Autoimmunity Reviews 18 (2019) 144–154

Contents lists available at ScienceDirect

Autoimmunity Reviews

journal homepage: www.elsevier.com/locate/autrev

STAT signaling as a marker of SLE disease severity and implications for clinical therapy

Aleš Goropevšek[a], Marija Holcar[b,c], Artur Pahor[d], Tadej Avčin[b,e,*]

[a] Department of Laboratory Diagnostics, University Medical Centre Maribor, Maribor, Slovenia
[b] Department of Allergology, Rheumatology and Clinical Immunology, University Children's Hospital, University Medical Center Ljubljana, Ljubljana SI-1525, Slovenia
[c] Unit for Special Laboratory Diagnostics, University Children's Hospital, University Medical Center Ljubljana, Ljubljana, Slovenia
[d] Department of Rheumatology, University Medical Centre Maribor and Faculty of Medicine, University of Maribor, Maribor, Slovenia
[e] Department of Pediatrics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

A B S T R A C T

The Janus kinase/signal transduction and activator of transcription (JAK–STAT) signaling pathway is implicated in the pathogenesis of autoimmune diseases, including systemic lupus erythematosus (SLE). While small-molecule JAK inhibitors (Jakinibs) are currently under investigation for SLE, results of recent studies suggest, that the efficacy of drugs such as methotrexate (MTX) may also be due to their ability to suppress phosphorylation of STAT proteins. A previously identified STAT5 phosphorylation (pSTAT5) and STAT1 protein expression »signature« in circulating CD4+ T cells of patients with SLE was associated with perturbed homeostasis between conventional (Tcon) and activated regulatory (aTreg) subset and with time-adjusted cumulative disease activity during follow-up.

Initial observations in SLE patient cohort were validated with additional markers of disease severity and patients were stratified according to medication status. Preliminary results show that lower CD4+ T-cell counts in patients with SLE are associated with higher pSTAT5 levels and Tcon homeostatic proliferation, which was previously found to drive lymphopenia associated autoimmunity. Relapsing disease was better predicted by pSTAT5 levels than CD4 counts. Further, significant correlation was found between mean pSTAT5 levels during follow- up and the markers of disease severity. As patients with SLE, also patients with rheumatoid arthritis (RA) not receiving methotrexate, had significantly higher increase in CD4+ T-cell pSTAT5 levels compared to patients not receiving this specific therapy. However, the difference in pSTAT5 between Tcon and aTreg was independent of treatment with MTX and significantly increased only in patients with SLE.

CD4 depletion, driving homeostatic proliferation of Tcon subset, is therefore associated with higher pSTAT5 levels, which confer worse prognosis in patients with SLE. While treatment with MTX may decrease overall pSTAT5 levels in CD4+ T-cells also from patients with RA, increased pSTAT5 levels in Tcon relative to aTreg subset are specific for SLE.

1. Introduction

Janus kinases (JAK) and signal transducer and activator of transcription (STAT) proteins are major components in interferon (IFN)-dependent gene expression and are responsible for signal transduction of over 50 cytokines, hormones and growth factors regulating key cellular processes such as survival, proliferation, and differentiation [1,2].

Aberrant JAK/STAT signaling was linked to the inflammatory conditions and autoimmune diseases, such as systemic lupus erythematosus (SLE) [3].

Genome-wide association studies (GWASs), enabled by next-generation sequencing allowed for linking of single nucleotide polymorphisms (SNPs) in affected populations with autoimmune disease phenotypes [4–7]. A SNP haplotype in the *STAT4* gene was first associated with susceptibility to both systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [8]. Recent integration of genome-wide epigenomics data with data from GWASs on SLE suggests that control of CD4+ T cell homeostasis in which STATs play a major role, may be an important component of the genetic contribution to SLE disease susceptibility [9–12]. Perturbed homeostasis between pathogenic and regulatory subsets of CD4+ T-cells was described in patients with SLE [13–15].

As demonstrated by recent studies, characterization of STAT signaling responses in relevant T cell subsets is likely to reveal new aspects of their homeostasis and immunopathology of SLE [16–18]. Results of studies utilizing phospho-specific flow cytometry in patients with SLE show that STAT signaling analyzes performed at the level of total T cells may fail to capture SLE- associated alterations specific to a given T cell subset [19,20]. Important involvement of T-cell subset specific STAT signaling in SLE disease severity was first suggested when robust changes in cytokine signal transduction, dependent on disease progression, were described in murine model of SLE [21].

The success of small-molecule JAK inhibitors (Jakinibs) in the

* Corresponding author at: Department of Allergology, Rheumatology and Clinical Immunology University Children's Hospital, University Medical Center Ljubljana Bohoričeva 20, SI-1525 Ljubljana, Slovenia.
E-mail address: tadej.avcin@kclj.si (T. Avčin).

https://doi.org/10.1016/j.autrev.2018.08.010
Received 8 August 2018; Accepted 13 August 2018
Available online 18 December 2018

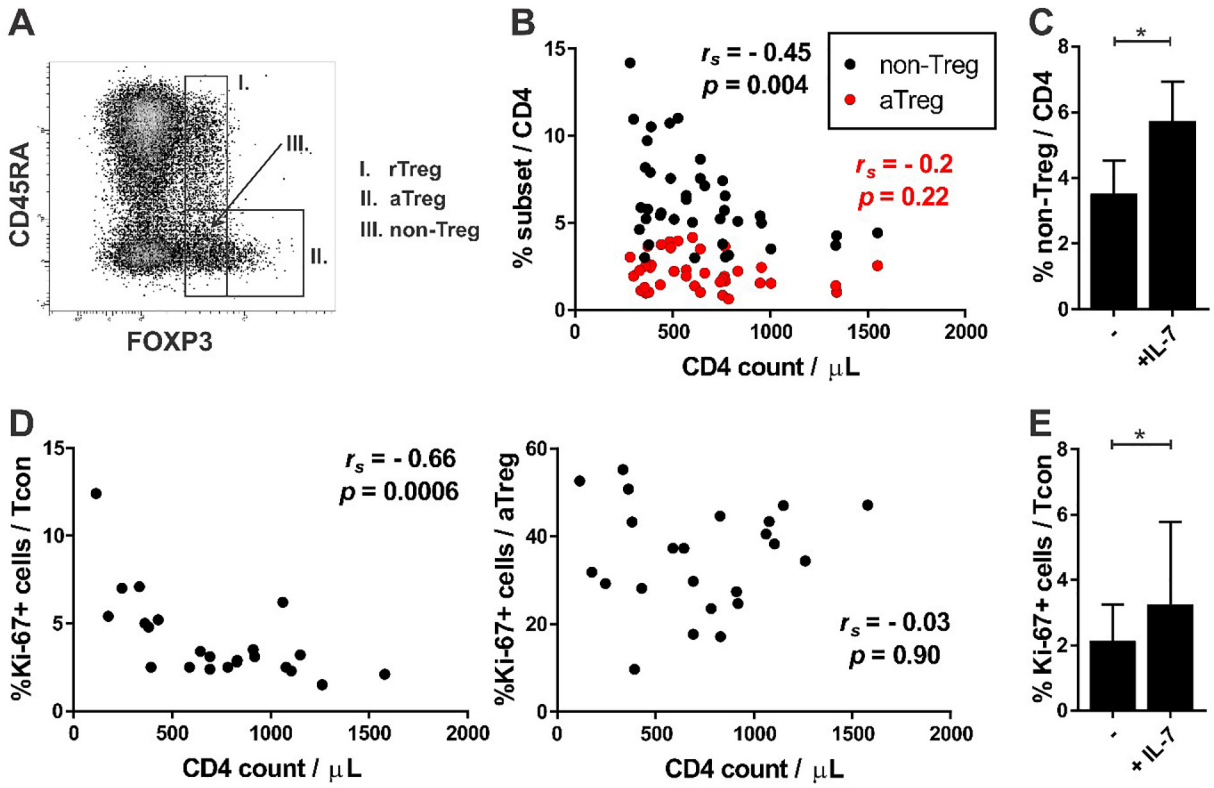

Fig. 1. Lover CD4 T-cell counts in blood samples from patients with SLE are associated with increased proliferation – Ki-67 expression in Tcon, but not aTreg subset. **(A)** FOXP3+ cells among gated CD4+ T cells were subdivided into 3 fractions based on CD45RA and the level of FOXP3 expression: I. CD45RA+ FOXP3lo resting Treg (rTreg), II. CD45RA– FOXP3hi acitvated Treg (aTreg) and III. CD45RA– FOXP3lo non-Treg subset as shown on representative dot plot from patient with SLE. **(B)** Correlation between CD4 T-cell counts (cells/μL) and percentage of non-Treg and aTreg among CD4+ T-cells from patients with SLE (n = 39). **(C)** Purified CD4+ T-cells from healthy donors (n = 6) were cultured for 3 days in the presence or absence of IL-7 (0.1 ng/mL). Bar graph shows percentage of the non-Treg subset among CD4+ T-cells for treated (IL-7) and untreated (−) samples. **(D)** Correlation between CD4 T-cell counts (cells/μL) and percentage of Ki-67+ cells among FOXP3– Tcon (left) and CD45RA– FOXP3hi aTreg (right) from patients with SLE (n = 23). **(E)** Purified CD4+ T-cells from healthy donors (n = 6) were cultured for 3 days in the presence or absence of IL-7 (0.1.ng/mL). Bar graph shows percentage of Ki-67+ cells among Tcon for treated (IL-7) and untreated (−) samples. rs, Spearman correlation coefficient; *p < .05.

treatment of RA demonstrates that intracellular signaling pathways can be targeted therapeutically to treat autoimmunity. Different Jakinibs are in preclinical development or in various phases of clinical trials in patients with SLE [22].

