients with PsA, 50 patients with Ps, and 23 HCs. The gating strategy that we used has been described in our recent report in great detail [11].

Memory Bregs expressed as percentage of total lymphocytes were decreased in PsA (2.77 ± 1.94 [mean % ± SD], $p < 0.0001$) and Ps (2.40 ± 1.42, $p < 0.0001$) compared to HCs (6.53 ± 1.56) (Fig. 1A). No statistically significant differences were found between PsA and Ps patients. Transitional Bregs expressed as percentage of total lymphocytes were also decreased in PsA and Ps compared to HCs (Fig. 1B). The mean percentage of transitional Bregs in PsA, Ps, and HCs were 0.71 ± 0.47, 0.72 ± 0.41, and 1.68 ± 0.48, respectively (PsA vs HCs $p < 0.0001$; Ps vs HCs $p < 0.0001$; PsA vs Ps, $p > 0.05$) (Fig. 1B). Flow cytometry dot plots of memory (blue color) and transitional (green color) Bregs from representative patients with PsA, Ps and HCs are shown in Fig. 1C. Absolute numbers of memory and transitional Bregs were also significantly decreased in PsA and Ps compared to controls ($p < 0.001$ for both) (Supplementary Fig. 1).

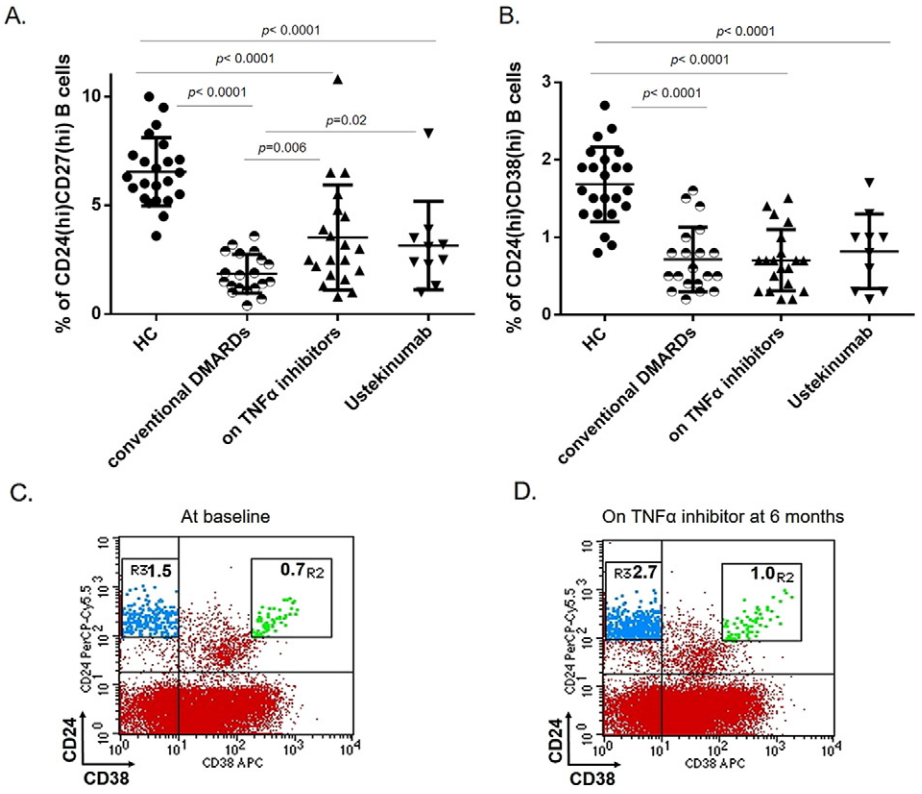

Fig. 3. A. Distribution of memory Bregs among Ps patients on conventional DMARDs, and biologics. Scatter dot-plot illustrations showing percentages of memory Bregs calculated as mean ± SD within total lymphocytes. Memory Bregs are significantly decreased in Ps patients on conventional DMARDs ($n = 20$), on TNFα inhibitors ($n = 20$), and on anti-IL-12/IL-23 MoAb (Ustekinumab) ($n = 10$) compared to healthy controls (HC, $n = 23$). However, memory Bregs are significantly increased in Ps patients treated with anti-TNFα agents and in patients treated with Ustekinumab compared to those on conventional DMARDs. B. Distribution of transitional Bregs among Ps patients on conventional DMARDs, and biologics. Memory Bregs are significantly decreased in patients with Ps on conventional DMARDs, and on biologics (TNFα inhibitors, and anti-IL-12/IL-23 MoAb [Ustekinumab]) compared to healthy controls (HC). There was no statistical difference between transitional Bregs from Ps patients treated with anti-TNFα biologicals and in patients treated with Ustekinumab compared to those on conventional DMARDs. C & D. Flow cytometric analysis of memory and transitional Bregs in a Ps patient (C) at baseline (month 0) and (D) on TNFα inhibitor 6 months later. PBMCs from a patient with Ps were stained with CD19, CD24, CD27 and CD38 MoAbs and analyzed by flow cytometry. Transitional Bregs (green color-gate R2) and memory Bregs (blue color-gate R3) are shown in percentages. Memory Bregs are increased from 1.5% to 2.7% and transitional Bregs are increased from 0.7% to 1%, whereas PASI score decreased from 15 to 6 during a 6 month treatment with etanercept, a TNFα inhibitor.

