Ex Vivo Signaling Protein Mapping in T Lymphocytes in the Psoriatic Arthritis Joints

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ABSTRACT. We assessed signaling protein mapping in total T cells, to analyze the proportions of T regulatory (Treg) and TCD4+ effector (Teff) cell phenotypes, and the respective interleukin 6Rα (IL-6Rα) expression in the inflammatory microenvironment of synovial fluid (SF) of patients with sustained psoriatic arthritis (PsA). Our approach was to measure the IL-6 level in SF using a multiplex bead immunoassay. Reverse-phase protein array was used to assess Janus kinase (JAK) 1 and JAK2, extracellular regulated kinase (ERK) 1 and 2, protein kinase Cδ (PKCδ), signal transducer and activator and transcription (STAT) 1, STAT3, and STAT5 phosphoproteins in total T cell lysates from SF of patients with PsA. Frequencies of CD4+IL-17A-F+IL-23+ CD4+ Th cells producing IL-17A and IL-17F (Th17) and CD4+CD25^{high} intracellular forkhead box transcription factor+ (FOXP3+) phenotypes, and the percentage of Treg– and Teff– cells were quantified in SF and matched peripheral blood (PB) of patients with PsA and PB of healthy controls (HC) by flow cytometry. Our results were the following: In PsA SF samples, a coordinate increase of JAK1, ERK1/2, STAT1, STAT3, and STAT5 phosphoproteins was found in total T cells in SF of PsA; where IL-6 levels were higher than in PB from HC. Expanded CD4+IL-17A–F+IL-23+ Th17, CD4+ CD25– Teff– and CD4+CD25^{high} FoxP3+ Treg subsets, showing similar levels of enhanced IL-6Rδ expression, were confined to PsA joints. In our studies, the transcriptional network profile identified by *ex vivo* signaling protein mapping in T lymphocytes in PsA joints revealed the complex interplay between IL-1, IL-6, and IL-23 signaling and differentiation of Th17 cells and CD4+Tregs in sustained joint inflammation in PsA. (J Rheumatol Suppl. 2015 Nov;93:48–52; doi:10.3899/jrheum.150636)

Key Indexing Terms:
PSORIATIC ARTHRITIS INTERLEUKIN 6Rα SIGNALING TH17 CELLS
T REGULATORY (TREG) CELLS JAK1/STAT3/STAT5 PHOSPHOPROTEINS

Cytokines exert their biologic effects through binding to specific receptors on the cell surface; signal transduction involves activation of Janus kinase (JAK) tyrosine kinase family members, leading to activation of the signal transducer

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and activator and transcription (STAT) family, which results in the phosphorylation of STAT proteins. STAT proteins then dimerize and translocate from the cytoplasm into the nucleus, where they bind to specific promoter regions of target genes[1]. STAT act upon the repertoire of enhancers to generate specific transcriptional responses, driving the distinct cell lineage specification[2].

Th cell differentiation occurs by T cell stimulation in the context of the cytokine milieu. Signals result in the specification of different Th lineages, such as regulatory T cell (Treg), Th type 1 (Th1), Th type 2 (Th2), and Th cell-producing interleukin (IL) 17 (Th17), occurring through the action of multiple regulatory elements[3,4].

Our recent research aimed to assess the chronic inflammatory process in clinically active joints of sustained psoriatic arthritis (PsA) by exploring the local factors involved in the regulation of the CD4+ T cell differentiation, in the joint milieu, at both cellular and intracellular level[5,6]. We explored the feasibility of a direct *ex vivo* analysis of distinct CD4+Tcell phenotypes in synovial fluid (SF) of patients with PsA[5]. The ultimate goal of the study was to identify the main pathways that might represent candidate biomarkers, as potential pharmacological targets of disease.

MATERIALS AND METHODS

Briefly, our study of T cell phenotypes was carried out in SF from 16 patients with PsA, and peripheral blood (PB) from 7 healthy subjects as controls (HC). SF samples were obtained from affected patients with clinically active joints (joint pain, tenderness, and swelling). The study was approved by the local Ethics Committee of the University Hospital of Padua (Italy) (number 52723; October 11, 2010), and all patients signed informed consent statements.

PB mononuclear cells (MC) and SFMC were isolated from the PB and SF by Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation.

For reverse-phase protein array (RPPA) analysis we isolated lymphocytes plated PBMC and SFMC for 2 h at 37°C with 5% CO_2, to allow the adhesion of monocytes on the bottom of the plate. The levels of JAK1 Y1022/Y1023, JAK2 Y1008, protein kinase C delta (PKCδ) T505, STAT 1 Y701, STAT3 Y705, and extracellular regulated kinase 1 and 2 (ERK1/2) T202/Y204 phosphoproteins were measured and compared with the levels obtained from T cells from PB of HC.

