Active JAK/STAT Signaling in Circulating Leucocytes Defines Distinct Immunologic Endotypes of Rheumatoid Arthritis

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BACKGROUND

In rheumatoid arthritis (RA) stratification is considered an important step towards the development of patient-tailored therapeutic concepts. The fact that less than 50% of RA patients experience a substantial improvement in response to any single biologic therapy has brought up the idea that yet unidentified subtypes of RA (endotypes) might exist. This concept is in line with distinct microscopic patterns of synovitis found in biopsies of RA joints.[1] Furthermore, a subset of RA patients has leucocytes with interferon driven gene expression, whereas most RA patients do not [2]. Interferons activate receptor associated Janus kinases leading to phosphorylation of STAT1 and STAT2. Other STAT family members are activated by cytokines such as IL-6 (STAT3) or IL-15 (STAT5). Therefore, the phosphorylation pattern of the different STAT molecules in circulating leucocytes might mirror the specific cytokine milieu of a given patient.

OBJECTIVE

To define endotypes of RA based on the phosphorylation patterns of the different STAT molecules in circulating leucocytes.

MATERIAL & METHODS

Cross-sectional study of 63 patients with established RA fulfilling the 2010 EULAR/ACR criteria (mean age: 64.5 ± 1.7 (SEM) years, female ratio: 0.79, Tab. 1). Ten healthy subjects served as a control group. Flow cytometry was performed to detect the phosphorylated forms of STAT1-6 in Monocytes, Granulocytes, B cells, naïve , effector , and memory T cells of the $CD4^+$ and CD4- lineage. All steps from blood draw to cell fixation were performed at 4°C to prevent auto-activation of leucocytes (Fig. 1). The geometric mean fluorescence intensity (gmMFI) of fluorochrome labeled antibodies against phosphorylated STATs (pSTATs) in the different leucocyte populations was used for statistical analysis. MFIs were correlated with disease activity measured by the cDAI. gmMFIs of populations with elevated STAT phosphorylation not associated with disease activity were analyzed by unsupervised hierarchical clustering. The resulting groups were validated by principal component analysis. Finally, criteria for patient assignment to specific groups by gmMFI were generated by calculating ROC-curves.

Table 1 Patient characteristics

Patient characteristics	
sample size	63
Gender	
Female, n (%)	50 (79.4 %)
Age, years ± SD	64.5 ± 1.7
disease duration, years ± SD	11.5 ± 9.3
Clinical parameters, n (%)	
disease status	
seropositive	47 (75 %)
RF IgM	29 (64.4 %)
aCCP	31 (72.1 %)
disease activity, cDAI	
Remission	13 (20.6 %)
low	24 (38.1 %)
moderate	15 (23.8 %)
high	11 (17.5 %)
Treatment, n (%)	
Glucocorticoids	20 (31.8 %)
sDMARDS	47 (74.6 %)
csDMARDS	43 (68.2 %)
MTX	34 (54.0 %)
Leflunomide	6 (9.5%)
others	3 (4.8 %)
tsDMARDS	4 (6.4 %)
bDMARDS	35 (55.6%)
TNFα inhibitors	10 (16.0 %)
IL-6 inhibitors	7 (11.1 %)
RTX	4 (6.3 %)
others	14 (22.2 %)

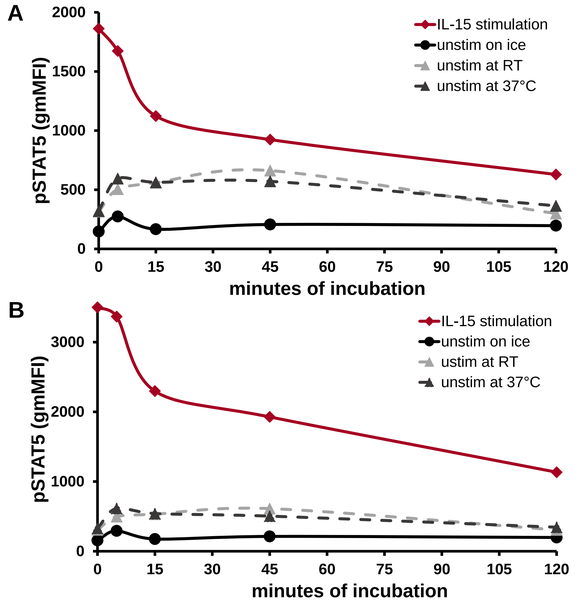


Figure 1 Validation of pSTAT signaling staining protocol. To determine the STAT phosphorylation stability under different temperatures, whole blood was drawn and kept on ice before analysis. 200 µl whole blood where stimulated with 20 ng IL-15 and incubated at 37°C for 10 minutes. Samples were washed three times with Phosphate buffered saline (PBS) to remove unbound cytokines and were incubated for five, 15, 45 minutes and two hours on ice, at room temperature (RT) and at 37°C respectively. After incubation the samples were fixed and treated as described in the estabished staining protocol. The samples were analysed using a FACSCanto platform (Becton Dickinson). Geometric means of the fluorescence intensities (gmMFI) were obtained using FlowJo software. gmMFIs over time were plotted for $CD4^-$ naïve- (**A**) and $CD4^+$ memory-T cells (**B**).

