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Impaired IL-6-induced JAK-STAT signaling in CD4^{+} T cells associates with longer treatment duration in giant cell arteritis

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ABSTRACT

Introduction: The IL-12-IFNγ-Th1 and the IL-6-IL-23-Th17 axes are considered the dominant pathogenic pathways in Giant Cell Arteritis (GCA). Both pathways signal via activation of the downstream JAK/STAT proteins. We hypothesized that phosphorylated STAT (pSTAT) signatures in circulating immune cells may aid to stratify GCA-patients for personalized treatment.

Methods: To investigate pSTAT expression, PBMCs from treatment-naive GCA-patients (n = 18), infection controls (INF, n = 11) and age-matched healthy controls (HC, n = 15) were stimulated in vitro with IL-6, IL-2, IL-10, IFN-γ, M-CSF or GM-CSF, and stained with CD3, CD4, CD19, CD45RO, pSTAT1, pSTAT3, pSTAT5 antibodies, and analyzed by flow cytometry. Serum IL-6, sIL-6-receptor and gp130 were measured by Luminex. The change in percentages of pSTAT3+CD4+T-cells was evaluated at diagnosis and at 3 months and 1-year of follow-up. Kaplan-Meier analyses was used to asses prognostic accuracy.

Results: Analysis of IL-6 stimulated immune cell subsets revealed a significant decrease in percentages of pSTAT3+CD4+T-cells of GCA-patients and INF-controls compared to HCs. Following patient stratification according to high (median>1.5 pg/mL) and low (median<1.5 pg/mL) IL-6 levels, we observed a reduction in the pSTAT3 response in GCA-patients with high serum IL-6. Percentages of pSTAT3+CD4+T-cells in patients with high serum IL-6 levels at diagnosis normalized after glucocorticoid (GC) treatment. Importantly, we found that patients with low percentages of pSTAT3+CD4+T-cells at baseline require longer GC-treatment.

Conclusion: Overall, in GCA, the percentages of in vitro IL-6-induced pSTAT3+CD4+T-cells likely reflect prior in vivo exposure to high IL-6 and may serve as a prognostic marker for GC-treatment duration and may assist improving personalized treatment options in the future.

1. Introduction

Giant cell arteritis (GCA) is a granulomatous disease that affects large and medium vessels of persons older than 50 years [1,2]. GCA is a heterogeneous disease in terms of symptoms, immune pathology and response to treatment [3]. Early diagnosis and timely start of treatment are essential to avoid ischemic complications such as blindness and stroke [4]. At the time of GCA diagnosis, interleukin (IL)-6-dependent acute-phase markers such as ESR and CRP are mostly elevated [5]. Unfortunately, current serological markers used for GCA diagnosis are not disease-specific nor reliable predictors of a prolonged disease course [6]. Thus, there is still a significant unmet need to discover diagnostic and prognostic markers for patients with GCA [5,7].

Although its exact pathophysiology is not yet fully understood, GCA is regarded as a T cell-mediated disease with an important role for vascular dendritic cells and macrophages contributing to the local granulomatous inflammation. One of the major pro-inflammatory cytokine clusters in the immunopathology of GCA is the IL-6-IL-23-Th17 axis. IL-6 signaling may occur in two different forms; classical or *trans*-signaling. Classical- and *trans*-signaling by IL-6 is achieved via binding of IL-6 to the membrane bound IL-6R or to soluble IL-6R (sIL-6R), respectively. The first pathway activates the JAK-STAT signaling in

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IL-6R bearing cells whereas the IL-6-sIL-6R complex activates the membrane-bound β-subunit gp130 and Janus Kinase (JAK)/signal transducers and activators of the transcription (STAT) pathway in all gp130+ cells [8–10]. In addition to the IL-6-IL-23-Th17 axis, in temporal artery biopsies (TAB), the involvement of the interleukin (IL)-12-IFNγ-Th1 axis also signaling via the JAK/STAT pathway was also demonstrated. More specifically, the IL-12-IFNγ-Th1 axis leads to phosphorylation of STAT1 (pSTAT1), whereas the IL-6-IL-23-Th17 axis induces STAT3 phosphorylation (pSTAT3) [11]. Previously, Shen-Orr et al. reported an age-related decrease in cytokine induced JAK/STAT responsiveness, particularly in T cells. Authors linked the chronic inflammation and reduced cell responsiveness in the JAK-STAT pathway to cardiovascular aging [12]. Importantly, this study suggested a treatment approach to overcome cardiovascular dysfunctions by targeting chronic inflammation and thereby improving cellular responsivity in the JAK/STAT signaling [12].

Glucocorticoids (GC) are the primary treatment for GCA. However, their long-term high-dose usage can result in severe GC-related adverse effects. Therefore, selective targeting of the JAK-STAT-pathway may provide a GC-sparing treatment strategy for GCA [13,14]. Recently, in the Giant-Cell Arteritis Actemra (GiACTA) trial, tocilizumab, an IL-6R antagonist, was found to be beneficial for GCA patients as 56% of patients achieved sustained GC-free remission after 52 weeks, which was a significant improvement compared to the placebo groups (14–18%) [15].However, 44% of patients treated with tocilizumab did not reach GC-free remission, thereby emphasizing the heterogeneity among GCA patients [15]. Since multiple immune pathways can be involved in GCA, investigations into other targeted therapies are highly needed. A recently published open-label study with the JAK inhibitor baricitinib showed promise as a new GC-sparing therapy option for GCA patients [16,17].However, at the individual level, it cannot yet be predicted who will benefit from these different treatment options. Therefore, the identification of novel robust and reliable biomarkers for patient stratification is a requirement for the implementation of personalized treatment in GCA.