Fluorescence activated cell sorter (FACS) analysis was assessed as described: T CD4+ lymphocytes were gated on the basis of physical characteristic of cells and expression of CD4 in the area of lymphocytes to identify the TH and Treg cells. Cells were acquired on FACS CANTO analyzer (Becton-Dickinson). The proportions of the CD4+CD25^{high}FoxP3+ Treg, or CD4+IL-17A–F+IL-23+ Th17 cells, and the fraction of the CD4+ Treg and TCD4+ effector (Teff) cell subsets expressing IL-6Rδ (CD126) were analyzed with FlowJo software in the SF of patients with PsA.

Statistical analysis provided mean and SD. Further, 2-tailed Student's t test was used to assess differences; and Spearman rank test was used to assess correlations between distinct variables.

RESULTS

RPPA technique on total T cells from SF. The levels of STAT1 Y701, STAT3 Y705, JAK2 Y1008, PKCδ T505ERK1/2T202/Y204, and JAK1 Y1022/Y1023 phosphoproteins were significantly higher on total T cells in SF of patients with PsA compared with T cells from PB of HC. A significant positive correlation between levels of STAT3 Y705 and STAT1 Y701, STAT3 Y705 and PKCδ T505, STAT1 Y701 and JAK1 Y1022/Y1023, JAK1 Y1022/Y1023 and PKCδ T505, was observed on total T cells in SF of PsA.

Flow cytometry analysis on SF CD4+T cells. Expansion of either CD4+CD25^{high}FoxP3+ Treg (Figure 1 A-C), or CD4+IL-17A–F+IL-23+ Th17 cells in SF of patients with PsA compared to PB of HC (Figure 1 D-F), was observed. The percentages of CD4+CD25^{high}IL-6Rα+Treg cells, compared to those of CD4+CD25-IL-6Rα+ Teff cells, were significantly higher both in PB of HC and in the SF of patients with PsA ($p < 0.01$).

Significant correlations were found either between CD4+CD25-IL-6Rα and both STAT 3Y705 (Figure 2A) and JAK1 Y1022/Y1023 (Figure 2B), or between CD4+CD25^{high}FoxP3+ and both STAT 3Y705 (Figure 2C) and ERK1/2Y204 (Figure 2D), in the SF of patients with PsA.

DISCUSSION

By RPPA analysis, levels of JAK1/ STAT1/ STAT3/ and PKCδ phosphorylation on T cells in the SF of patients with PsA were significantly higher when compared to PB of HC; further, in SF of PsA, levels were highly correlated with each other[5,6], showing a typical pattern of IL-6 stimulation[7,8]. IL-6-induced activation and expansion of CD4+ Teff cells can also be supported by the relation of CD4+CD25-IL-6Rα+ fraction with STAT3Y705 and JAK1 Y1022/Y1023 on SF T cells. Results suggest a prominent role of IL-6, IL-21, and IL-23 in local differentiation and activation of CD4+ Teff cells, working in synergy with IL-1β[5,6]. In particular, these findings unravel the *in vivo* role of IL-6Rα signaling in driving Th17 cell lineage development, as demonstrated by the higher proportion of CD4+ IL-17A–F+ IL-23R+ Th17 cells in the joints of patients with PsA. In addition, JAK1/ STAT1 may participate in the polarization of Th17 cells, by inducing *in vivo* transition to Th17/Th1, IL-17A, and interferon (IFN)-γ+ double-producing cells[4]. Phenotypic analysis also revealed expansion of CD4+ CD25^{high} FOXP3+, supporting development of truly Treg cells, indicating the concomitant expansion of Treg and Th17 cells, as already reported in psoriasis and in arthritis, at the local site of inflammation[5,9].

Among cytokines already identified in PsA joints, either the common γ receptor chain (γC) cytokines, such as IL-2, IL-7, IL-15, and IL-21; or the heterodimeric IL-23 (p40, p19), belonging to the IL-12 family/IL-6 superfamily of cytokines, may act in synergy to induce the distinctive pattern of signaling-pathway activation. In T cells, the γC cytokine activation of STAT pathways is mainly dependent on JAK1[10,11,12]. On CD4+ T cells JAK1 was indeed found to represent a crucial pharmacological target of synovial inflammation[13,14]. Interestingly, IL-6 blockade also represents a promising approach in PsA[15].

