Regulatory T-cell dysfunctions are associated with increase in tumor necrosis factor α in autoimmune hemolytic anemia and participate in Th17 polarization

Marion Ciudad,[1,2] Sethi Ouandji,[1,2] Baptiste Lamarthée,[2] Claudie Cladière,[1,2] Thibault Ghesquière,[1,2] Martin Nivet,[1,2] Marine Thébault,[1,2] Romain Boidot,[3] Agnès Soudry-Faure,[4] Sandy Chevrier,[3] Corentin Richard,[3] Thibault Maillet,[5] François Maurier,[6] Hélène Greigert,[1,2] Coraline Genet,[2] André Ramon,[2] Malika Trad,[2] Valérie Predan,[1] Philippe Saas,[2] Maxime Samson,[1,2] Bernard Bonnotte[1,2] and Sylvain Audia[1,2]

[1]Department of Internal Medicine and Clinical Immunology, Referral Center for Adult Autoimmune Cytopenia (CeReCAI) - Dijon University Hospital, Dijon; [2]Université de Bourgogne, INSERM, UMR1098, RIGHT, Dijon; [3]Unit of Molecular Biology, Georges-François Leclerc Cancer Center, Dijon; [4]Department of Clinical Research and Innovation (DRCI), Clinical Research Unit-Methodological Support Network (USMR), Dijon Bourgogne University Hospital, Dijon; [5]Department of Internal Medicine - Centre Hospitalier de Mâcon, Groupe Hospitalier Bourgogne Méridionale, Macon and [6]Department of Internal Medicine, Groupe Hospitalier UNEOS, Metz, France

Correspondence: S. Audia
sylvain.audia@u-bourgogne.fr

Received: February 3, 2023.
Accepted: July 25, 2023.
Early view: August 3, 2023.

https://doi.org/10.3324/haematol.2023.282859

Abstract

Warm autoimmune hemolytic anemia (wAIHA) is a rare acquired autoimmune disease mediated by antibodies targeting red blood cells. The involvement of CD4 T-helper cells has been scarcely explored, with most findings extrapolated from animal models. Here, we performed quantification of both effector T lymphocytes (Teff) and regulatory T cells (Treg), associated with functional and transcriptomic analyses of Treg in human wAIHA. We observed a shift of Teff toward a Th17 polarization concordant with an increase in serum interleukin-17 concentration that correlates with red blood cell destruction parameters, namely lactate dehydrogenase and bilirubin levels. A decrease in circulating Treg, notably effector Treg, associated with a functional deficiency, as represented by their decrease capability to inhibit Teff proliferation, were also observed. Treg deficiency was associated with a reduced expression of Foxp3, the master transcription factor known to maintain the Treg phenotype stability and suppressive functions. Transcriptomic profiling of Treg revealed activation of the tumor necrosis facto (TNF)-α pathway, which was linked to increased serum TNF-α concentrations that were twice as high as in controls. Treg transcriptomic profiling also suggested that post-translational mechanisms possibly accounted for Foxp3 downregulation and Treg dysfunctions. Since TNF-α participates in the rupture of immune tolerance during wAIHA, its inhibition could be of interest. To this end, the effects of fostamatinib, a SYK inhibitor, were investigated *in vitro*, and we showed that besides the inhibition of erythrocyte phagocytosis by monocytes, fostamatinib is also able to dampen TNF-α production, thus appearing as a promising multitargeting therapy in wAIHA (*clinicaltrials gov. Identifier: NCT02158195*).

Introduction

Warm autoimmune hemolytic anemia (wAIHA) is a rare acquired autoimmune disease due to antibodies targeting red blood cell (RBC) antigens.[1-3] Anti-RBC antibodies are mostly immunoglobulin (Ig)G, underlying a class-switch recombination, a mechanism involving cooperation between B and T cells. Until now, the involvement of helper CD4 T cells (Th) has been scarcely studied in human wAIHA, and most of the conclusions have been derived from animal models. In mice, a Th1 polarization has been first observed,[4] with an improvement of the disease by Th2 cytokines such as interleukin (IL)-4.[5] On the opposite, human wAIHA was first thought to be associated with a Th2 skewing as suggested by an increased production of IL-4 while interferon (IFN)-γ was reduced.[6] However, when CD4 T cells from patients were stimulated with specific RBC antigens, IFN-γ secretion was enhanced whereas IL-4 was not detected, arguing for a Th1 polarization.[7] This was secondly amended by the fact that IL-17 and Th17 were

increased and associated with wAIHA severity.[8,9]
A defect in regulatory T cells (Treg), together with a pro-inflammatory T-cell response, are commonly observed in autoimmune diseases.[10] During immune thrombocytopenia (ITP), the most prevalent autoimmune cytopenia, such abnormalities have been observed,[11-13] with a negative correlation between Th17 and Treg frequencies.[13] To date, the results remain controversial in wAIHA. In animals, whereas the role of Treg has been shown in a xenogeneic model in which Treg depletion enhanced the occurrence of wAIHA while their adoptive transfer prevented its establishment,[14] it was not confirmed in a non-xenogeneic model.[15] In humans, only one study reported on a decrease in circulating Treg, but without assessing their function.[16]
With the aim to better understand the pathophysiology of human wAIHA, and thus opening the way to new therapies, a better comprehensive appraisal of its global T-cell immune response is required. Here we provide the first concurrent evaluation of circulating effector T lymphocytes (Teff) and Treg, associated with Treg function and transcriptomic analyses.