Considering the heterogeneity of GCA patients and the potential beneficial impact of therapies targeting the JAK/STAT signaling pathway, we evaluated the pSTAT3 as well as the pSTAT1 and pSTAT5 responsivity to various inflammatory cytokines in vitro as a direct intracellular marker of activation in T cells, B cells and monocytes. We hypothesized that impaired pSTAT responsiveness of $CD4^+$ T cells may help to identify GCA subsets with longer treatment duration and which are eligible for IL-6R or JAK/STAT inhibitor treatment.

2. Materials & methods

2.1. Patient sample collection and characteristics

Newly diagnosed GCA patients were, after receiving informed consent, prospectively followed according to a fixed study protocol. GCA patients were diagnosed based on a positive temporal artery biopsy (TAB) and/or a positive [^{18}F]-fluorodeoxyglucose-positron emission tomography-computed tomography (FDG-PET-CT) for LV-GCA. Both a cross-sectional and longitudinal analysis was performed on in vitro cytokine-induced phospho-STATs (pSTATs) in circulating immune cells. For the cross-sectional study, samples of GCA (n = 18) at baseline, and age-matched healthy controls (HC, n = 15) were included. Hospitalized patients, diagnosed with pneumonia or a urinary tract infection, were included as infection controls (INFs, n = 11). To assess group differences in terms of gender, we conducted Fischer's exact test, which revealed no statistically significant differences between HC-GCA ($p > 0.999$), HC-INF ($p = 0.111$) and GCA-INF ($p = 0.128$). For the longitudinal analyses of pSTATs, samples from 11 GCA patients were included at baseline (treatment-naïve), and at 3 months and 1 year of therapy. All GCA patients started treatment with glucocorticoids (GCs), according to the BSR guidelines [18]. In case of relapse, a higher daily GC dose was administered and either methotrexate (MTX), tocilizumab (TCZ) or leflunomide (LEF) was added to the treatment regimen. GCs were tapered if there were no clinical signs and symptoms of disease activity and when CRP and ESR levels had normalized. Basic laboratory measurements of CRP and ESR were collected at all time points.

All procedures complied with the declaration of Helsinki. The study was approved by the institutional review board of the University Medical Center Groningen (UMCG, files METc2012/375 for HC and METc2010/222 for GCA patients). Patient characteristics of both the cross-sectional and longitudinal studies can be found in Table 1.

2.2. Assessment of serological markers

Levels of serum IL-6 (lower limit of detection: 0.854 pg/mL), soluble IL-6 receptor (sIL-6R, lower limit of detection: 178 pg/mL) and soluble gp130 (lower limit of detection:371 pg/mL) at diagnosis were measured with Human premix Magnetic Luminex screening assay kits (R&D Systems, Abingdon, UK) according to the manufacturer's protocol. CRP levels were determined using the Cobas 8000 modular analyzer (Roche, Basel, Switzerland). ESR (Westergren method) was determined by the XN-9000 (Sysmex, Kobe, Japan).

2.3. Phosphoflow and fluorescent cell barcoding (FCB)

For the cross-sectional study, frozen PBMCs from GCA patients, matched HCs and INF controls were thawed and re-suspended to 5×10^6 cells/mL in RPMI1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% FCS and 50 mg/mL gentamycin (Gibco, Scotland, UK). The cell suspension was aliquoted into 9 separate polypropylene tubes (5×10^5 cells per tube), labeled with BUV 395-*anti*-CD19 (BD), and stimulated with either IL-6 (25 ng/mL, Peprotech, Rocky Hill, CT, USA), IFN-γ (50 ng/mL, Peprotech), IL-2 (50 ng/mL, R&D Systems), IL-10 (100 ng/mL, Peprotech), M-CSF (100 ng/mL, Peprotech) or GM-CSF (100 ng/mL, Peprotech). As a negative control, 2 cell samples remained without stimulation.

For the longitudinal study, frozen PBMCs from GCA patients sampled at three timepoints (baseline, 3 months, 1year) were thawed and re-suspended as described above. The cell suspension was aliquoted into separate polypropylene tubes (5×10^5 cells per tube), labeled with BUV 395-*anti*-CD25(BD), and stimulated either with IL-6 (25 ng/mL) or IL-2 (50 ng/mL). As a negative control, one cell sample from each time point remained without stimulation.

For both studies, samples were incubated for 30 min at 37 °C and 5% CO_2. After stimulation, cells were fixed with Cytofix (BD) for 10 min. Subsequently, cells were washed and permeabilized in perm buffer III (BD) containing a different concentration and/or combination of Pacific Blue (PB) and/or Pacific Orange (PO) dyes (Invitrogen, Carlsbad, CA, USA) to enable FCB of each original tube. After incubation, the different FCB samples were combined into one FACS-tube and stained with the following antibodies: AF647-*anti*-phospho-STAT1 (pSTAT1) (BD; 562070, clone; 4a), PE-*anti*-pSTAT3 (BD; 612569, clone; 4/P-STAT3), AF488-*anti*-pSTAT5 (BD; 562075, clone; 47/Stat5(pY694)), BUV737-*anti*-CD3 (BD; 612750, clone; UCHT1), Pe-CY7-*anti*-CD4 (BD; 348809, clone; SK3), and BV711-*anti*-CD45RO (BD; 563723, clone; UCHL1). After staining, cells were analyzed on the FACSymphony™ (Becton Dickinson) flow cytometer. A detailed graphical representation of the protocol can be found in Supplementary Fig. 1.