Treatment of MRL/*lpr* mice with tyrphostin AG490, a selective JAK2 inhibitor, significantly inhibited the phosphorylation of STAT1, improved renal function, reduced proteinuria and suppressed histological lesions of the kidneys [23]. CEP-33779 – another JAK2 inhibitor, prevented the development of nephritis in NZB/ W F1 mice [24]. In addition to slowing down the course of experimental lupus nephritis, pleiotropic beneficial effects of pharmacologic inhibition of the JAK/STAT pathway in murine lupus were shown with oral use of tofacitinib, a drug that preferentially inhibits JAK1 and JAK3 [25,26].

In addition, results of recent studies suggest, that the efficacy of drugs such as methotrexate (MTX) may also be due to their ability to suppress JAK/STAT signaling [27,28].

On the other hand, treatment with homeostatic cytokines, such as low-dose IL-2 is being considered as a therapeutic approach in patients with SLE [29–31].

A previously identified basal activation – phosphorylation of STAT5 (pSTAT5) in circulating CD4+ T cells of patients with SLE was dependent on another homeostatic cytokine IL-7. Such STAT5 signaling signature was associated with perturbed homeostasis between subsets of CD4+ T-cells and with time-adjusted cumulative disease activity during follow-up [32].

The initial observations in SLE patient cohort were further validated by the phospho-flow protocols to analyze the STAT5 signaling in T-cells in peripheral blood of patients with SLE and the pSTAT5 signatures were compared with those from patients with RA. In particular, a better understanding of CD4+ T-cell depletion was assessed as a pathological event potentially driving such signaling changes and homeostatic proliferation of CD4+ T-cell subsets. STAT5 signaling was monitored during follow-up of patients, which were stratified according to the medication status and pSTAT5 levels after stimulation with recombinant cytokines were used to examine responsiveness to kinase inhibitor.

2. CD4 counts and homeostasis of CD4+ T-cell subsets

Systemic lupus erythematosus (SLE) is a prototype systemic autoimmune disorder with a broad clinical spectrum, unpredictable course and many life threatening manifestations. Peripheral blood mononuclear cells (PBMCs) from patients with SLE carry gene expression signature of exposure to interferons [33,34]. Moreover, SLE patients with hematologic involvement, including lymphopenia, are more likely to show the IFN signature [33]. Administration of IFN- alpha in mice results in lymphopenia [35], which is in patients with SLE associated with several clinical and immunologic manifestations [36].

As shown first in spontaneous animal models of autoimmunity, lymphopenia with associated compensatory homeostatic proliferation enables differentiation of autoreactive T cells into memory/effector cells [37]. It was also demonstrated that such homeostatic proliferation in response to IL-7 can release autoreactive CD4+ cells from inhibitory networks, eventually leading to autoimmune disease [38].

Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion [39]. Also in patients with SLE significant negative correlation between CD4 counts and increased IL-7 levels was recently found [32].

The excess IL-7, which was shown to support proliferation of autoreactive T cells and progression of autoimmunity also in lupus-predisposed mice [40], contributes to the expansion and activation of autoreactive FOXP3- conventional T cells (Tcon) via a direct effect on cells that express the IL-7R complex at moderate to high levels [41]. Proliferation of FOXP3 + regulatory CD4 + T cells (Treg), characterized by lower CD127 (IL-7Ralpha) expression [42], in response to lymphopenia is on the other hand not controlled by IL-7 levels [43].

As only a minority of subjects develop autoimmune disease also during lymphopenia, other factors, such as Treg depletion [44,45], may contribute to the development of autoimmune pathology under lymphopenic conditions. FOXP3 + Treg cells, critical in maintenance of peripheral self-tolerance [46], have shown impaired in vivo proliferative potential in some human autoimmune diseases [47].

The absence of Tregs in knock-out or antibody depletion mouse models leads to systemic autoimmunity with characteristics similar to systemic lupus erythematosus (SLE), including glomerulonephritis and the development of DNA-specific antibodies [48]. In recent studies even increased frequencies of FOXP3 + cells, which however lack the suppressive function (CD45RA- FOXP3lo non-Treg cells) were described in patients with SLE [49]. In addition, high FOXP3 (FoxP3hi) expressing, CD45RA- activated Treg (aTreg) subpopulation, which is most suppressive in vitro and highly proliferative in vivo, was shown to be decreased in SLE patients [49].

When subsets of FOXP3 + CD4 + T-cells defined recently by Miyara et. al [49] were analyzed (Fig. 1A), a significant negative correlation between CD4 counts and percentage of non- Treg, but not aTreg subset from patients with SLE was found (Fig. 1B).

As significantly higher levels of IL-7 were also found in patients with SLE [32], influence of in vitro treatment with recombinant human IL-7 on percentages of different CD4 + FOXP3 + T cell subsets from healthy donors was examined. Increased percentage of non-Treg among CD4 + T-cells was seen after IL-7 treatment (Fig. 1C).

CD4 + T cell reconstitution following lymphopenia in SLE may be characterized by enhanced peripheral proliferation of Tcon subsets but not aTreg cells, which could also explain relative increase in non-Treg subset in patients with lover CD4 counts. To understand the relationship between CD4 T-cell depletion and homeostatic proliferation of Tcon and Treg, cell proliferation was assessed in each subset by quantifying the expression of Ki-67, which is a critical protein for cell division and is expressed exclusively by proliferating/cycling cells [50]. In Fig. 1D, we show correlations between the percentage of Ki67 + Tcon and Ki67 + aTreg cells and CD4 absolute cell counts, which was statistically significant only for Tcon population. These results suggest that cells in Tcon compartments are more actively cycling when CD4 numbers are diminished in patients with SLE.

When influence of in vitro IL-7 treatment on proliferation – Ki67 expression of different CD4 + T cell subsets from healthy donors was examined, increased proliferation in FOXP3- Tcon was seen after IL-7 treatment (Fig. 1E). This suggested that higher levels of IL-7, associated with (CD4) lymphopenia, could be responsible for both the relative increase in non-Treg subset and increased SLE Tcon homeostatic proliferation found in patients with lower CD4 counts.

Of note, IFN-alpha was also recently shown to preferentially promote activation of conventional – effector T-cells while inhibiting activated Treg expansion and function [51]. IFNs and homeostatic cytokines, such as IL-7 act on T-cells, through their intracellular signaling pathways that lead from surface receptors, through STAT proteins, which upon being activated-phosphorylated at specific tyrosine residues, translocate to the nucleus and control numerous gene programs critical for the T-cell function [52,53]. While homeostatic cytokines that use the common gamma chain signal mainly through STAT5 [54], STAT1 plays a role in the anti- proliferative effects delivered by type 1 or type 2 IFNs [55].

An IL-7 dependent STAT5 phosphorylation (pSTAT5) 'signature' in circulating CD4 + T cells from patients with SLE was identified, which was significantly higher in Tcon than aTreg subset. In that study, STAT5-regulated Bcl-2 protein expression and proliferation, assessed by Ki-67 expression, were positively correlated with pSTAT5 and significantly increased in Tcon, but not aTreg subset [32].

In another study increased STAT1 protein expression (median fluorescence intensity – MFI) was found in circulating CD4 + T cells from patients with SLE, which was positively correlated with disease activity. The highest STAT1 expression was found in aTreg subset, which demonstrated the highest STAT1 phosphorylation responses among SLE CD4 T cells and significant decrease in proliferation marker Ki-67 expression after IFN-alpha stimulation. While percentage of Ki-67 + aTregs was significantly decreased in patients with SLE and was negatively correlated with CD4 T cell STAT1 expression, mean of aTreg counts in follow-up samples from patients were negatively correlated with mean follow-up STAT1 protein levels [56]. Results of these two studies clearly indicated involvement of augmented STAT1 signaling in perturbed aTreg homeostasis and a role of imbalanced STAT5 phosphorylation in conferring survival and proliferative advantage to Tcon over aTreg. STAT5 signaling changes could represent a possible marker of SLE disease severity as cumulative CD4 T-cell pSTAT5 levels during follow-up were positively correlated with time-adjusted cumulative disease activity [32].

3. Homeostatic STAT5 signaling as a marker of SLE disease severity

When homeostatic STAT5 signaling was analyzed in whole blood samples from patients with SLE, significant negative correlation was found between lower CD4 counts and higher CD4 + T-cell pSTAT5 levels (Fig. 2A), which could both confer worse prognosis in patients. It was therefore investigated whether patients with higher pSTAT5 levels in CD4 + T-cells or patients with lower CD4 counts would experience a more aggressive disease course using an approach described recently [57,58]. The SLE cohort, composed of 38 patients with no moderate/severe disease activity at enrolment (no BILAG score A or B in any system) was followed up to 1000 days after their CD4 count and pSTAT5 levels in blood CD4 + T cells were determined. During follow up an episode was defined as a discrete disease flare if it met all 3 of the following prospectively-defined criteria: **1.** new BILAG score A or B in any system, **2.** clinical impression of active disease by the reviewing physician and **3.** increase in immunosuppressive therapy as a result.