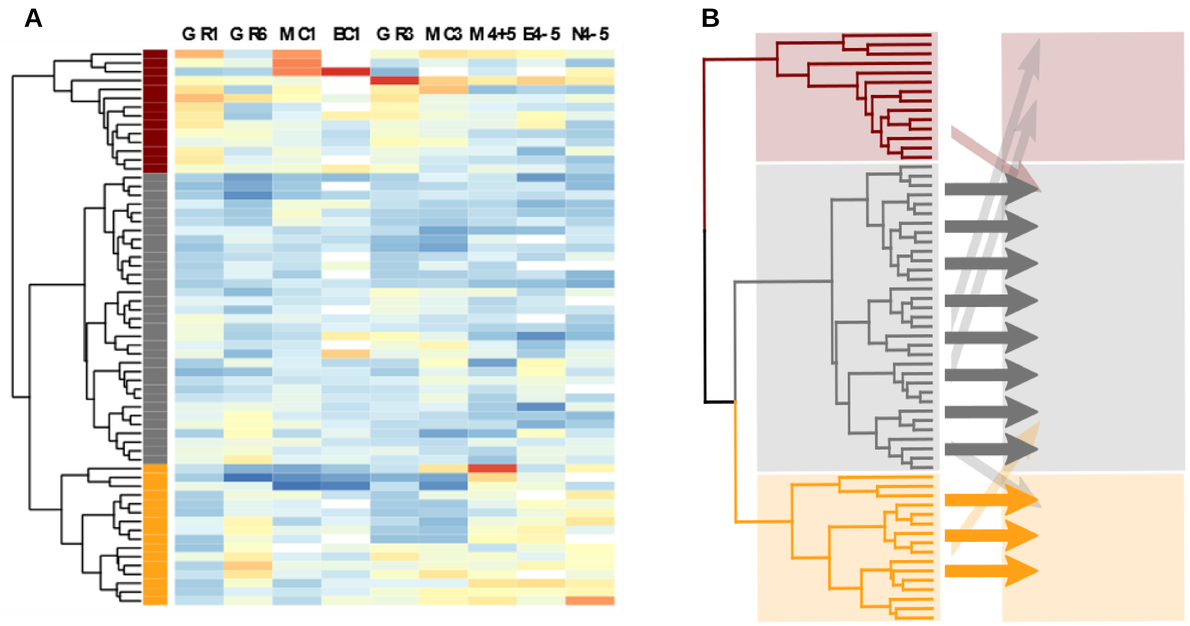
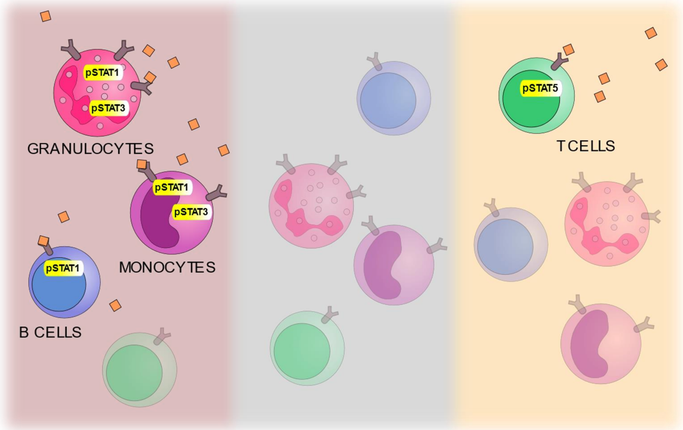


Figure 2 Cluster Analysis of pSTAT Signaling in RA patients. gmMFIs obtained from flow cytometric analysis were clustered by hierarchical clustering to detect different STAT phosphorylation patterns in RA patients. **A** Hierachical clustering of the scaled gmMFI was performed and visualized with a heatmap**.** Colours beside the cluster tree indicate the assignment of the patients to either one of the three clusters (red, yellow and grey) **B** Whole blood from 16 patients was retested after three to six months. 11 patients could be reasigned to their initial cluster using gmMFI cut-offs generated from the initial analysis. gmMFI values for phosphorylated STATs are plotted as followed: GC1: pSTAT1 in Granulocytes, GC6: pSTAT6 in Granulocytes, MC1: pSTAT1 in Monocytes, BC1: pSTAT1 in B cells, GC3: pSTAT3 in Granulocytes, MC3: pSTAT in Monocytes, M4+5: pSTAT5 $CD4^+$ memory T cells, E4-5: pSTAT5 in $CD4^-$ effector T cells, N4-5: pSTAT5 in $CD4^-$ naïve T cells.

RESULTS

Pronounced ex vivo phosphorylation of STAT1-6 in any leucocyte population was detected in 30 of 63 (48%) RA patients. Active STAT5 signaling in Monocytes, naïve $CD4^+$ T cells and $CD4^+$ effector T cells was significantly associated with disease activity. Unsupervised hierarchical cluster analysis of RA patients based on pSTAT gmMFIs not associated with disease activity resulted in 3 groups: 1) Patients with active STAT1 and STAT3 signal in Monocytes and Granulocytes (n=14/63, 22%), red) Patients with active STAT5 signal in naïve $CD4^-$ T cells, $CD4^-$ effector T cells and $CD4^+$ memory T cells (n=16/63, 25%, yellow) (Fig. 2A). Patients without active STAT signal in any leucocyte population (n=33/63, 52%, grey). cDAI, CRP, ESR, current treatment, RF and ACPA status did not differ significantly between the groups. To test if the assignment to a group changed over time, we performed a second analysis of STAT phosphorylation after 3-6 months. 69 percent of the patients tested (11/16) were re-assignment to their initial group (Fig. 2B).



We identified three distinct RA endotypes based on active STAT signals. Whether patients within different endotypes respond differently to a given therapy will be subject to further research.

Figure 3 pSTAT signaling in lymphocytes. Summary of the detected STAT phosphorylation patterns in lymphocytes, indicating activated and not activated lymphocyte subgroups. Backgroud colours resemble clusters derived from hierarchical cluster analysis (Fig. 2).

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