3.2. CD19(+)CD27(+)CD24(high) memory Bregs inversely correlated with psoriasis activity and severity index (PASI) score

In PsA, no significant association was detected between Bregs and CRP, swollen joint count, or tender joint count. However, patients with oligoarthritis had slight (not significant) increase in memory Bregs compared to polyarthritis and spondylitis group.

In Ps, there was an inverse correlation of memory Bregs with disease severity. The percentage of memory Bregs from patients with Ps negatively correlated with PASI score ($r = -0.3$, $p = 0.032$) (Fig. 2). There was no correlation between transitional Bregs and PASI score ($r = -0.116$, $p > 0.05$). There was a positive correlation between transitional Bregs and disease duration in Ps ($r = 0.37$, $p = 0.03$).

3.3. Ps patients on TNFα inhibitors or IL-12/IL-23 inhibitor had increased memory Breg frequencies compared to Ps patients on conventional DMARDs

When patients with Ps were subdivided according to treatment regimen, we found that patients on TNFα inhibitors or IL-12/IL23 inhibitor (Ustekinumab) had increased memory Bregs compared to Ps patients on conventional disease-modifying antirheumatic drugs (DMARDs) (Fig. 3A). The mean percentage of memory Bregs (Fig. 3A) in Ps treated with conventional DMARDs alone ($n = 20$), with TNFα inhibitors ($n = 20$), or with anti-IL-12/IL-23 MoAb (Ustekinumab) ($n = 10$) and in HCs ($n = 23$), were 1.85 ± 0.88, 3.52 ± 2.40, 3.15 ± 1.79 and 6.53 ± 1.56, respectively (Ps DMARDs vs Ps anti-TNFα, $p = 0.006$; Ps DMARDs vs Ps anti-IL-12/IL-23, $p = 0.02$; Ps anti-TNFα vs Ps anti-IL-12/IL-23, $p > 0.05$; HCs vs all groups $p < 0.0001$). Transitional Bregs were not increased in Ps patients treated with biological compared to those treated with conventional DMARDs alone (Fig. 3B). Fig. 3C and D illustrate a representative example of changes of memory and transitional Bregs percentages in a patient with Ps before and after 6 months of treatment with etanercept, a TNFα inhibitor. Memory Bregs increased for 1.5% to 2.7% whereas PASI score decreased from 15 to 6. Transitional Bregs also increased.