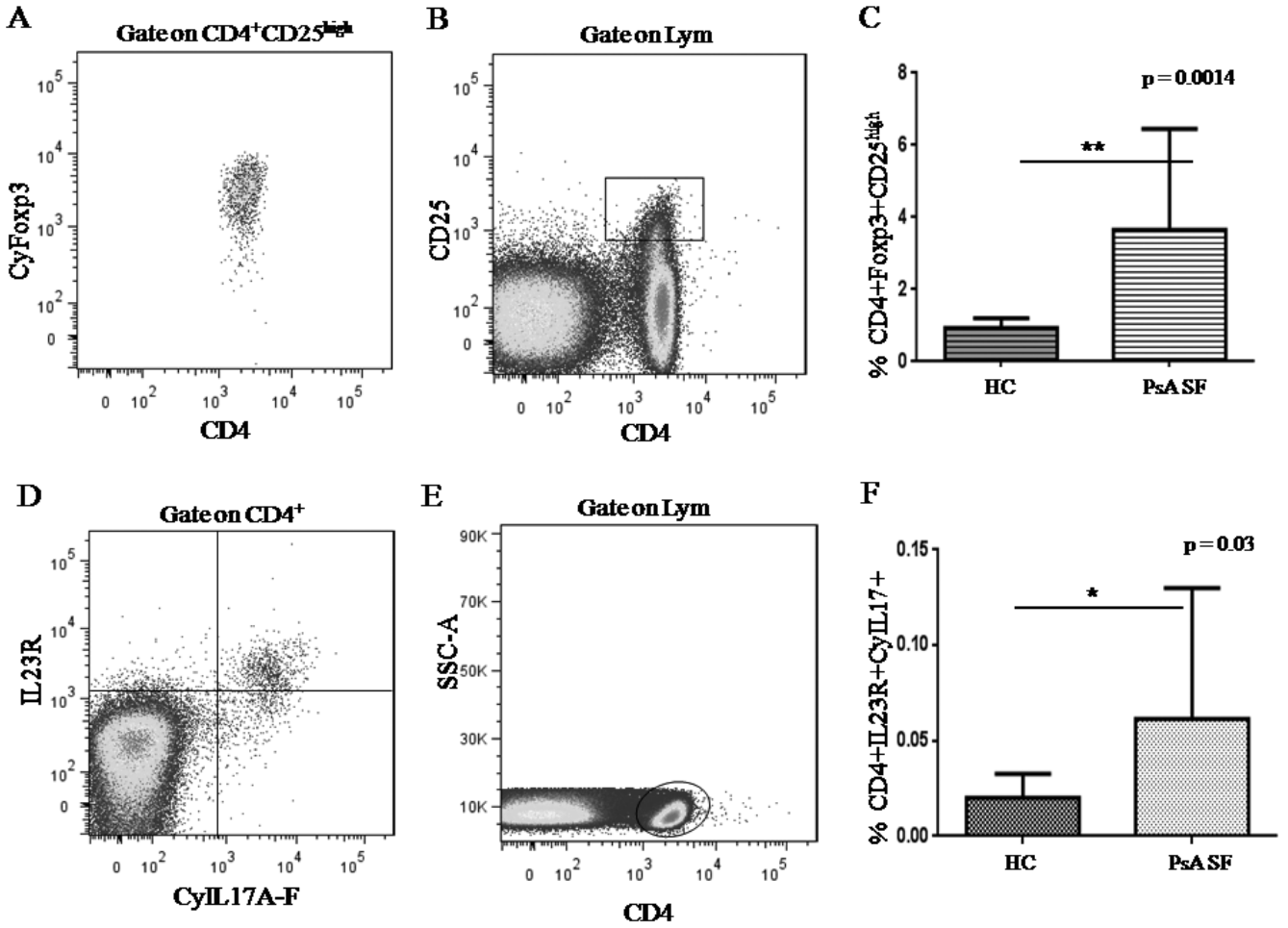

Figure 1. Representative dot plots showing the percentage of CD4+CD25^{high}Foxp3 cells (A) gated on CD4+CD25^{high} (B) in SF of patients with PsA. C. Box-and-whisker plot comparing results obtained from HC and PsA patients' peripheral blood. Representative dot plots show the percentage of CD4+IL23R+IL17A–F+ (D) cells gated on CD4+ (E) in SF of patients with PsA. F. Box-and-whisker plot comparing results obtained from HC and PsA patients' peripheral blood. In Figure 1C and 1F, data are represented as mean ± SD, and significance was calculated by Student t test (PsA SF, n = 16; HC peripheral blood, n = 7). Cy: intracytoplasmic; HC: healthy controls; IL: interleukin; PsA: psoriatic arthritis; R: receptor; SF: synovial fluid; SSC-A: side scatter intensity value.

The mechanisms of IL-10/ suppressor cytokine signaling 3 (SOCS3)-unrestrained STAT3 phosphorylation found in arthritis is not yet known. Low SOCS3 levels in CD4+ T cells have been found in rheumatoid synovitis[16].