For investigating the IL-6R expression on T and B cells, we included HC (n = 3), GCA (n = 6), INF controls (n = 3) PBMC samples. Samples were stained with BB515-*anti*-IL-6R (BD, 564623, clone; M5) together with cell phenotyping markers and STATs.

Data were plotted using the Kaluza software package (Beckman Coulter). For FCB analysis, cells from different stimulation tubes were identified based on their FCB signature, gated separately, and analyzed as individual samples for the expression of pSTAT1, pSTAT3 and pSTAT5. Unstimulated samples were used for setting the linear gates to

Table 1
Characteristics of patients and control groups from both the cross-sectional and longitudinal studies.

	Cross-sectional study			Longitudinal study		
Study groups	HCs	GCA	INF	GCA baseline	GCA 3 months	GCA 1-year
N	15	18	11	11	11	11
Age, years, mean (SD)	67 (9.5)	68 (9.6)	78 (12.07)	68 (7.3)	–	–
Female, n (%)	10 (67)	11 (61)	3 (27)	10 (90)	–	–
GCA symptoms (cranial/systemic/combined), n	NA	9/7/2	NA	4/6/1	–	–
Fulfilled ACR 2022 criteria, n (%)	NA	16 (88.9)	NA	10 (90.9)	–	–
Medication use, (GC/MTX/LEF/TCZ), n	NA	NA	NA	NA	11/3/0/0	11/5/0/0
ESR (mm/hr.), median (IQR)	6 (3–8.75)	73.5 (49.5–99)	98 (72–109)	81 (56–95)	11.5 (9-23)	24 (14–41)
CRP (mg/L), median (IQR)	1.95 (0.55–5)	41 (23.75–90.5)	76 (61–109)	62 (26–104.5)	2.95 (1.93–4.05)	9.3 (3.3–10.75)
IL-6 (pg/mL), median (IQR)	0.05 (0.027–0.102)	1.59 (0.89–3.6)	11.62 (4-15)	2.02 (0.956–9.866)	NA	0.28 (0.16–2.24)

delineate positive and negative populations. For the gating strategy see Supplementary Fig. 2.

2.4. Statistical analysis

In the cross-sectional analyses, statistical differences between 2 groups, tested by Mann Whitney U, are shown only if the Kruskal Wallis test showed significant differences between the non-paired study groups (3 or more groups). In the longitudinal analysis, the follow-up samples were analyzed by the Wilcoxon Signed Rank test. P values < 0.05 were considered statistically significant. For correlation analyses, the Spearman test was used. Data were analyzed with GraphPad Prism 9.2.0.

3. Results

1. Differences in responsivity of peripheral blood immune cells to cytokine-induced JAK/STAT signaling detected by analysis of intracellular phosphorylated STAT proteins in GCA patients, healthy controls and infection controls

First, we investigated in vitro induced pSTAT expression in $CD4^+$ T cells (CD45RO+ & CD45RO-), $CD8^+$ T cells, B cells ($CD19^+$) and monocytes of 18 treatment-naive GCA patients at diagnosis, 11 INF controls and 15 age-matched HC (Table 1). Analysis of stimulation with different cytokines demonstrated that each cytokine preferentially phosphorylates STAT1, STAT3 or STAT5 (Fig. 1), which is in line with the literature and supports the validity of our phosphoflow and FCB methods [17,18]. For example, IL-2 stimulation mainly initiated pSTAT5 in T cells, GM-CSF stimulation induced pSTAT5 in monocytes, and IL-10 specifically activated STAT3 in all cell subsets. IL-6 mainly induced pSTAT3, although it appears that pSTAT1 and pSTAT1/pSTAT3 heterodimers were also induced. M-CSF stimulation in vitro did not phosphorylate any of the STATs in the investigated immune cell subsets.

In general, cytokine responsiveness in immune cells of both GCA-patients and HCs appeared to be higher compared to INF controls. The percentages of monocytes expressing pSTAT5 after GM-CSF, IFN-γ stimulation was higher in GCA patients and HCs compared to INF controls (Fig. 1). In addition, enhanced percentages of pSTAT1+ cells were observed in monocytes of GCA patients and HCs upon stimulation with IFN-γ when compared to those of INF controls (Fig. 1). Furthermore, B