3.4. IL-10 production by Bregs was defective in PsA and Ps

As IL-10 production has long been considered the defining Bregs feature and in order to analyze the functional capacity of Bregs, we analyzed intracellular IL-10 in total and sub-gated memory and transitional Bregs in PsA, Ps and HCs. The percentages of IL-10-producing total B cells were decreased in PsA (1.38 ± 0.54, $p < 0.001$) and Ps (1.05 ± 0.71, $p < 0.0001$) compared to HCs (3.07 ± 0.77, $n = 8$ each group) with no difference between PsA and Ps (Fig. 4A). We next addressed the question of whether the reduced IL-10 production in PsA and Ps is associated with a decrease in Bregs or was rather due to an intracellular functional defect. To assess this, we analyzed PsA and Ps patients with comparable to HC percentages of memory Bregs and transitional Bregs and found that IL-10 production was defective in both PsA and Ps. A representative example is shown in Fig. 4B. We next examined the extent of IL-10 production from memory and transitional Bregs. When B cells were subgated phenotypically in memory and transitional subsets, IL-10 production was decreased in PsA and Ps, expressed as percentages of positive cells and as mean fluorescence intensity (MFI) compared to healthy controls (Supplementary Fig. 2). Basal IL-10 production in HCs mainly derived from memory Bregs, and increased after ODN2006 stimulation of cells. In PsA and Ps no basal IL-10 production and very little IL-10 production after ODN2006 stimulation of cells was noted.

3.5. IL-10-producing B cells were inversely correlated with IL-17A-producing CD3(+) T cells and IFNγ-producing CD3(+) T cells

Since Bregs inhibit Th17 and Th1 cell differentiation, we next assessed IL-17A and IFNγ-producing T cells in PsA and Ps. The percentage of IL-17A-producing CD3(+) T cells was increased in PsA (1.88 ± 0.75) and Ps (1.77 ± 0.56) compared to HCs (0.60 ± 0.26, $p < 0.001$ for both, $n = 8$ for each group) (Fig. 5A). The percentage of IL-10-producing B cells were inversely correlated with the percentages of IL-17A-producing CD3(+) T cells in PsA ($r = -0.99$, $p = 0.001$) and in Ps ($r = -0.95$, $p = 0.017$). Similarly, IFNγ-producing CD3(+) T cells