Receiver operator characteristic (ROC) curves were generated to test the ability of CD4 + T cell pSTAT5 and CD4 counts to discriminate between those with and without relapse by day +1000. The area under the curve (AUC) for pSTAT5 MFI in CD4 + T cells was 0.91 (Fig. 2B), and 0.78 for the CD4 count. A cut-off point of 404.5 pSTAT5 MFI in CD4 + T cells demonstrated a test sensitivity of 82% and specificity of 90% with a likelihood ratio of 8.65.

With the use of the selected cut-off point of pSTAT5 MFI 404.5, the cumulative incidence of flares was estimated for patients with pSTAT5 MFI > 404.5 and pSTAT5 MFI < 404.5 in CD4 + T-cells. When Kaplan Meier log-rank test was used to compare tendency to disease relapse between the 2 groups of patients a clear correlation between SLE group defined by higher pSTAT5 MFI and relapsing disease was found. 69.7 % SLE patients with pSTAT5 MFI higher than 404.5 had flared by 500 days after enrolment, compared to 8.7 % of patients with lower pSTAT5 MFI in their CD4 + T-cells (Fig. 2C).

No significant association with CD4 count (Fig. 2D), or other clinical/laboratory parameters was seen (Table 1), though patients with pre-existing disease in higher pSTAT5 MFI group had more immunosuppression before enrolment. In addition, mycophenolate mofetil and rituximab were used/required as induction therapy only in this

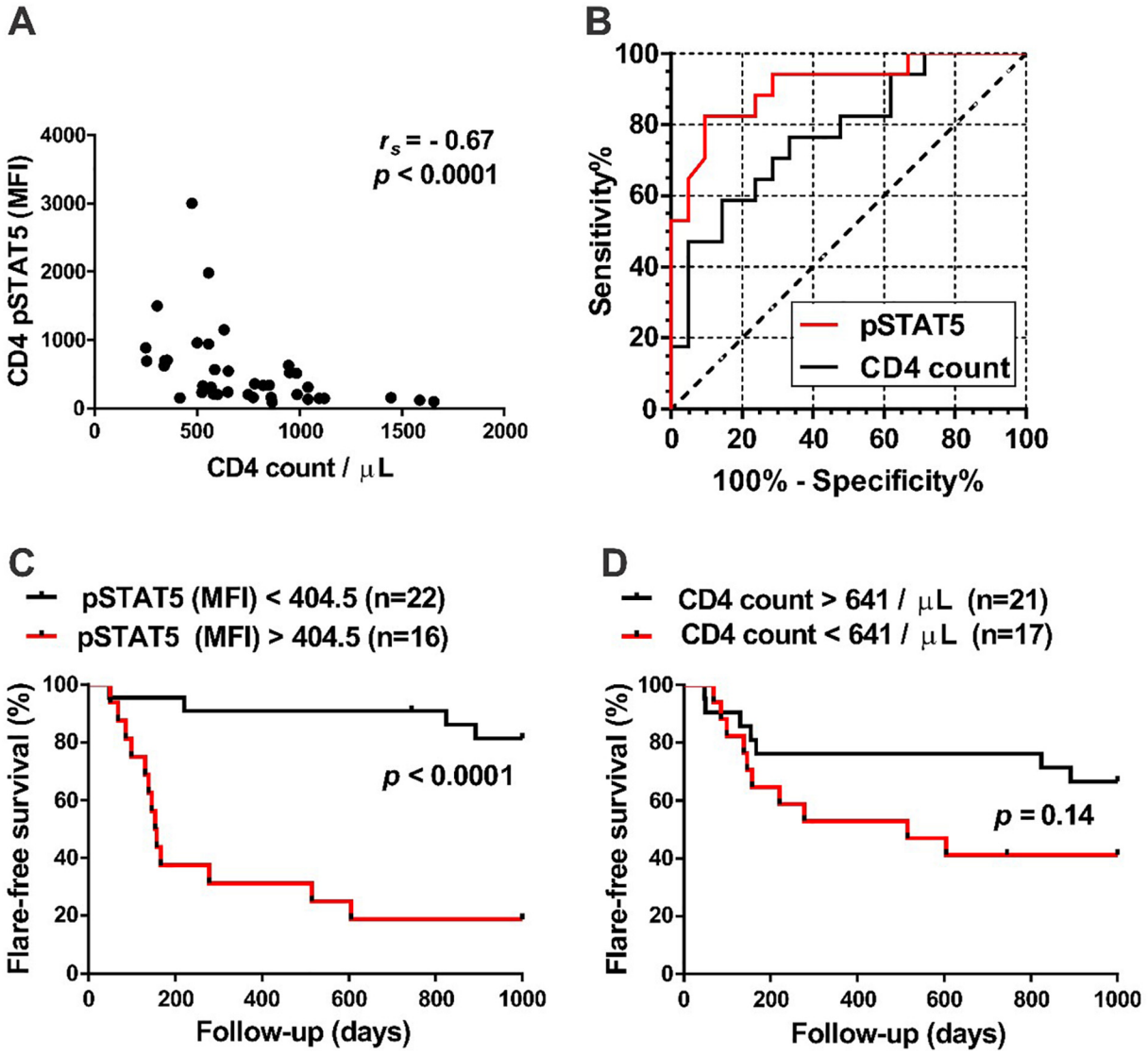

Fig. 2. Patients with SLE in subgroups defined based on the levels of STAT5 phosphorylation (pSTAT5) in CD4+ T-cells have significantly different disease courses (A) Correlation between CD4 T-cell counts (cells/μL) and pSTAT5 (median fluorescence intensity MFI) in CD4+ T-cells from SLE patients (n = 38). **(B)** ROC curves depict the sensitivity and specificity of pSTAT5 MFI in CD4+ T cells (AUC 0.91) and CD4 T-cell counts / μL (AUC 0.78) as a test to identify those at risk for disease flare during follow-up. **(C)** Survival curve (Kaplan-Meier plot) showing shorter time to first flare in the group 1 of SLE patients with higher CD4+ T-cell pSTAT5 MFI (> 404.5). **(D)** Survival curve (Kaplan–Meier plot) in subgroups of SLE patients defined by different CD4 T-cell counts at the start of follow- up. rs, Spearman correlation coefficient;

group (Table 2). This would be expected in a group with an increased tendency to disease relapse, and suggests that it is not a relative lack of prior therapy that has resulted in the increased flare frequency in the high pSTAT5 MFI group.

Collectively, our findings indicated that, while CD4 depletion is a critical factor driving homeostatic proliferation of Tcon cells, associated STAT5 activation may predispose individual patients to more aggressive disease.

4. Effects of drug therapy on STAT5 signaling and CD4 counts

Effects of specific therapy on the phosphorylation status of STAT5 and CD4 counts were examined in patients with SLE. As a first screen, patients were grouped according to whether they were receiving a particular treatment or not, regardless of whether a patient was on monotherapy or not. All patients, except one in the clinical trial were on some form of drug therapy (refer to Table 2). The effects of the systemic steroids (mehylprednisolone or prednisolone), antimalarial drugs (hydroxychloroquine), cyclophosphamide (CYC), methotrexate (MTX), mycophenolate mofetil (MMF) and azathioprine (AZA), were assessed. As significantly higher levels of pSTAT5 were found only in CD4+ T-cell subset of lymphocytes from patients with SLE [32], effects of specific therapy on increase in CD4+ T-cell pSTAT5 relative to other lymphocytes (Δ CD4 pSTAT5) were examined in patients with SLE. Significantly lower Δ CD4 pSTAT5 MFI values were observed only in the samples of patients on MTX (Fig. 3A), compared to those patients with SLE not taking this specific therapy. Significantly lower CD4+ T-cell counts, but not pSTAT5 levels, were on the other hand observed in samples from patients on CYC therapy, compared to those patients not taking this specific cytotoxic therapy (Fig. 3B).

Phosphoramide mustard is believed to be the active alkylating metabolite of CYC, which forms DNA crosslinks both between and within DNA strands [59]. DNA damage induced foci of phosphorylated histone pH2AX (γ-H2AX) [60], which occurs at sites of DNA double-strand breaks (DSBs), were therefore examined immediately ex vivo in T lymphocytes of peripheral blood from patients with SLE treated with

Table 1
SLE patient cohort demographics, clinical and laboratory data.