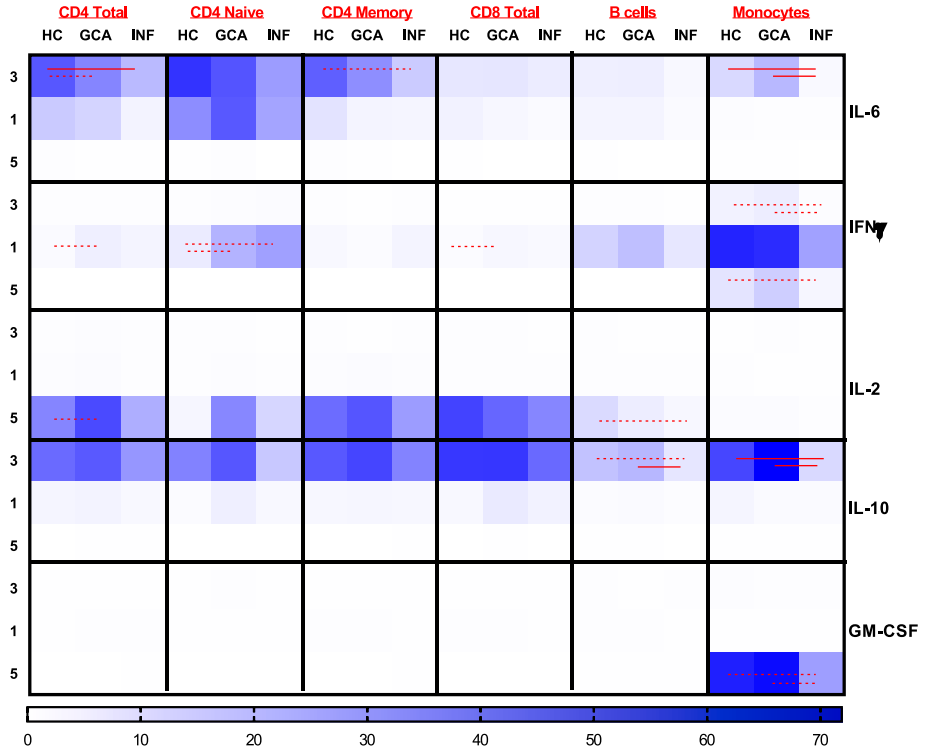

Fig. 1. Overall cytokine-induced Pstat signatures in treatment-naïve GCA patients, healthy controls (HC) and infection controls (INF). Heatmap representation of cytokine (IL-6, IFN-γ, IL2, IL-10, GM-CSF) stimulations and Pstat1/3/5 response in CD4Total, $CD4^+$CD45RO-(CD4 Naïve), $CD4^+$CD45RO+(CD4 Memory), CD8 Total, B cells. Lines were used for reporting significant difference between groups (red dashed line: $p \leq 0.05$), (red solid line: $p < 0.01$). For statistical analysis, Mann-Whitney *U* test was used. The scale bar represents the percentages of cytokine induced pSTATs in the indicated cell types.

cells from GCA patients exhibited increased pSTAT3 expression upon stimulation with IL-10 compared to B cells of IFN controls (Fig. 1).

We observed a few important differences in cytokine responsiveness between GCA patients and HCs (Fig. 1). The percentages of pSTAT1+ $CD4^+$ and $CD8^+$ T cells, in response to IFN-γ, were higher in GCA patients than in HCs. Previously, we showed that the percentage of pSTAT1+ cells after IFN-α stimulation was also significantly upregulated in $CD8^+$ T cells of GCA patients [19]. Interestingly, the percentage of IL-6-induced pSTAT3+ $CD4^+$ T cells of GCA patients was significantly lower in comparison to HCs. However, we did not detect differences in the mean fluorescence intensity (MFI) of IL-6-induced pSTAT3 expression in $CD4^+$ T cells among the different study groups (Supplementary Fig. 3A). These results prompted us to further focus on the effects of IL-6 signaling in GCA patients since IL-6 is a proven central cytokine in GCA pathogenesis and target of treatment in GCA (Fig. 1). The median percentages of immune cells positive for pSTATs, along with interquartile ranges, for all studied groups are provided in Supplementary Tables 1–5.

2. Low percentages of IL-6-induced pSTAT3+ CD4 T cells in treatment naïve GCA-patients is associated with high levels of serum IL-6

Considering the previously reported positive association between IL-6, CRP and ESR [5], we postulated that prolonged exposure to high levels of IL-6 in vivo may have an impact on the Pstat3 signature in GCA patients. To test this, we measured membrane bound IL-6 receptor expression on immune cells from HCs (n = 3), treatment-naïve GCA patients (n = 6) and INF controls (n = 3). We detected high IL-6R expression on $CD4^+$ T cells (Supplementary Figure Fig. 4A). Moreover, we found that pSTAT3 expression associates with IL-6R expression on $CD4^+$ T cells from GCA patients after in vitro IL-6 stimulation (Supplementary Figure Fig. 4B). Next, we measured IL-6, soluble IL-6 receptor (sIL-6R) and soluble gp130 levels, which are key players in both the classical and trans signaling pathway, in serum samples collected from baseline GCA patients (n = 16), HCs (n = 11) and INF controls (n = 13). Serum levels of soluble gp130 and sIL-6R were similar between study groups, whereas serum IL-6 levels in GCA patients, and INF controls, were elevated compared to HCs (Fig. 2A). We next correlated