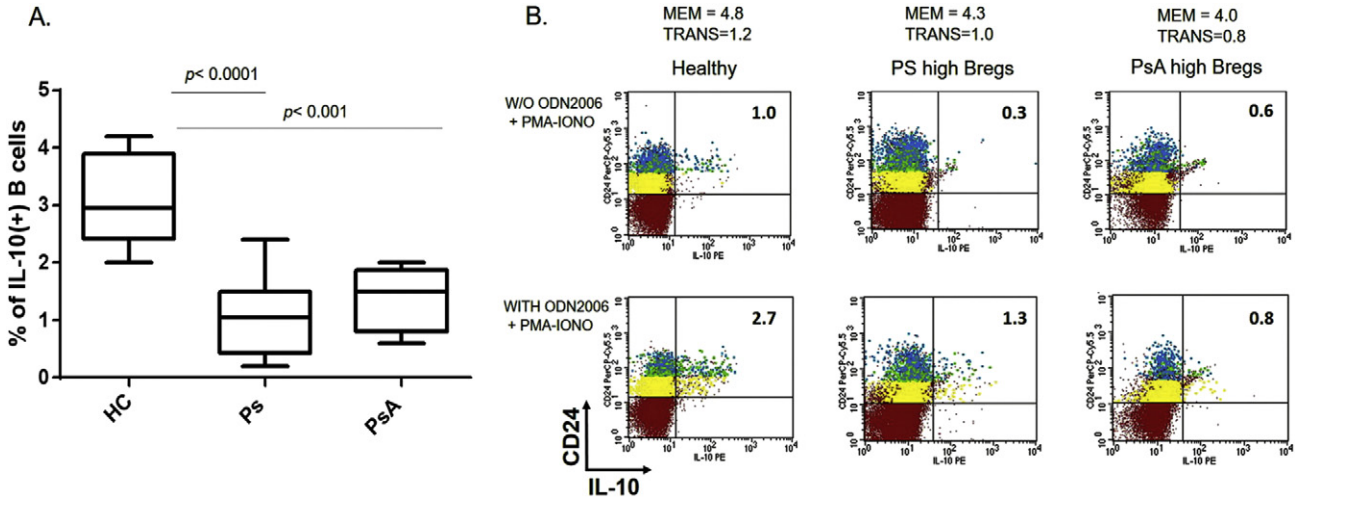

Fig. 4. A. Frequency of IL-10 expressing B cells in PsA, Ps and HCs. Box and whiskers min to max graphical representation showing significant reduction in the percentages of IL-10-producing B cells following a 24 h incubation with a TLR-9 agonist (ODN2006), in patients with PsA ($n = 8$) and Ps ($n = 8$) compared with HCs ($n = 8$). B. Flow cytometric analysis of intracellular IL-10 expression by Bregs PBMCs from PsA patients, Ps patients and HCs were analyzed for IL-10 expression by flow cytometry. Representative dot plots from selected PsA and Ps patients with comparable to HC percentages of phenotypic memory and transitional Bregs are illustrated. IL-10 expression following ODN2006 stimulation and re-stimulation with PMA plus ionomycin is observed within CD24(+) B cells. CD24 (high) memory Bregs (blue), CD38 (high) transitional Bregs (green) and CD24(low) B cells (yellow) are indicated. The percentage of IL-10(+) cells is shown on top right corner of each plot. In cultures without ODN2006, (basal) IL-10 is produced by memory Bregs from healthy controls. Basal IL-10 production is barely detected in PsA and Ps patients. In cultures with ODN2006, IL-10 is expressed by transitional and memory Bregs. IL-10 is also produced by a proportion of CD24(low) B cells. In PsA and Ps, IL-10 production is significantly reduced.