	Group1 [a]	Group2 [a]	p	adj p
Cohort size	16	22	na	na
Age (years)	38.2 (2.0)	39.9 (2.7)	0.74	ns
Gender	16F / 0 M	19F / 3 M	0.12	ns
Ethnicity	16 Slovene	23 Slovene	na	na
Disease duration (months)	54 (11)	116 (29)	0.07	ns
Age at diagnosis (years)	33.8 (1.7)	32.4 (2.5)	0.78	ns
No. ACR criteria	5.6	5.3	0.31	ns
Serum Cr t0 (μmol/L)	70 (4)	83 (8)	0.12	ns
Serum Cr final (μmol/L)	77 (5)	87 (7)	0.19	ns
CRP t0 (mg/L)	8 (2)	5 (1)	0.30	ns
ESR t0 (mm/h)	39 (4)	30 (3)	0.08	ns
WBC t0 (x109/L)	5.9 (0.5)	6.4 (0.5)	0.61	ns
Neutrophils t0 (x109/L)	3.9 (0.5)	3.8 (0.4)	0.80	ns
Lymphocytes t0 (x109/L)	1.40 (0.19)	1.83 (0.12)	0.02	ns
CD4% t0 (%)	44.7 (2.4)	47.9 (2.2)	0.36	ns
ANA status	16/16	21/22	0.39	ns
Anti-dsDNA	12/16	12/23	0.20	ns
Anti-ENA	9/16	6/22	0.07	ns
Low C3/4	9/16	7/22	0.13	ns
aPL pos	8/16	5/22	0.08	ns
Renal involvement	10/16	15/22	0.72	ns
Pre-existing disease	15/16	22/22	0.23	ns
Current therapy t0	15/16	22/22	0.23	ns
Prednisolone dose t0 (mg/day)	9.7 (2.3)	6.9 (1.0)	0.54	ns
SLEDAI t0	2.5 (0.5)	1.7 (0.3)	0.14	ns

p value refers to the comparison of Group1 (CD4 pSTAT5 MFI > 404.5) versus Group2 (CD4 pSTAT5 MFI ≤404.5), adj p = Bonferroni adjusted *p*-value, ns = $p > .05$, na = not applicable, t0 = at time zero enrollment, M = male, F = female, No. ACR criteria = mean number of ACR (American College of Rheumatology) classification criteria met, Serum Cr = serum creatinine, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, WBC = white blood cell count, CD4% = percentage of CD4+ T-cells among lymphocytes, Anti-dsDNA = Anti-double-stranded DNA antibodies, aPL pos = Anti-phospholipid antibody positive, ENA = extractable nuclear antigen, ANA = antinuclear antibody, Low C3/4 = hypocomplementemia at any timepoint during follow-up, Renal involvement = biopsy-proven renal SLE involvement, Pre-existing disease = diagnosis of SLE made prior to enrollment, Current therapy t0 = any therapy being taken at time of enrollment, Prednisolone dose t0 = (equivalent) prednisolone dose being given at time of enrollment bleed, SLEDAI t0 = SLEDAI-2 K disease activity index at time of enrollment.

[a] Mean (Standard error of mean) or the number of patients / whole Group.

CYC. When imaging flow cytometry was used to analyze STAT5 signaling in T lymphocytes showing nuclear γ-gH2AX foci, they were in contrast to SLE T-cells in general not characterized by STAT5 phosphorylation and nuclear localization (Fig. 3C).

In chronic HCV infection, γ-H2AX+ T lymphocytes were shown to be highly differentiated with shortened telomeres and failed to activate JAK/STAT pathways in response to IFN-α, IL- 2 or IL-6 [61]. Lower pSTAT5 nuclear localization, found in SLE T-cells showing nuclear γ-gH2AX foci is consistent with their failure to respond to homeostatic cytokines by phosphorylation of STAT5, which may also be a part of a broader defect in senescent T lymphocytes in response to exogenous cytokine signals. As DNA damage induced by treatment with alkylating agents, such as cyclophosphamide is also accompanied by inhibition of telomerase and, as a consequence, a shortening of telomere lengths [62], defining the differentiated-senescent T-cell subset affected by the failure to respond to homeostatic cytokines could be important.

Imaging flow cytometry was therefore used to analyze STAT5 signaling in CD4+ T-cell subsets from patients with SLE treated with CYC. In Treg cells, characterized by nuclear FOXP3 expression, higher levels of STAT5 phosphorylation and nuclear localization were seen after stimulation with recombinant human IL-2 compared to Tcon (Fig. 3D).

Therefore, although comparably lower levels of basal homeostatic STAT5 signaling were seen in SLE γ-H2AX+ T-cells, Treg subset was in patients treated with CYC not affected by the failure to respond to homeostatic cytokine IL-2.

It was shown that patients rendered lymphopenic by cyclophosphamide-based chemotherapy regimens for cancer do not experience selective depletion of Tregs, but demonstrate a relative increase in the frequency of Tregs compared to normal individuals, resulting from efficient homeostatic peripheral expansion of Treg compartment, which appears to out-compete the non- regulatory CD4+ pool [63]. Effector – activated regulatory T cells (aTregs) recover rapidly also after allogenic bone marrow transplantation and aldehyde dehydrogenase expression was shown to drive human regulatory T cell resistance to post-transplantation cyclophosphamide [64].

In patients with multiple sclerosis treated with the lymphocyte-depleting monoclonal antibody alemtuzumab T-cell recovery was driven by homeostatic proliferation, leading to the generation of highly proliferative (Ki67+), oligoclonal, memory-like CD4 T cells [65]. However increased aTregs were reported also after alemtuzumab therapy [65].

In our patients with SLE, lower CD4 counts in peripheral blood were associated with Tcon homeostatic proliferation, which was however not accompanied by increased frequencies of aTregs among FOXP3+ CD4+ T-cells (Fig. 1B,D).

Together with findings from the aforementioned studies this data suggest that homeostatic proliferation may drive lymphopenia-associated autoimmunity also in humans. However Treg/Tcon homeostasis may be affected differently in lymphopenia associated with different autoimmune diseases / treatments.

5. Imbalanced STAT5 signaling during follow-up of patients with SLE

When we compared homeostatic STAT5 signaling between subsets of CD4+ T-cells, levels of basal STAT5 phosphorylation and pSTAT5 nuclear localization were higher in Tcon than in FOXP3+CD4+ T-cells (Fig. 4A).

T cell STAT5 signaling status was examined also in samples from patients with SLE collected at various times during the 3 years of follow up to investigate whether imbalanced activation of STAT5, potentially driving homeostatic proliferation of pathogenic Tcon, but not Treg cells, was maintained over time. We found that STAT5 signaling signature was surprisingly preserved, as pSTAT5 levels remained at higher level in Tcon compared to FOXP3+ subset of CD4+ T- cells during entire follow up (Fig. 4B).

Inhibition of cytokine induced STAT activation was shown for glucocorticoids before [66]. Although majority of our patients with SLE were treated with corticosteroids also during follow-up (Table 2), the daily dose of methyl (prednisolone) did not seem to influence the constitutive activation of STAT5 in patients. Different modes of additional immunosuppressive therapy, including intravenous pulse CYC treatment, were not associated with a change in basal pSTAT5, which was maintained at high levels in patients CD4+ T cells for years (Fig. 4B).

Disease severity in patients with SLE was assessed by the mean annual steroid dose [67], which was for each patient calculated by determining the total prednisone (or equivalent) dose, then dividing by the time period (in years). Significant positive correlation was found between mean levels of Δ CD4 pSTAT5 in samples obtained at various times from the same patient and his mean annual steroid dose, calculated after 3 years of follow up (Fig. 4C).

It was shown in a recent study that STAT5 may also have a role in pathogenesis of IL-2-induced glucocorticoid resistance [68]. However, Δ CD4 pSTAT5 was not significantly correlated to the daily steroid dose in treated patients (Fig. 4D) and mean pSTAT5 levels during follow-up were associated with adjusted mean SLEDAI, which is also used as SLE disease severity index [32,67].

Table 2
SLE patient cohort therapy data.

		Group1 *a*	%	Group2 [a]	%	p	adj p
Prior therapy	Cyclophosphamide	8/16	50	8/22	36	0.40	ns
	Rituximab	1/16	6	0/22	0	0.23	ns
	Mycophenolate Mofetil	2/16	13	0/22	0	0.09	ns
	Azathioprine	7/16	44	12/22	54	0.51	ns
	Methotrexate	3/16	19	2/22	9	0.73	ns
	(Hydroxy) chloroquine	9/16	56	6/22	27	0.07	ns
	Cyclosporine	2/16	13	2/22	9	0.74	ns
	(Methyl) prednisolone	15/16	94	22/22	100	0.23	ns
Current therapy t0	(Methyl) prednisolone	14/16	88	18/22	82	0.64	ns
	Rituximab	1/16	6	0/22	0	0.23	ns
	Mycophenolate Mofetil	3/16	19	0/22	0	0.03	ns
	Azathioprine	7/16	44	5/22	23	0.31	ns
	Methotrexate	0/16	0	3/22	14	0.12	ns
	Cyclophosphamide	2/16	13	1/22	5	0.78	ns
	(Hydroxy)chloroquine	9/16	56	7/22	32	0.13	ns
Induction therapy	Cyclophosphamide	5/16	31	2/22	9	0.08	ns
	Rituximab	1/16	6	0/22	0	0.23	ns
	Mycophenolate Mofetil	3/16	19	0/22	0	0.03	ns
	Azathioprine	5/16	31	8/22	36	0.74	ns
	Methotrexate	0/16	0	3/22	14	0.12	ns
	(Hydroxy)chloroquine	7/16	44	7/22	32	0.45	ns
	Cyclosporine	1/16	6	2/22	9	0.75	ns
	(Methyl) prednisolone	15/16	94	22/22	100	0.23	ns
Follow-up therapy	Cyclophosphamide	2/16	13	1/22	5	0.37	ns
	Rituximab	1/16	6	1/22	5	0.82	ns
	Mycophenolate Mofetil	3/16	19	2/22	9	0.38	ns
	Azathioprine	9/16	56	7/22	32	0.13	ns
	Methotrexate	1/16	6	3/22	14	0.46	ns
	(Hydroxy)chloroquine	9/16	56	18/22	82	0.09	ns
	Cyclosporine	1/16	6	1/22	5	0.82	ns
	(Methyl) prednisolone	16/16	100	19/22	86	0.12	ns

[a] Number of patients / whole Group and % = percentage, p value refers for the comparison of Group1 (CD4 pSTAT5 MFI > 404.5) versus Group2 (CD4 pSTAT5 MFI ≤404.5), adj p. = Bonferroni adjusted p-value, ns = $p > .05$, t0 = at time zero enrollment, Prior therapy = therapy administered prior to enrollment, Current therapy = therapy being given at time of enrollment, Induction therapy = therapy given before enrollment to control previous disease flare, Follow-up therapy = therapy given at any timepoint during subsequent follow-up.