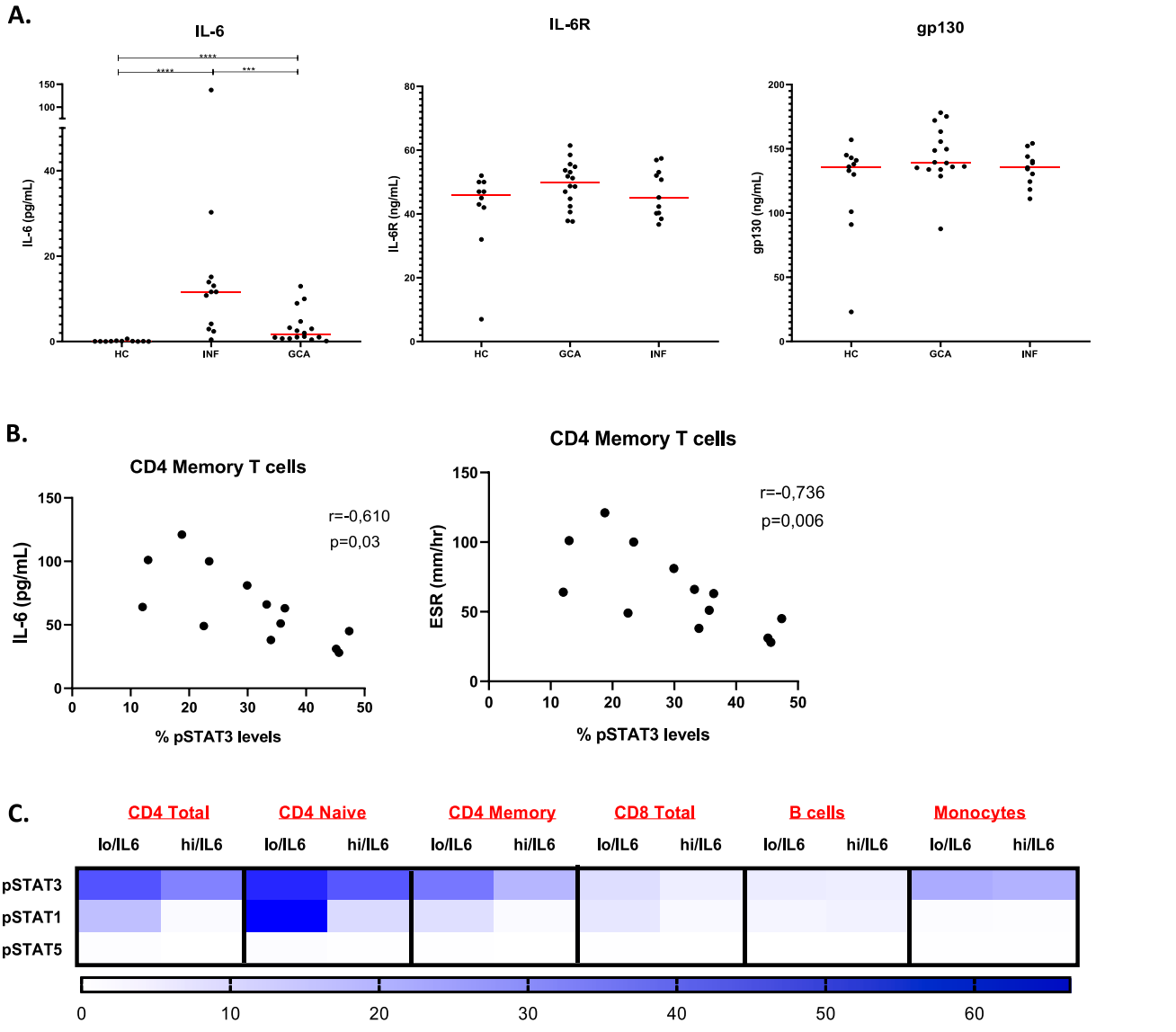

Fig. 2. High IL-6 serum levels at diagnosis associate with decreased percentages of IL-6 induced Pstat3+ CD4 T cells. A. Serum IL-6, IL-6R, gp130 levels of HC, INF and treatment naïve GCA patients **B.** Association of baseline serum CRP, IL-6 and ESR levels in GCA with Pstat3 percentages in $CD4^+$ memory T cells. **C.** Heatmap representation of the influence of serum IL-6 levels on Pstat signatures in GCA patients. Bars in heatmap figures represent percentages of Pstat1, 3 and 5 in the indicated cell types. For correlation analysis, the Spearman test was used.

inflammation markers and pSTAT3 expression for each cell subset. We found that percentages of IL-6-induced pSTAT3+ CD4 memory T cells were negatively associated with baseline ESR ($p = 0.006$, $r = -0.736$) and serum IL-6 levels ($p = 0.03$, $r = -0.610$) (Fig. 2B), whereas no significant correlation was observed with CRP, Sil-6R and gp130. Next, patients were stratified according to serum IL-6 levels (below median: ≤1.5 pg/Ml, above median>1.5 pg/Ml) and correlations with pSTAT expression were evaluated. Indeed, we observed a decrease of pSTAT3 percentages in $CD4^+$ T cells of GCA patients with high serum IL-6 levels (Fig. 2C). The median percentages of immune cells positive for pSTATs with interquartile ranges in GCA patients with low and high IL-6 serum levels IL-6 stimulation can be found in Supplementary Table 6. As we detected some higher MFI in a small subset of GCA patients (Supplementary Fig. 3A), we investigated if this was linked to lower serum IL-6 levels but no differences were found in MFI of IL-6 induced pSTAT3 in $CD4^+$ after stratification of GCA patients according to serum IL-6 levels (Supplementary Fig. 3B). We also looked more in detail at INF controls asking the question if serum IL-6 levels have an influence on pSTAT3 percentages. Although there was no significant difference between INF controls with low and high IL-6 levels, we demonstrated lower pSTAT3 percentages in $CD4^+$ T cells of INF controls with high IL-6 serum levels (Supplementary Fig. 5). Additionally, we checked the correlation between IL-6, CRP ESR and percentages of pSTAT3 in INF controls after IL-6 stimulation. The results show a clear negative correlation between CRP and percentage of pSTAT3+ $CD4^+$ T cells (both naïve and memory). (Supplementary Fig. 5).

3. In GCA patients with high serum IL-6, percentages of IL-6 stimulated pSTAT3+ CD4 memory T cells normalize after treatment.