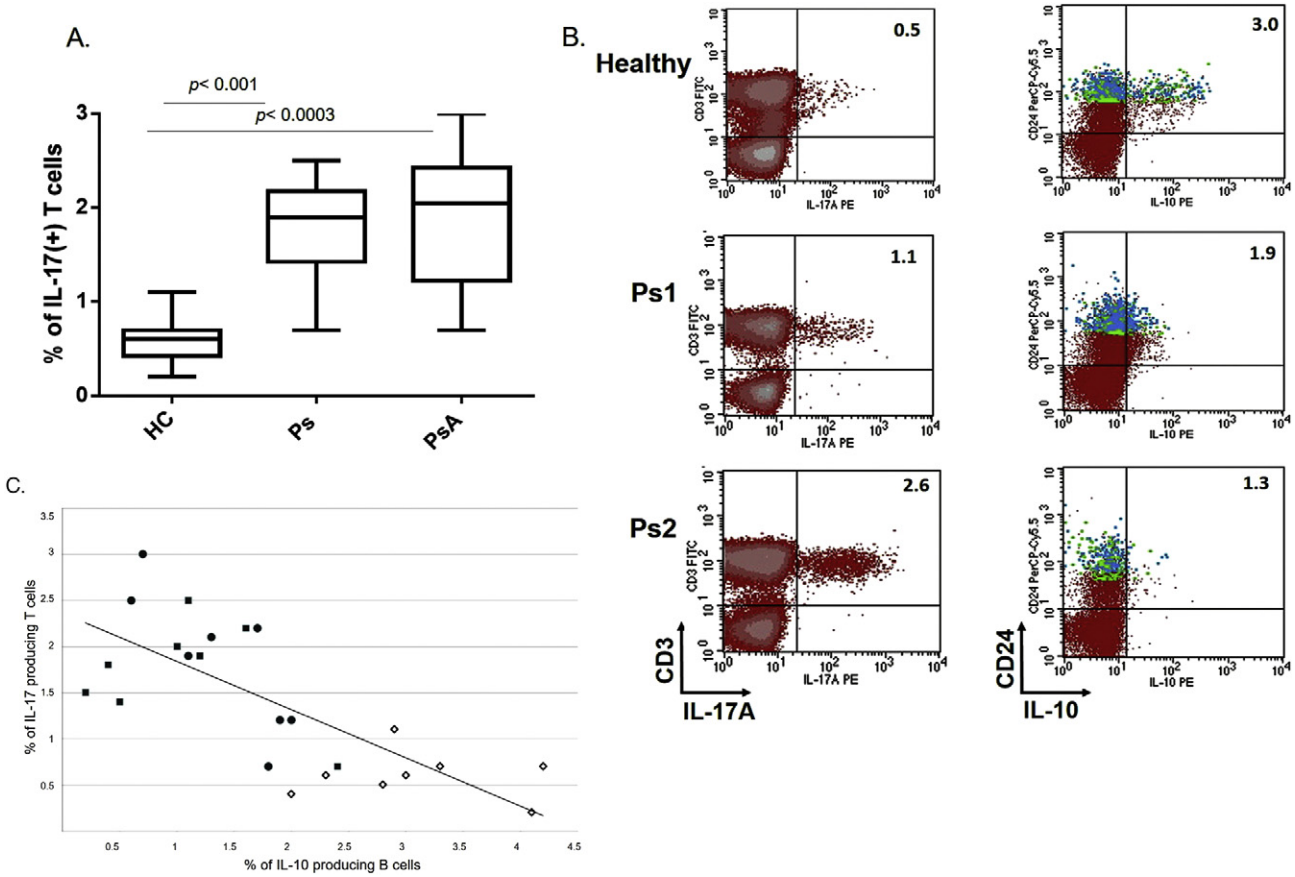

Fig. 5. A. Frequency of IL-17-producing T cells in PsA, and Ps. Box and whiskers min to max graphical representation showing significant reduction in the percentages of IL-17-producing T cells after a 6-h PMA and ionomycin stimulation in patients with PsA ($n = 8$) and Ps ($n = 8$) compared to HCs ($n = 8$). The percentage of IL-17-producing T cells is higher in patients with PsA or Ps compared to HCs. B. Flow cytometric analysis of IL-17-producing T cells and IL-10-producing Bregs. PBMCs from PsA patients ($n = 8$), Ps patients ($n = 8$) and HCs ($n = 8$) were analyzed for IL-17 expression by flow cytometry. IL-17 was mainly produced from CD3(+) following 6-h stimulation with PMA and ionomycin. IL-10 production following 24-h ODN2006 treatment and PMA/ionomycin re-stimulation was also assessed in the same patients and observed within CD24(+) B cells. CD24 (high) memory Bregs (blue) and CD38 (high) transitional Bregs (green) are indicated. The percentages of IL-17(+) and IL-10(+) cells are shown on top right corners of each plots. The percentage of IL-17-producing CD3(+) T cells is increased in PsA and Ps compared to HCs. In contrast, the percentage of IL-10-producing B cells is decreased in the same patients. C. Inverse correlation of IL-10-producing B cells with IL-17A-producing T cells in PsA and Ps. A negative correlation between IL-10-producing B cells and IL-17-producing T cells is shown in PsA (dark squares) (Pearson $r = -0.99$, $p = 0.001$) and in Ps (dark circles) (Pearson $r = -0.95$, $p = 0.017$).

were increased in PsA and Ps and inversely correlated with IL-10-producing B cells ($r = -0.814$, $p < 0.001$) (Supplementary Fig. 3).