6. IL-7 dependent differences in levels of STAT5 activation between subsets of CD4+ T-cells from patients with SLE

As significant differences in Δ CD4 pSTAT5 MFI values were observed only in the samples of patients on MTX (Fig. 3A), compared to those patients with SLE not taking this specific therapy, the effects of MTX therapy on the phosphorylation status of STAT5 were examined also in patients with RA. Significantly lower Δ CD4 pSTAT5 levels were found also in the samples of patients with RA on MTX (Fig. 5A), compared to those patients with RA not taking this specific therapy.

Whether CD4+ T-cell activation of STAT5 was disease specific was examined by comparing the basal phosphorylation profiles of whole blood samples from patients with SLE and RA. Δ CD4 pSTAT5 values were significantly higher in patients with SLE only when compared with patients with RA on MTX, but not compared to those patients with RA not taking this specific therapy (Fig. 5A).

However, when pSTAT5 levels in CD4+ T-cell subsets were analyzed, the differences in pSTAT5 levels between Tcon and aTreg subset (Δ Tcon/aTreg pSTAT5) were significantly higher in patients with SLE when compared with patients with RA, both on MTX, and not taking this specific therapy (Fig. 5B).

ROC curves were generated to test the ability of STAT1 protein levels (MFI) in CD4+ T-cells, Δ CD4 pSTAT5 and the Δ Tcon/aTreg pSTAT5 to discriminate between patients with SLE and disease controls – patients with RA. The AUC for Δ pSTAT5 in CD4+ T cells was 0.69, for the STAT1 protein levels in CD4+ T cells 0.58 and for the Δ Tcon/aTreg pSTAT5 0.82 (Fig. 5C). Therefore, the differences in levels of STAT5 activation between Tcon and aTreg subset may be more specific for SLE than increased overall STAT1 and pSTAT5 levels in CD4+ T-cells. Such differences in pSTAT5 levels between CD4+ T-cell subsets were dependent on homeostatic cytokine IL-7, as incubation of whole blood samples from patients with SLE with neutralizing anti-IL7, but not anti-IL-2 antibodies resulted in significant reduction of Δ Tcon/aTreg pSTAT5 values (Fig. 5D).

7. STAT5 phosphorylation responses and kinase inhibitors

As significantly lover Δ CD4 pSTAT5 MFI, but not Δ Tcon/aTreg pSTAT5 values (Fig. 5A,B) were observed in the samples of patients on MTX, which is, as shown by recent studies, a JAK kinase inhibitor [27,28], in vitro effects of another kinase inhibitor Imatinib mesylate (IM) were analyzed.

Despite the relatively high specificity of IM treatment towards the BCR-ABL fusion protein, off-target multikinase inhibitory effects occur and it was shown recently, that IM inhibits STAT5 phosphorylation in response to IL-7 and promotes T cell lymphopenia in chronic myelogenous leukemia (CML) [69].

When whole blood samples from patients with SLE were treated with recombinant human IL-7, lover levels of pSTAT5 were indeed found in CD4+ T-cells after preincubation with IM (Fig. 5E). However, even after treatment with IM, stimulation with different concentrations of IL-7 resulted in significantly lower levels of pSTAT5 in CD25+FOXP3+ Treg population compared to other – conventional CD4+ T-cells (Fig. 5F).

IM was shown to enhance tumor immunity by depleting functionally mature – aTreg cells, which are increased in patients with CML [70]. As the difference in STAT5 activation between Tcon and Treg subsets, seen in patients with SLE ex vivo, was found also after in vitro treatment with IM in response to IL-7, the aTreg subset, characterized by the lowest pSTAT5 levels [32] may be affected by IM treatment even in response to physiological levels of ligand stimulation. By applying the phosphoflow technique it was shown before that it can be used to

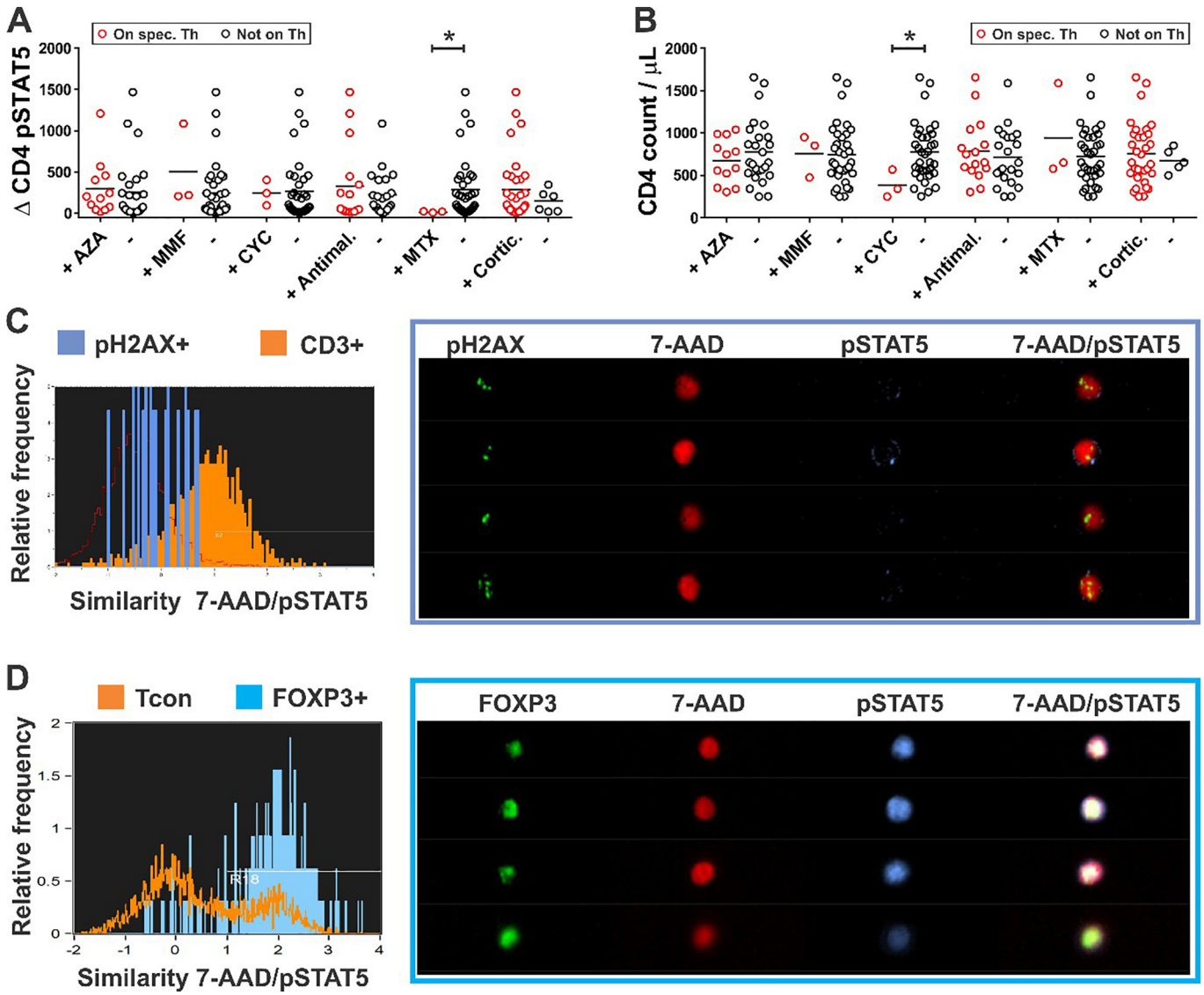

Fig. 3. Differences in CD4 T-cell pSTAT5 and CD4 T-cell counts in the blood samples of patients with SLE, grouped according to specific therapy (A and B)
Scatter dot plots, which depict increase in CD4+ T-cell pSTAT5 levels (Δ CD4 pSTAT5, calculated as the difference in pSTAT5 MFI between CD4+ T-cells and other – CD4 negative lymphocytes) and CD4 T-cell counts (cells/µL) are plotted to compare patients with SLE on azathioprine (AZA), mycophenolate mofetil (MMF), intravenous cyclophosphamide (CYC), hydroxy-chloroquine (antimal.), methotrexate (MTX), and, mehylprednisolone or prednisolone (cortic.), versus those not on these specific therapies: only significant differences (*$p < .05$) are shown.
Image flow cytometry analysis of whole blood leukocytes from patient with SLE treated with pulsed intravenous CYC: representative histograms of 7-AAD/pSTAT5 Similarity scores, correlating 7-AAD nuclear stain with the pSTAT5 signal in the gated pH2AX+ population (blue) compared to all CD3+ T-cells (orange), is shown left. The example images are shown right: pSTAT5 (blue) staining is of low intensity and is not localized to the nucleus stained with 7-AAD (red) in gated CD3+ T-cells showing nuclear pH2AX foci (green).
Image flow cytometry analysis of whole blood leukocytes from patient with SLE treated with pulsed intravenous CYC after stimulation with recombinant human IL-2 for 15 min: representative histograms of 7-AAD/pSTAT5 Similarity scores of the FOXP3+ population (blue) compared to FOXP3- Tcon (orange), gated among CD4+ T-cells. The higher the Similarity score, the more nuclear localization is visualized in the example images of cells shown right: pSTAT5 (blue) is localized to the nucleus stained with 7-AAD (red) in gated CD4+ T-cells showing nuclear FOXP3 expression (green).