Notably, the inhibition of SOCS3 expression by IL-1β signaling was recently shown to enhance and sustain JAK-STAT interaction and tyrosine-phosphorylation of STAT3 on CD4+ T cells, by a mechanism dependent on the transcription factor nuclear factor-κB[17]. Intriguingly, IL-1β/IL-1R is an essential pathway for the development of Treg cells with Th17 plasticity, the CD4+CD25^{high} FOXP3^{low}Treg cells that produce IL-17, in a STAT3-dependent manner[18].

Distinct inflammatory signals may have altered the epigenetic regulation of Foxp3 expression and Treg suppressive function[19]. The IL-6R/STAT3 and Toll-like receptor2/myeloid differentiation primary response 88 inflammatory pathways may cause the loss of functional suppression[8,9], the ablation of Foxp3 expression on Treg[19]. However, the *in vivo* role of STAT3 in Treg is still complex, suggesting the involvement of STAT3 in maintenance of Treg phenotype and function[20], by regulating the expression of genes contributing to the suppressor function of Treg cells, such as *Ccr6*, *Il1r1*, *Il6ra*, or *Prf1*[21]. The environmental expression of STAT3 or STAT1 on Treg, by cooperating with FOXP3, also regulates selective Treg suppressive functions in distinct local Th17 or Th1 polarizing conditions[3]. Serum amyloid A was indeed shown to induce the ERK-mediated proliferation and enhance suppressive capacity of Treg[22], in keeping with ERK pathway involvement in Treg development[23].

The *ex vivo* transcriptional network profile in T lymphocytes in the PsA joints reveals a complex interplay between IL-1, IL-6, and IL-23 in the control of Teff and Treg cell

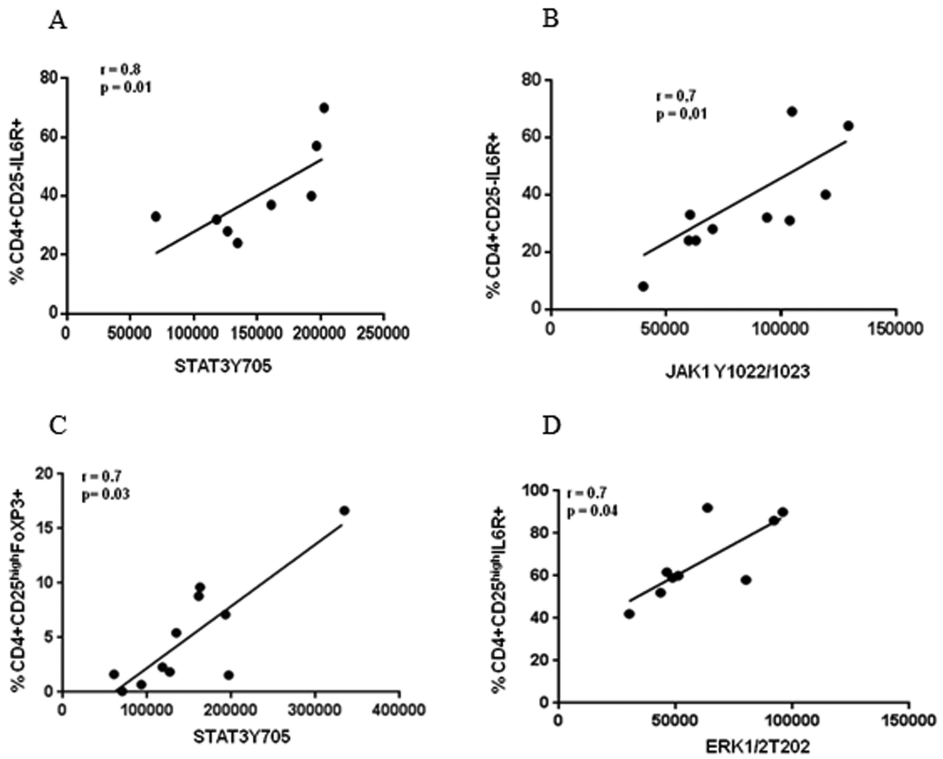

Figure 2. Correlation between either the frequency (%) of CD4+CD25–IL-6Rβ+ Teff cells and the levels of STAT3 Y705 (A), or JAK1 Y1022/Y1023 (B), or percentage of CD4+ $CD25^{high}$ IL6Rα+ Treg cells and the levels of STAT3 Y705 (C), and ERK1/2 Y204 (D), on total T cells in synovial fluid of patients with psoriatic arthritis. Significance was calculated by Spearman's rank test. ERK: extracellular regulated kinase; JAK: Janus kinase; IL: interleukin; PKCδ: protein kinase C delta; R: receptor; STAT: signal transducer and activator and transcription.

development, which may drive the transition from innate to adaptive immune response and sustained joint inflammation in PsA.

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