3.6. The activation of p38 MAPK and STAT-3 was impaired in B cells from PsA and Ps patients

We have recently shown that IL-10 production by B cells stimulated through TLRs is dependent on p38 MAPK and STAT3 activation and have reported an impaired activation of p38 MAPK and of STAT3 in patients with SSc [11]. In addition, Ca(2+) signaling contributes to B-cell and plasmablast IL-10 expression [20]. Thus, we addressed the activation status of p38 MAPK and STAT3 in Bregs from Ps and PsA patients. IL-10 inducing stimuli, such as PMA plus Ca(2+) Ionophore (Ionomycin), IgM (BCR stimulator) and ODN2006 (TLR9 stimulator) were used to activate (phosphorylate) both molecules that are part of a signaling cascade whereby p38 MAPK induces serine phosphorylation of STAT-3 which then translocates to the nucleus in order to induce gene expression [21,22].

P38 MAPK and STAT3 activation were decreased in B cells from PsA and Ps patients compared to HCs. A representative illustration of p38 MAPK and STAT-3 phosphorylation within PBMCs, and gated for CD20(+) B cells, are shown in Fig. 6. The phosphorylation of p38 MAPK was impaired in CD20(+) B cells from 5 patients with PsA and 5 patients with Ps compared to HCs (Fig. 6A). More specifically, over 65% of p-p38(+) B cells were consistently detected in stimulated HCs compared to <25% p-p38(+) B cells in PsA and to <37% p-p38(+) B cells in Ps and ($p < 0.05$ for all groups). STAT-3 was constitutively phosphorylated in approximately 30% of B cells from HCs and was induced to >50% of B cells following PMA and ionomycin activation. In patients with PsA and Ps, STAT-3 was constitutively phosphorylated in 15–22% of B cells and was induced to 22–27% of B cells following PMA and ionomycin activation (Fig. 6).