analyze the difference in drug susceptibilities between CD4+ T-cell subsets. A significant difference in drug sensitivity between effector T cells and Tregs [71] was found. At a clinically relevant concentration of 50 ng/mL tofacitinib, IL-2-induced STAT5 phosphorylation was significantly inhibited in effector CD4+CD25-FoxP3- T cells, while only partially blocked in the CD4+CD25high+FoxP3+ regulatory T cells. The IC50 was 2 times higher for Tregs than for effector T cells. As discussed by the authors, these differences in sensitivity for tofacitinib can be explained by a different distribution of the IL-2 receptor α, β and γ-c chains or a variation in the recruitment of JAK1 and JAK3 molecules. Tregs might also have more JAK3 and/or JAK1 molecules and need more tofacitinib to inhibit all molecules [71].

8. Conclusion

Phosphospecific flow cytometry is a valuable tool to monitor the JAK-STAT signaling pathway in patients with SLE. Decreased CD4 T-cell counts in patients with SLE were associated with homeostatic proliferation of Tcon subset and increased pSTAT5 levels. Observations of the longitudinal studies implicate basal activation of STAT5 in CD4+ T-cells as a possible marker of severe disease course in SLE.

Correction of IL-7-dependent Tcon/aTreg STAT5 signaling

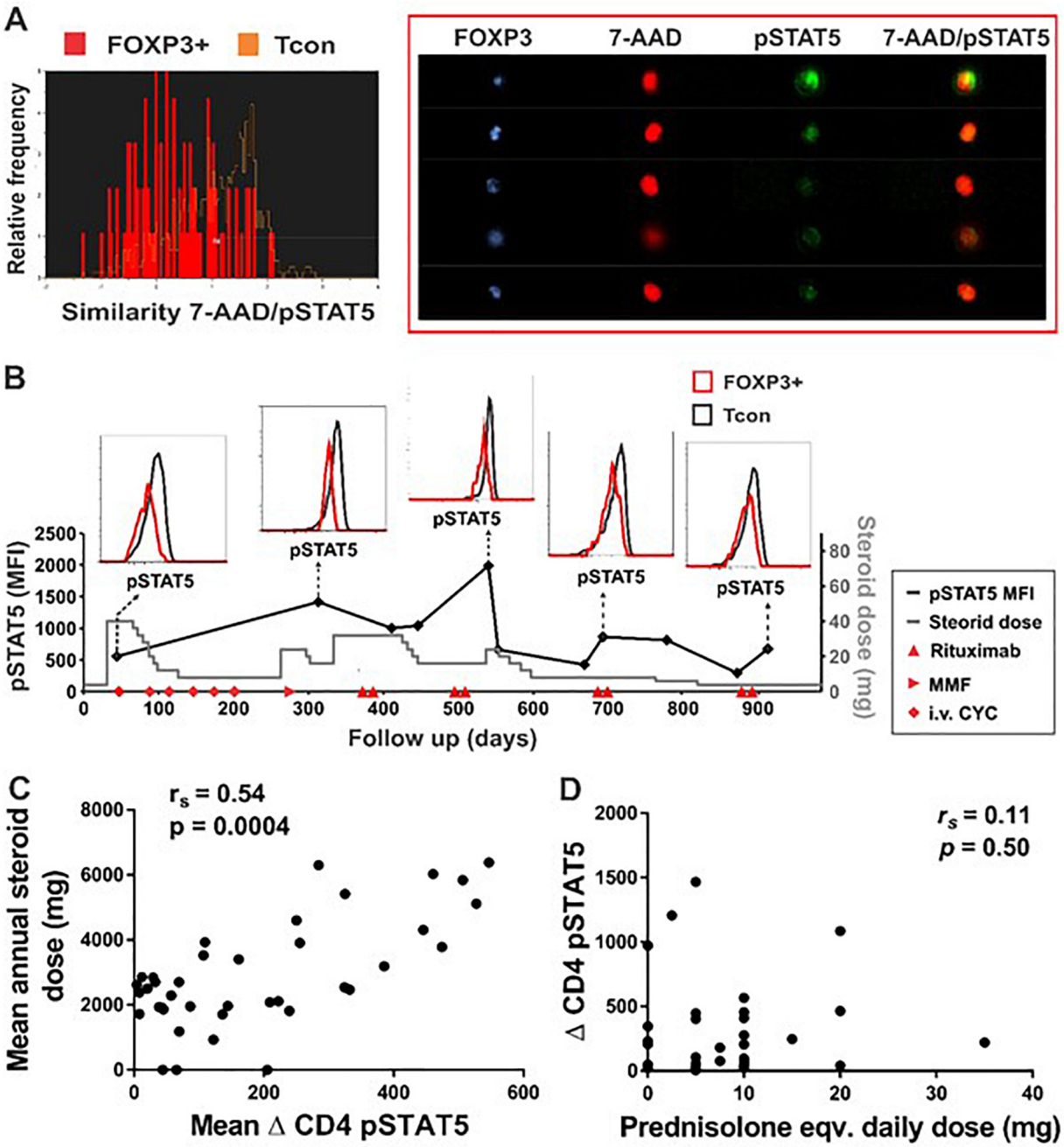

Fig. 4. Increased pSTAT5 in CD4+ T-cells from patients with SLE during follow-up is associated with disease severity. (A) Image flow cytometry analysis of whole blood leukocytes from patient with SLE: representative histogram depicts lower 7-AAD/pSTAT5 Similarity scores for the FOXP3+ population (red) compared to FOXP3- Tcon (orange), gated among CD4+ T-cells. The example images are shown right: pSTAT5 (green) staining is of low intensity and is not localized to the nucleus stained with 7-AAD (red) in CD4+ T-cells with nuclear FOXP3 expression (blue). **(B)** CD4+ T-cell pSTAT5 levels (MFI, black line), examined on indicated days during follow-up of SLE patient, treated with indicated doses of methylprednisolone (grey line) and additional immunosuppressive therapy (red triangles on x-axis): intravenous cyclophosphamide, mycophenolate mofetil (start of therapy is shown) and rituximab therapy. Representative histograms of pSTAT5 levels in FOXP3– Tcon (black) and in FOXP3 + CD4+ T-cells (red) are shown above. **(C)** Correlation between index of disease severity Mean annual steroid dose (mg) and mean follow-up Δ CD4 pSTAT5 from patients with SLE (n = 38). **(D)** Correlation between Δ CD4 pSTAT5 and daily (equivalent) prednisolone dose (mg) for patients with SLE (n = 38). rs, Spearman correlation coefficient.

imbalances could restore homeostasis between Tcon and suppressive-effector aTreg by selectively affecting proliferation of pathogenic Tcon subsets, and may represent a promising therapeutic approach in SLE. Further work is needed to examine the role of other STAT signaling changes in perturbed SLE Treg/Tcon homeostasis and in disease progression to identify potential therapeutic targets. We speculate that through the use of phosphospecific flow cytometry the effects of immunosuppressive therapy, including JAKinibs, on Tcon/Treg subsets can be assessed in different autoimmune diseases, possibly enabling optimization of the therapy for individual patients.

Acknowledgement

This work was partially supported by the Slovenian Research Agency grant L7-8274 and by the University Medical Center Ljubljana research grant 20180093.