Next, we investigated the IL-6 induced pSTAT3 response of naïve and memory CD4 T cells before and after the initiation of treatment. This analysis was performed in 11 GCA patients at baseline (treatment-naïve), and at 3 months and 1 year of therapy (Table 1). In $CD4^+$ T cells, sensitivity to IL-6 stimulation, as measured in percentages of pSTAT3+ cells, increased after 3 months and 1 year of treatment (Fig. 3). Our subgroup analysis demonstrated that $CD4^+$ T cells of GCA patients with high serum IL-6 levels at baseline had low percentages of pSTAT3, but the percentages increased after 3 months and normalized after 1 year of treatment (Fig. 3). GCA patients with low baseline IL-6 levels who presented with relatively high pSTAT3 percentages at baseline maintained similar pSTAT3 percentages during follow-up, while patients with high baseline IL-6 levels and low pSTAT3 percentages showed increased percentages (normalized levels) at 1-year of treatment.

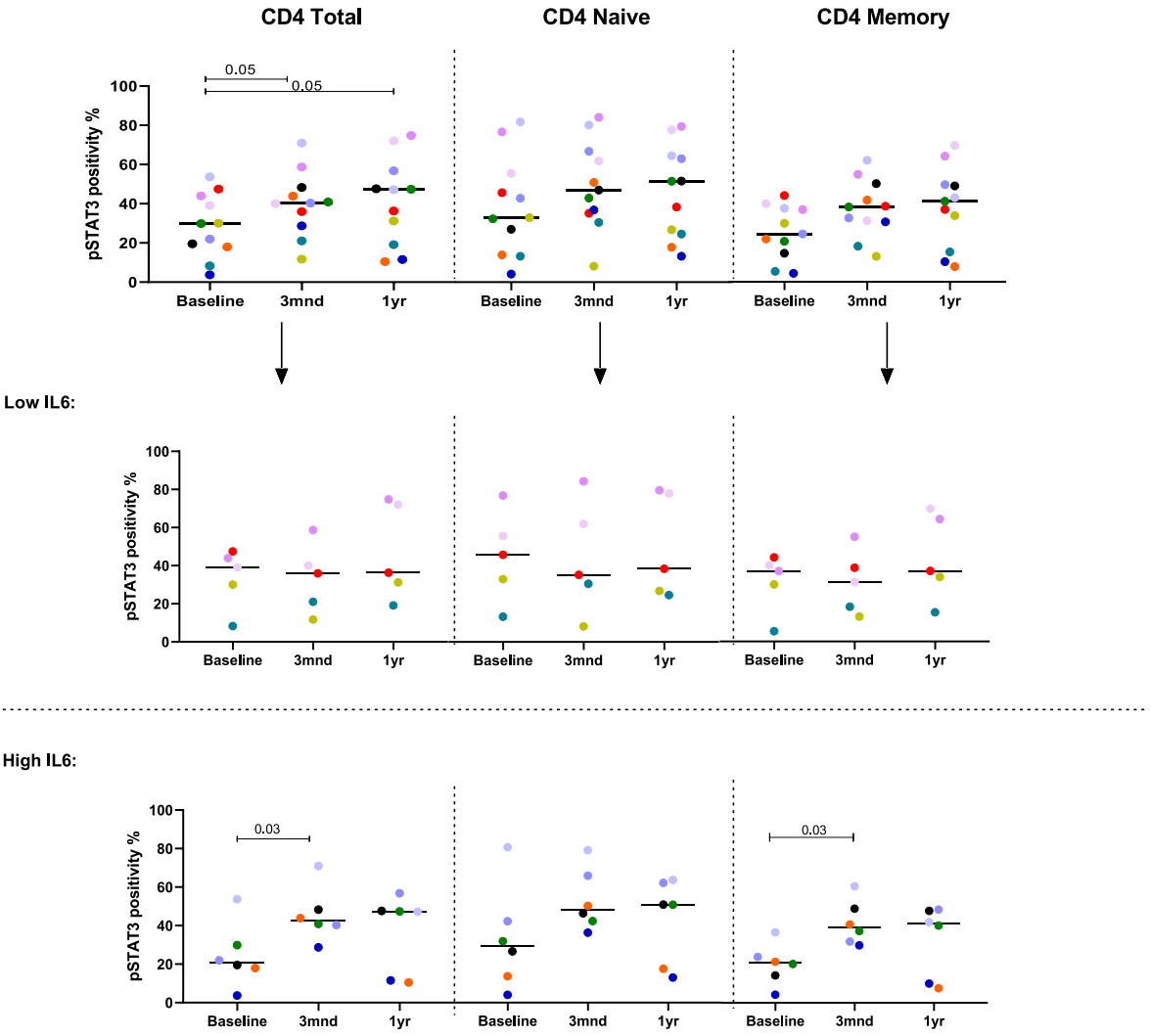

Fig. 3. Percentages of IL-6 induced pSTAT3+ CD4 (naïve and memory) T cells tend to differ in GCA patients with low and high levels of serum IL-6, but normalize after treatment. Data are shown at baseline and after 3 months and 1-year of follow-up. Wilcoxon tests were used for statistical analysis. Each color depicts one GCA patient with corresponding 3 months and 1 year follow-up.