We also examined the effects of BCR-specific stimulation using polyclonal IgM and ODN2006. Since H2O2 deactivates phosphatases and enhances the strength of B cell signaling, we included this stimulant in our experiments [11,23]. The phosphorylation of p38 MAPK and STAT-3 was impaired irrespective of whether B cells were stimulated by BCR or TLR-9 (Supplementary Fig. 4).

4. Discussion

Our study is the first to report that Bregs are numerically decreased and functionally impaired, as they could not produce IL-10, in patients with PsA and Ps. Furthermore, memory Bregs were inversely correlated with PASI scores in Ps. During the completion of our study, another study in patients with Ps, which did not include PsA patients, reported a decrease of IL-10-producing Bregs but did not find a correlation with disease severity score [24]. In a mouse model of Ps, induced by

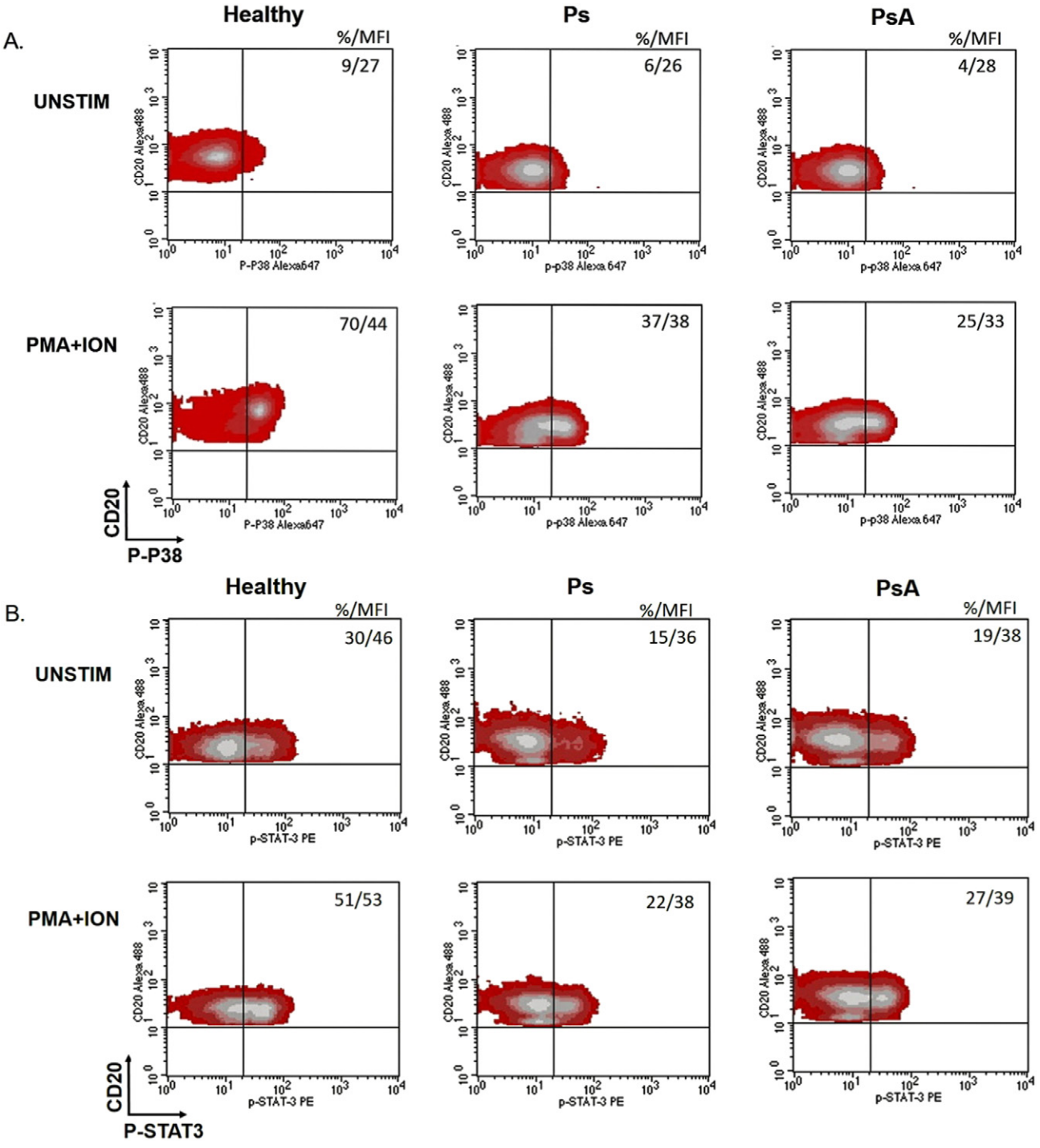

Fig. 6. Impaired phosphorylation of p38 MAPK and STAT-3 in B cells from PsA and Ps. PBMCs from PsA patients, Ps patients and HCs were stimulated with PMA plus ionomycin for 15 min and p38 MAPK (A) and STAT-3 (B) phosphorylation was assessed in CD20(+) B cells by flow cytometry. The percentage of p-p38(+) cells and the mean fluorescence intensity (MFI) is indicated. Representative density plots of five independent experiments show an impaired activation of p38 MAPK(A) and STAT-3 (B) in both PsA and Ps.

imiquimod, a TLR7/8 ligand, where the IL-23/IL-17 axis plays a pivotal role [25], splenic IL-10-producing Bregs (B10 cells) were greatly reduced [26]. In this model, CD19 deletion resulted in more severe skin inflammation and complete depletion of IL-10-producing CD1d(high)CD5(+) Bregs [26], whereas adoptive transfer of B10 cells from wild-type mice to imiquimod-treated CD19 −/− animals reduced skin inflammation and IFNγ and IL-17A production [26]. In humans, IL-10 administration appears to have a favorable effect on PsA and Ps: IL-10, administered subcutaneously for 28 days in patients with PsA in a double-blind study, improved psoriasis and decreased T cell and macrophage infiltrations in synovial tissue [27].

Our finding of reduced and dysfunctional Breg cells in PsA and Ps is of pathogenic significance for both diseases. We found an inverse correlation between IL-10-producing B cells with IL-17A-producing T cells and between IL-10-producing B cells and IFNγ-producing T cells. Mice lacking IL-10-producing B cells exhibited markedly increased Th1 and Th17 cells and decreased Tregs and exacerbated arthritis [28]. In addition, transfer of wild type Bregs to arthritic IL-10 −/− mice reduced

Th1 and Th17 cells, increased Tregs and inhibited inflammation [28]. These findings were supported by knock-out mice lacking IL-10-producing B cells that developed severe collagen-induced arthritis with increased levels of Th1 and Th17 cells [13]. Similar findings were reported in humans. In humans, CD19(+)CD24(high)CD38(high) B cells inhibited differentiation of naïve T cells into Th1 and Th17 cells and converted CD4(+)CD25(−) into Tregs partly through IL-10 [13,14]. In patients with active rheumatoid arthritis (RA), CD19(+)CD24(high)CD38(high) Bregs were reduced compared to patients with inactive disease and healthy controls and failed to inhibit Th1 or Th17 cell development or to induce Tregs [14].