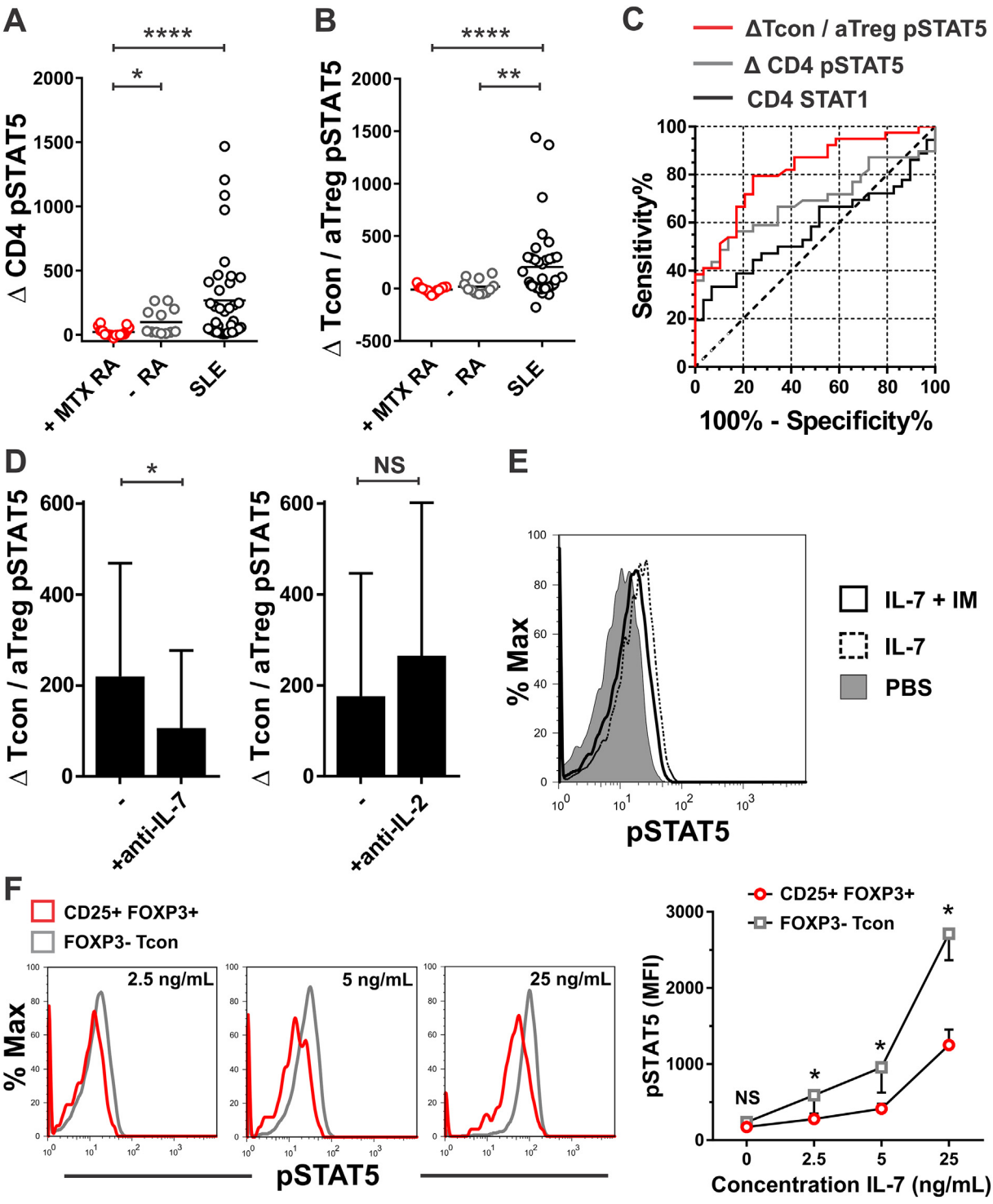

Fig. 5. IL-7 dependent differences in levels of pSTAT5 between Tcon and aTreg subset from patients with SLE (A and B) Scatter dot plots, which depict Δ CD4 pSTAT5 and the difference in pSTAT5 MFI between Tcon and aTreg subset (Δ Tcon/aTreg pSTAT5) are plotted to compare patients with RA on methotrexate (MTX) and those not on this specific therapy to patients with SLE: only significant differences (*$p < .05$) are shown. **(C)** ROC curves depicting compared discriminatory utilities with respect to diagnostic outcome (SLE vs RA): the AUC for STAT1 protein MFI levels in CD4+ T cells (CD4 STAT1) was 0.58, for Δ CD4 pSTAT5 0.69, and for the Δ Tcon/aTreg pSTAT5 0.82. **(D)** Bar graph shows Δ Tcon/aTreg pSTAT5 in whole blood samples from patients with SLE, incubated with anti-IL-7 antibodies (left, $n = 7$) and anti-IL-2 antibodies (right, $n = 6$) for 30 min as compared to basal – untreated samples. **(E)** Representative histograms show pSTAT5 levels in gated CD4+ T-cells after stimulation with recombinant human IL-7 (2,5 ng/mL – 15 min.) with or without previous incubation with IM (3 μM – 60 min.) compared to unstimulated cells (PBS). **(E)** Left: representative histograms show pSTAT5 levels in gated CD25 + FOXP3+ cells (red) and FOXP- Tcon (grey) after incubation with IM and stimulation with different concentrations of recombinant human IL-7. Right: pSTAT5 MFI after IL-7 challenge: means and SD for a given IL-7 concentration are shown. CD25 + FOXP3+ Treg population was characterized by significantly lower pSTAT5 MFI levels compared to Tcon after stimulation with different concentrations of IL-7 ($n = 6$). NS, Not significant; *$p < .05$, **$p = .001$—0.01, ****$p < .0001$.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.autrev.2018.08.010.