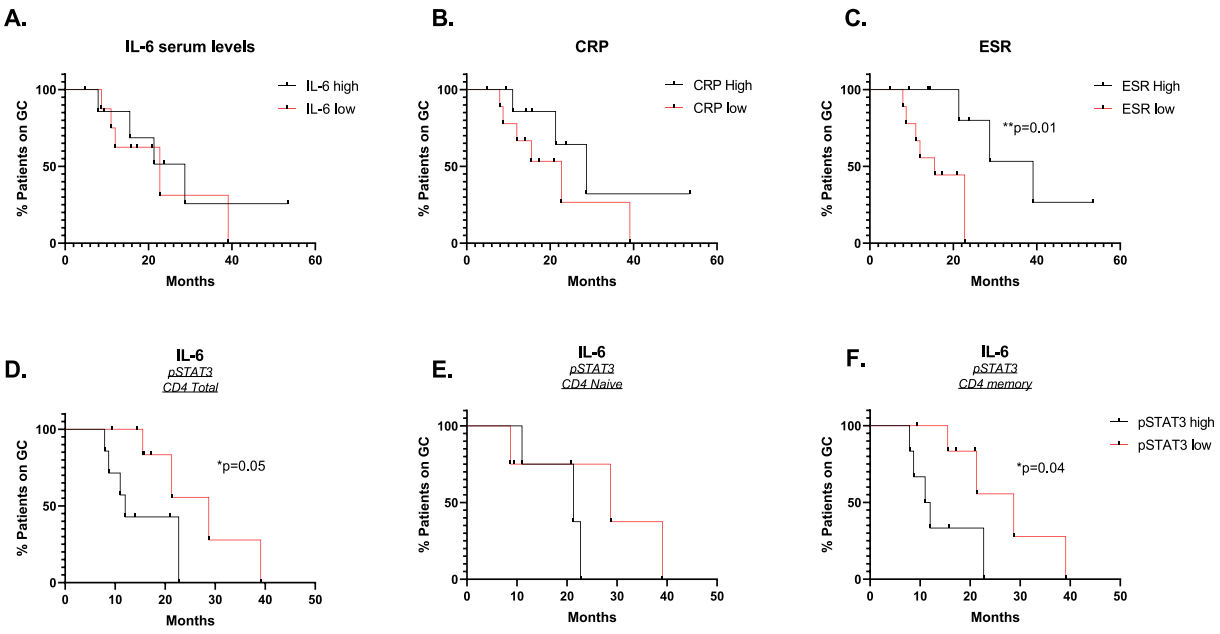

Fig. 4. Low IL-6-induced pSTAT3 responses in CD4$^+$ T cells associate with long-term GC-requirement in GCA patients. The log Rank test was used for analyzing the percentage of GCA with low and high IL-6, CRP, ESR, pSTAT3 at baseline and their duration of GC treatment.

4. Low percentages of pSTAT3 expressing CD4$^+$ T cells after IL-6 stimulation predict long-term GC-requirement in GCA-patients

The time to GC-free remission is an estimate for the severity of the disease course in GCA [5]. To investigate whether analysis of pSTAT3 expression in CD4$^+$ T cells of GCA patients can act as a useful prognostic marker for future treatment response, the time to GC-free remission during follow-up was determined. Next, we assessed the effect of low (below median) and high levels (above median) of baseline inflammation markers (CRP, ESR, IL-6) and CD4 T cell pSTAT3 expression on the disease course (Fig. 4). IL-6 and CRP failed to predict disease duration in GCA (Fig. 4A and B, respectively). On the other hand, high baseline levels of ESR ($p = 0.01$ Fig. 4C), low pSTAT3 expression in total CD4$^+$ T cells ($p = 0.05$ Fig. 4D) and CD4 memory ($p = 0.04$, Fig. 4F) cells predicted long-term GC-requirement. Thus, our results show that analysis of CD4 T cell pSTAT3 responses may help to identify GCA-patients with a longer disease duration.

4. Discussion

In this study, we assessed the responsiveness of various cytokine-induced JAK-STAT signaling in circulating immune cell subsets to explore the value of intracellular pSTATs in further defining GCA heterogeneity and to explore its use in patient stratification for suitable GC-sparing treatment options. As main finding of the study, our analysis of pSTAT signatures in GCA, HCs and INF controls demonstrated that IL-6-induced pSTAT3 responsivity was lower in CD4$^+$ T cells of GCA patients compared to HCs. The decreased pSTAT3 response in GCA patients CD4$^+$ T cells was associated with high serum IL-6 levels at the time of diagnosis. Additionally, we further substantiated that at baseline pSTAT3 responsivity was impaired in GCA-patients with high serum IL-6 levels as compared to patients with low serum IL-6. Moreover, impaired IL-6-induced pSTAT3 signaling in CD4$^+$ T-cells was subsequently found to be associated with an unfavorable long-term disease course. The data combined suggest that analysis of IL-6-induced pSTAT3 responsiveness of CD4$^+$ T cells may be used to identify patients who will likely experience a longer disease course and who will benefit most from tocilizumab treatment or treatment with inhibitors of the JAK/STAT pathway such as tofacitinib, baricitinib or upadacitinib.

We also demonstrated that, similar to GCA patients, INF controls exhibited decreased pSTAT3 responses to in vitro IL-6 stimulation. This can be explained by the high serum IL-6 levels in INF controls, as these were even higher than in GCA. Additionally, the immune cells of INF controls showed lower sensitivity to cytokine stimulation than the immune cells of GCA patients after IL-2, IL-10, IFN-α and GM-CSF stimulations, an observation which may be explained by the higher prior exposure of INF control cells to systemic inflammatory cytokines. Currently, blood markers for GCA diagnosis have low diagnostic accuracy [19,20]. Especially, distinguishing GCA patients from patients with infections is still difficult because in both patient groups acute-phase proteins (CRP, IL-6) are elevated. Therefore, more discriminative markers need to be identified. In rheumatoid arthritis (RA), the expression of STAT3-regulated genes in circulating CD4$^+$ T cells was shown to identify early arthritis [21]. Our results point to the possible utility of intracellular markers of the JAK-STAT pathway to distinguish GCA patients from individuals with acute infection and possibly also other inflammatory diseases.