Our study shows that phenotypic characterization of Bregs (based on generally accepted surface markers such as CD38, CD24 and CD27) does not provide an accurate estimation of IL-10 producing Bregs. Firstly, because not all memory and transitional "Bregs" are able to express IL-10. Moreover, not all IL-10 production stems from CD19(+)CD24(high)CD38(high) (transitional Bregs) or CD19(+)CD27(+)CD24(high) (memory Bregs). Thus, a precise estimation of IL-10 Bregs must involve the functional capacity of B cells to produce to IL-10 and to phosphorylate associated molecules rather than just to examine phenotypic markers. This assumption is supported by our data and findings of recent studies demonstrating the ability of B cell subsets other than CD19(+)CD24(high)CD38(high) and CD19(+)CD27(+)CD24(high) [such as CD24(low) and CD25(high)FoxP3(high)] to also produce a significant amount of IL-10 [16]. The recent identification of IL-10-independent Bregs, such as those expressing PD-L1, TGF-β or IDO [29], further underline the need to abandon phenotypic markers of Bregs which are widely used in research protocols and may lead to inaccurate estimations of Bregs in PsA and Ps, as well as in other autoimmune diseases. This has become evident in our study, as only 20% phenotypically characterized "Bregs" where real IL-10 producers. Signaling cascade investigation could provide more information regarding the functional capacity of potentially specialized Bregs. To this end, we found a defective p38 MAPK and STAT3 activation (serine 727 phosphorylation) in Bregs from PsA and Ps patients. This is in accordance with previous reports showing that p38 and STAT3 activation is required for IL-10 production by B cells, and inhibition of STAT3 activation abrogated B cell IL-10 production [30,31]. We also have recently reported defective p38 and STAT-3 phosphorylation using several IL-10 inducing agonists such as PMA plus Ionomycin, IgM (BCR) and ODN2006 (TLR9) in Bregs in systemic sclerosis [11].

The role of B cells in the pathogenesis of PsA and Ps is slowly emerging. Lymphoid aggregates with features of tertiary lymphoid organs, such as distinct T cell and B cell-rich aggregates, high endothelial venules and capable of inducing local antibody response [32] are present in PsA synovial membrane and regressed in patients with complete response to treatment [6]. B cells are also present in the skin of PsA patients [33] and in tenosynovium of a patient with dactylitis, another characteristic clinical feature of PsA [34]. Finally, a recent study showed that autoantibodies against a peptide that shares sequence homology with autoantigens, present in skin and enthesis, were detected in 85% of patients with PsA but not in healthy controls [8], and these antibodies bind to TLR2. TLRs activate innate immune cells, and TLR2 agonists promote Th17 differentiation [9,10]. B cells in PsA synovial membrane may function as antigen-presenting cells, as has been shown in experimental arthritis [35–37]. The findings of synovial fluid B cells from patients with PsA that show signs of activation, such as increased expression of HLA-DR, HLA-DQ, CD40, and CD86, and decreased expression of CD23 compared to peripheral blood, are in accordance with this view [38]. Very limited data of anti-B cell treatment in PsA and Ps appears promising. In small studies, one with PsA [39] and another with psoriatic spondyloarthritis [40], rituximab, an anti-CD-20 monoclonal antibody that depletes B cells, exhibited efficacy. It should be mentioned here that Bregs are resistant to rituximab [41]. In a patient with cryoglobulinemia and psoriasis, rituximab, apart from marked improvement of cryoglobulinaemic manifestations, modesty improved psoriasis [42]. On the other hand, there are few case reports of psoriasis induced by rituximab in patients with rheumatoid arthritis [43,44].

Our finding that TNFα inhibitors increased Bregs but not to the level of healthy controls is of interest and may relate to responsiveness to treatment. In a recent study in PsA, B cell numbers were decreased and restored in responders to etanercept treatment [45]. These findings further support a role for Bregs in the pathogenesis of PsA and Ps. Large multi-center studies on prospectively collected samples are needed to delineate the exact role of Bregs in PsA and Ps.

In conclusion, our findings demonstrating that IL-10-producing Bregs were decreased in PsA and Ps and inversely correlated with IL-17A-producing and IFNγ-producing T cells, clearly implicate Bregs as regulators in the pathogenesis of these diseases.

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Contributions

All authors were involved in drafting the article or revising it critically for important intellectual content. All authors approved the final version of the paper. Professor LI Sakkas had full access to all of the data in the study and takes responsibility for the integrity of the data and their accuracy.

Competing interest

None.

Study conception and design

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Acquisition of data

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Analysis and interpretation of data

A Mavropoulos, C Liaskos, DP Bogdanos and LI Sakkas.

Drafting of manuscript

A Mavropoulos, DP Bogdanos, LI Sakkas.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2017.04.010.

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