References

[1] Stark GR, Darnell Jr. JE. The JAK-STAT pathway at twenty. Immunity 2012;36:503–14.
[2] Levy DE, Darnell Jr. JE. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 2002;3:651–62.
[3] Villarino AV, Kanno Y, Ferdinand JR, O'Shea JJ. Mechanisms of Jak/STAT signaling in immunity and disease. J Immunol 2015;194:21–7.
[4] Altshuler D, Daly MJ, Lander ES. Genetic mapping in human disease. Science 2008;322:881–8.
[5] Parkes M, Cortes A, van Heel DA, Brown MA. Genetic insights into common pathways and complex relationships among immune-mediated diseases. Nat Rev Genet 2013;14:661–73.
[6] Ghodke-Puranik Y, Niewold TB. Immunogenetics of systemic lupus erythematosus: a comprehensive review. J Autoimmun 2015;64:125–36.
[7] Rullo OJ, Tsao BP. Recent insights into the genetic basis of systemic lupus erythematosus. Ann Rheum Dis 2013;72(Suppl. 2):56–61. ii.
[8] Remmers EF, Plenge RM, Lee AT, et al. STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 2007;357:977–86.
[9] Hnisz D, Abraham BJ, Lee TI, et al. Super-enhancers in the control of cell identity and disease. Cell 2013;155:934–47.
[10] Parker SC, Stitzel ML, Taylor DL, et al. Chromatin stretch enhancer states drive cell-specific gene regulation and harbor human disease risk variants. Proc Natl Acad Sci U S A 2013;110:17921–6.
[11] Vahedi G, Kanno Y, Furumoto Y, et al. Super-enhancers delineate disease-associated regulatory nodes in T cells. Nature 2015;520:558–62.
[12] Vahedi G, Takahashi H, Nakayamada S, et al. STATs shape the active enhancer landscape of T cell populations. Cell 2012;151:981–93.
[13] Tucci M, Lombardi L, Richards HB, Dammacco F, Silvestris F. Overexpression of interleukin-12 and T helper 1 predominance in lupus nephritis. Clin Exp Immunol 2008;154:247–54.
[14] Venigalla RK, Tretter T, Krienke S, Max R, Eckstein V, Blank N, et al. Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25high,CD127 −/low regulatory T cells in patients with active systemic lupus erythematosus. Arthritis Rheum 2008;58:2120–30.
[15] Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, et al. Global natural regulatory T cell depletion in active systemic lupus erythematosus. J Immunol 2005;175:8392–400.
[16] Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441:235–8.
[17] Chaudhry A, Rudra D, Treuting P, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. Science 2009;326:986–91.
[18] Kluger MA, Melderis S, Nosko A, et al. Treg17 cells are programmed by Stat3 to suppress Th17 responses in systemic lupus. Kidney Int 2015;89:158–66.
[19] Huang X, Guo Y, Bao C, Shen N. Multidimensional single cell based STAT phosphorylation profiling identifies a novel biosignature for evaluation of systemic lupus erythematosus activity. PLoS One 2011;6:e21671.
[20] Rasmussen TK, Andersen T, Bak RO, et al. Overexpression of microRNA-155 increases IL-21 mediated STAT3 signaling and IL-21 production in systemic lupus erythematosus. Arthritis Res Ther 2015;17:154.
[21] Hale MB, Krutzik PO, Samra SS, Crane JM, Nolan GP. Stage dependent aberrant regulation of cytokine-STAT signaling in murine systemic lupus erythematosus. PLoS One 2009;4:e6756.
[22] Schwartz DM, Kanno Y, Villarino A, Ward M, Gadina M, O'Shea JJ. JAK inhibition as a therapeutic strategy for immune and inflammatory diseases. Nat Rev Drug Discov 2017 Dec;16(12):843–62.
[23] Wang S, Yang N, Zhang L, et al. Jak/STAT signaling is involved in the inflammatory infiltration of the kidneys in MRL/lpr mice. Lupus 2010;19(10):1171–80.
[24] Markopoulou A, Kyttaris VC. Small molecules in the treatment of systemic lupus erythematosus. Clin Immunol 2013;148(3):359–68. https://doi.org/10.1016/j.clim.2012.09.009.
[25] Ripoll È, de Ramon L, Draibe Bordignon J, et al. JAK3-STAT pathway blocking benefits in experimental lupus nephritis. Arthritis Res Ther 2016;18(1):1–12.
[26] Thacker SG, Abdalrahman Z, Sciumè G, Tsai WL, Anna M. Tofacitinib ameliorates murine lupus and its associated vascular dysfunction. Arthritis Rheumatol 2017;69(1):148–60.
[27] Thomas S, Fisher KH, Snowden JA, Danson SJ, Brown S, Zeidler MP. Methotrexate is a JAK/STAT Pathway Inhibitor. PLoS One 2015 Jul 1;10(7):e0130078.
[28] Chinnaiya K, Lawson MA, Thomas S, Haider MT, Down J, Chantry AD, et al. Low-dose methotrexate in myeloproliferative neoplasm models. Haematologica 2017 Sep;102(9):e336–9.
[29] Humrich JY, von Spee-Mayer C, Siegert E, Alexander T, Hiepe F, Radbruch A, et al. Rapid induction of clinical remission by low-dose interleukin-2 in a patient with refractory SLE. Ann Rheum Dis 2015 Apr;74(4):791–2.
[30] von Spee-Mayer C, Siegert E, Abdirama D, Rose A, Klaus A, Alexander T, et al. Low-dose interleukin-2 selectively corrects regulatory T cell defects in patients with systemic lupus erythematosus. Ann Rheum Dis 2016 Jul;75(7):1407–15.
[31] He J, Zhang X, Wei Y, Sun X, Chen Y, Deng J, et al. Low-dose interleukin-2 treatment selectively modulates CD4(+) T cell subsets in patients with systemic lupus erythematosus. Nat Med 2016 Sep;22(9):991–3.
[32] Goropevšek A, Gorenjak M, Gradišnik S, Dai K, Holc I, Hojs R, et al. STAT5 phosphorylation in CD4 T cells from patients with SLE is related to changes in their subsets and follow-up disease severity. J Leukoc Biol 2017 Jun;101(6):1405–18.
[33] Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, et al. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. Proc Natl Acad Sci U S A 2003;100:2610–5.
[34] Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, et al. Interferon and Granulopoiesis Signatures in Systemic Lupus Erythematosus Blood. J Exp Med 2003;197:711–23.
[35] Lin Q, Dong C, Cooper MD. Impairment of T and B cell development by treatment with a type I interferon. J Exp Med 1998 Jan 5;187(1):79–87.
[36] Vilá LM, Alarcón GS, McGwin Jr. G, Bastian HM, Fessler BJ, Reveille JD. Lumina Study Group. Systemic lupus erythematosus in a multiethnic US cohort, XXXVII: association of lymphopenia with clinical manifestations, serologic abnormalities, disease activity, and damage accrual. Arthritis Rheum 2006 Oct 15;55(5):799–806.
[37] King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. Cell 2004 Apr 16;117(2):265–77.
[38] Calzascia T, Pellegrini M, Lin A, Garza KM, Elford AR, Shahinian A, et al. CD4 T cells, lymphopenia, and IL-7 in a multistep pathway to autoimmunity. Proc Natl Acad Sci U S A 2008 Feb 26;105(8):2999–3004.
[39] Napolitano LA, Grant RM, Deeks SG, Schmidt D, De Rosa SC, Herzenberg LA, et al. Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. Nat Med 2001;7:73–9.
[40] Gonzalez-Quintial R, Lawson BR, Scatizzi JC, Craft J, Kono DH, Baccala R, et al. Systemic autoimmunity and lymphoproliferation are associated with excess IL-7 and inhibited by IL-7Rá blockade. PLoS One 2011;6:e27528.
[41] Monti P, Scirpoli M, Maffi P, Ghidoli N, De Taddeo F, Bertuzzi F, et al. Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory T cells. J Clin Invest 2008;118:1806–14.
[42] Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4 T reg cells. J Exp Med 2006;203:1701–11.
[43] Le Campion A, Pommier A, Delpoux A, Stouvenel L, Auffray C. Martin Bet al. IL-2 and IL-7 determine the homeostatic balance between the regulatory and conventional CD4+ T cell compartments during peripheral T cell reconstitution. J Immunol 2012;189:3339–46.
[44] Hsieh CS, Liang Y, Tyznik AJ, Self SG, Liggitt D, Rudensky AY. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. Immunity 2004;21:267–77.
[45] McHugh RS, Shevach EM. Cutting edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. J Immunol 2002;168:5979–83.
[46] Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008;133:775–87.
[47] Carbone F, De Rosa V, Carrieri PB, Montella S, Bruzzese D, Porcellini A, et al. Regulatory T cell proliferative potential is impaired in human autoimmune disease. Nat Med 2014 Jan;20(1):69–74.
[48] Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, et al. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol Rev 2006;212:8–27.
[49] Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity 2009 Jun 19;30(6):899–911.
[50] Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984;133:1710–5.
[51] Golding A, Rosen A, Petri M, Akhter E, Andrade F. Interferon-alpha regulates the dynamic balance between human activated regulatory and effector T cells:implications for antiviral and autoimmune responses. Immunology 2010 Sep;131(1):107–17.
[52] Bromberg JE Darnell Jr. The role of STATs in transcriptional control and their impact on cellular function. Oncogene 2000;19:2468–73.
[53] Karin M, Hunter T. Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. Curr Biol 1995;5:747–57.
[54] Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by γc family cytokines. Nat Rev Immunol 2009;9:480–90.
[55] Bromberg JF, Horvath CM, Wen Z, Schreiber RD, Darnell Jr. JE. Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. Proc Natl Acad Sci U S A 1996;93:7673–8.
[56] Goropevšek A, Gorenjak M, Gradišnik S, Dai K, Holc I, Hojs R, et al. Increased levels of STAT1 protein in blood CD4 T Cells from systemic lupus erythematosus patients are associated with perturbed homeostasis of activated CD45RA(−)FOXP3(hi) regulatory subset and follow-up disease severity. J Interferon Cytokine Res 2017 Jun;37(6):254–68.
[57] McKinney EF, Lyons PA, Carr EJ, Hollis JL, Jayne DR, Willcocks LC, et al. A CD8+ T cell transcription signature predicts prognosis in autoimmune disease. Nat Med 2010;16:586–91.
[58] Lee JC, Lyons PA, McKinney EF, Sowerby JM, Carr EJ, Bredin F, et al. Gene expression profiling of CD8+ T cells predicts prognosis in patients with Crohn disease and ulcerative colitis. J Clin Invest 2011;121:4170–9.
[59] Hall AG, Tilby MJ. Mechanisms of action of, and modes of resistance to, alkylating agents used in the treatment of haematological malignancies. Blood Rev 1992;6:163–73.
[60] Mah LJ, El-Osta A, Karagiannis TC. gammaH2AX: a sensitive molecular marker of

DNA damage and repair. Leukemia 2010;24:679–86.
[61] Hoare M, Shankar A, Shah M, Rushbrook S, Gelson W, Davies S, et al. γ-H2AX +CD8+ T lymphocytes cannot respond to IFN-α, IL-2 or IL-6 in chronic hepatitis C virus infection. J Hepatol 2013 May;58(5):868–74.
[62] Liu M, Hales BF, Robaire B. Effects of four chemotherapeutic agents, bleomycin, etoposide, cisplatin, and cyclophosphamide, on DNA damage and telomeres in a mouse spermatogonial cell line. Biol Reprod 2014 Apr 3;90(4):72.
[63] Zhang H, Chua KS, Guimond M, Kapoor V, Brown MV, Fleisher TA, et al. Lymphopenia and interleukin-2 therapy alter homeostasis of CD4+CD25+ regulatory T cells. Nat Med 2005 Nov;11(11):1238–43.
[64] Kanakry CG, Ganguly S, Zahurak M, Bolaños-Meade J, Thoburn C, Perkins B, et al. Aldehyde dehydrogenase expression drives human regulatory T cell resistance to posttransplantation cyclophosphamide. Sci Transl Med 2013 Nov 13;5(211):211ra157.
[65] Jones JL, Thompson SA, Loh P, Davies JL, Tuohy OC, Curry AJ, et al. Human autoimmunity after lymphocyte depletion is caused by homeostatic T-cell proliferation. Proc Natl Acad Sci U S A 2013 Dec 10;110(50):20200–5. (11025).
[66] Hu X, Li WP, Meng C, Ivashkiv LB. Inhibition of IFN-gamma signaling by glucocorticoids. J Immunol 2003;170:4833–9.
[67] Avcin T, Benseler SM, Tyrrell PN, Cucnik S, Silverman ED. A followup study of antiphospholipid antibodies and associated neuropsychiatric manifestations in 137 children with systemic lupus erythematosus. Arthritis Rheum 2008;59:206–13.
[68] Goleva E, Kisich KO, Leung DYM. A role for STAT5 in the pathogenesis of IL-2-induced glucocorticoid resistance. J Immunol 2002;169:5934–40.
[69] Thiant S, Moutuou MM, Laflamme P, Sidi Boumedine R, Leboeuf DM, Busque L, et al. Imatinib mesylate inhibits STAT5 phosphorylation in response to IL-7 and promotes T cell lymphopenia in chronic myelogenous leukemia patients. Blood Cancer J 2017 Apr 7;7(4):e551.
[70] Noguchi S, Nishikawa H, Saitoh H, et al. Tyrosine kinase inhibitor imatinib enhances tumor immunity by depleting functionally mature regulatory T cells. Blood 2015;126:2219.
[71] Sewgobind VD, Quaedackers ME, van der Laan LJ, Kraaijeveld R, Korevaar SS, Chan G, et al. The Jak inhibitor CP-690,550 preserves the function of CD4CD25FoxP3 regulatory T cells and inhibits effector T cells. Am J Transplant 2010;10:1785–95.