In GCA, the IL-6-IL-23-Th17 axis has been linked to disease activity, and this may be reflected by the aberrant phosphorylation of STAT3 in CD4$^+$ T-cells of GCA patients. In this study, initially, we showed a high expression of cell surface IL-6R by CD4 T cells, which correlated with pSTAT3 expression in response to IL-6 stimulation in vitro. We observed no differences between study groups for both sgp130 and sIL-6R levels in serum, but we found elevated IL-6 levels in GCA and INF patients compared to HCs, in line with our previous observations [5]. As the IL-6 induced pSTAT3 expression percentages in both GCA and INF samples were lower compared to HCs, we hypothesized that chronic exposure to circulating IL-6 may influence the IL-6 induced pSTAT3 responsiveness in vitro. Indeed, we documented an impaired IL-6-induced pSTAT3 response in CD4$^+$ T cells of GCA patients with high IL-6 levels. It is unlikely that the reduced STAT3 phosphorylation in GCA patients is due to a defect in the STAT3 molecule, as pSTAT3 percentages after IL-10 stimulation were robustly increased in GCA patients, and no differences with HCs were seen. Therefore, it is more likely that impaired IL-6 induced pSTAT3 responses observed in our study are the result of chronic stimulation and exhaustion of important components of the JAK/STAT signaling pathway [22]. Moreover, in a similar study in RA by Isomaki et al., a decreased phosphorylation of STAT3 after IL-6

stimulation in $CD4^+$ T cells in RA patients with high plasma IL-6 levels and constitutive STAT3 phosphorylation were reported [23]. Overall, these data suggest that a direct cellular marker of activation such as pSTAT3 may be a marker of immune modulation in GCA and further analysis of the mechanisms of the IL-6 signaling pathway in $CD4^+$ T cells of GCA patients is required.

Although some studies have shown a positive association of CRP and ESR levels with relapsing disease in GCA patients [22], other studies failed to confirm the prognostic values of these markers [24]. Therefore, novel diagnostic and prognostic biomarkers are needed to better characterize GCA patients. Importantly, here we showed that high ESR at diagnosis in combination with low pSTAT3 expression in $CD4^+$ T cells may aid in predicting a non-favorable disease course in GCA patients. Consequently, we found that baseline ESR levels and pSTAT3 expressing $CD4^+$ T cells from GCA patients were strongly negatively correlated. The discrepancy between other studies and ours, regarding the association of CRP and ESR with relapsing disease prognosis, can be due to the lack of defined criteria for 'low' and 'high' levels of systemic inflammation markers. The definition and its utilization in classification of GCA patients clearly requires further validation in additional cohort studies.

In GCA patients with high serum IL-6 levels, percentages of pSTAT3+ CD4 T-cells increased (normalized) after treatment initiation. Patients with low serum IL-6 levels showed no change in the percentages of CD4+pSTAT3+, as these levels were not different from HCs. Our results may suggest that GCA patients with low pSTAT3 expression at the time of diagnosis may recover their pSTAT3 response after treatment, but they remain at higher risk for GC-related adverse events due to prolonged GC treatment. Therefore, those patients might be eligible candidates for starting tocilizumab or JAK/STAT inhibitor treatment at the time of diagnosis to avoid cumulative high dose GC use. During follow-up, three patients were treated with TCZ. Two of them had high pSTAT3 percentages at baseline. One patient needed long term low dose GC for 21 months, the other one achieved treatment-free remission after 12 months. The patient with low pSTAT3 at baseline needed GC treatment for 17 months. Whether tocilizumab or JAK inhibitors can prevent prolonged GC treatment in GCA patients and whether the pSTAT3 profile can predict the response to GC-sparing biologicals remains to be investigated.

This study has several notable strengths. The FCB technique for analyzing pSTAT profiles in different conditions in several immune cell subsets simultaneously allowed us to investigate the downstream signaling pathways of selected cytokines that are involved in GCA immunopathogenesis. Another strength is the fact that we included treatment-naïve patients who were prospectively followed up at fixed intervals, which allowed us to perform a prognostic analysis. One of the limitations is the small number of patients included in the cross-sectional study and longitudinal study, which may have impacted the power of our subgroup analysis. Thus, this study should be regarded as an exploratory investigation of a new approach in GCA, using a cellular biomarker reflecting prior in vivo cytokine stimulation.

In conclusion, our findings suggest that pSTAT3 responsivity in $CD4^+$ T cells of GCA-patients is impaired by prior exposure to IL-6 in vivo. Moreover, patients with low pSTAT3 percentages at baseline will require a prolonged treatment with GC. Whether these patients will benefit from treatment with tocilizumab remains to be investigated. Overall, this study provides evidence for the importance of the JAK/STAT pathway for patient stratification and may assist in improving personalized treatment options in the future.

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Author statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication.

Contributors: IE and WA conceived and designed the study. IE and WA acquired the data. IE, WA, PH, AB, EB, MS and YvS were involved in the data analysis and/or interpretation. IE and WA drafted the manuscript, and all authors revised it critically for important intellectual content. All authors gave final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. IE accepts full responsibility for the finished work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

The authors have declared nothing to disclose.

Declaration of competing interest

The other authors have declared nothing to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2024.103215.

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