Methods

Patients

Healthy controls (HC) and patients with newly diagnosed wAIHA were proposed to participate. The diagnosis of wAIHA was defined as hemoglobin <11 g/dL, with a low haptoglobin level and a positive direct antiglobulin test (DAT) for IgG +/- complement (C3d). The diagnosis of primary wAIHA was retained after exclusion of lymphoproliferative malignancies, other autoimmune diseases, primary immunodeficiency and infections, as recommended.[17,18] All patients were included before receiving immunomodulating drugs, especially steroids.

Study approval

All patients gave a written informed consent in accordance with the Declaration of Helsinki before participating to this prospective study (*clinicaltrials gov. Identifier: NCT02158195*). The research was approved by the Institutional Review Board and the Independent Ethics Committee (Comité de Protection des Personnes, CPP Est 1).

Cell isolation, culture and storage

A more detailed methods is provided in the *Online Supplementary Appendix.* Whole blood from patients or HC was collected for peripheral blood mononuclear cells (PBMC) isolation to perform immunophenotyping, cell culture and storage.

T-cell proliferation suppression assay

Teff ($CD4^+CD25^-$) and Treg ($CD4^+CD25^{hi}$) were purified by magnetic cell isolation. Labeled Teff were activated with anti-CD2/CD3/CD28 microbeads and cultured for 4 days with or without Treg (Teff/Treg ratio=2/1). Proliferation was measured by flow cytometry based on CellTrace dilution.

Assessment of monocyte function during hemolysis

Monocytes were isolated based on CD14 expression and cultured for 24 hours either alone or stimulated with heme or RBC coated with either anti-glycophorin A or in the presence of isotype control. RBC collected from one patient with relapsing wAIHA were also used. The effect of R406, the active metabolite of fostamatinib was also investigated. Flow cytometry was used to quantify TNF-α production and RBC phagocytosis.

RNA sequencing, gene ontology terms, Treg signature and index computations

Fluorescent-activated cell sorting (FACS) of Treg ($FVS780^-CD3^+CD4^+CD25^{hi}CD127^-$) from four primary wAIHA and four sex- and age-matched HC were used for RNA sequencing (RNAseq). RNAseq was processed in one batch. Only protein-coding transcripts and genes were included in the downstream analysis. Genes with a *P* value below 0.05 and normalized read count greater than 25 were considered as significantly differentially expressed. RNAseq data are available in the Gene Expression Omnibus database (accession number: GSE195791).
Treg transcriptomic identity was assessed by referring to the Treg signature genes.[19] Tumor necrosis factor (TNF) index was calculated for each subject by averaging the normalized expression of all genes belonging to TNF pathway (normalized expression *vs*. mean expression of all HC) similarly to published data on Treg signature.[19,20]

Cytokine assays

IFN-γ, IL-4, IL-17 and TNF-α were quantified in culture supernatants using multiplex immunoassay. High sensitivity enzyme-linked immunosorbant assay was performed for serum IL-17A.

Statistics

Statistical analyses and graphs were performed with Prism v9.3.0 (GraphPad Software), R Studio (1.4.1717) and Heatmaper. Data are reported as the median with interquartile range (IQR) and were compared using Mann-Whitney test for independent data, Wilcoxon signed-rank test for paired conditions, and Spearman's rank correlation test for correlation analyses, unless otherwise specified. *P* value <0.05 was considered significant.

Results

Patients

Twenty-two patients with wAIHA were enrolled at diagnosis, before initiation of any immunomodulatory drugs

Table 1. Baseline characteristics of warm autoimmune hemolytic anemia patients and healthy controls.

Characteristics	HC N=30	wAIHA N=22	*P*
Age in years, median (IQR)	67 (58-77)	65 (53-76)	0.95
Sex ratio, female/male	18/12	15/7	0.63
Primary AIHA, N (%)	NA	17 (77)	NA
Hemoglobin, g/dL, median (IQR)	14. (13.2- 15.2)	7.4 (6.4-9.5)	<0.0001
Reticulocytes, x10^9/L, median (IQR)	NA	189 (139-324)	NA
Leukocytes, x10^9/L, median (IQR)	5.8 (4.9-7.0)	5.8 (4.6- 8.5)	0.97
Lymphocytes, x10^9/L, median (IQR)	1.6 (1.2-2.1)	1.3 (0.7-2.5)	0.31
Platelets, x10^9/L, median (IQR)	224 (204-296)	191 (162-314)	0.47
Haptoglobin, g/L, median (IQR)	NA	0 (0.0-0.2)	NA
Direct antiglobulin test, N (%) IgG alone IgG and C3d	NA	22 (100) 15 (68) 7 (32)	NA

wAIHA: warm autoimmune hemolytic anemia; HC: healthy controls; IQR: interquartile range; NA: not applicable.

(Table 1). Their median age was 65 (IQR, 53-76) years with a female/male ratio of 2.1. Secondary wAIHA was diagnosed in five patients (22,7%), associated with lupus (n=1), ITP (n=1) or B-cell lymphoproliferation (B-cell monoclonal lymphocytosis, n=2; indolent marginal zone B cell, n=1). As none of the measured parameters were different between primary and secondary AIHA, the data set was kept as a single group (*Online Supplementary Table S3*). Patients were compared to 30 HC, with a median age of 67 years (IQR, 58-77) (*P*=0.9) and a female/male ratio of 1.5 (*P*=0.6). Hemoglobin was lower in wAIHA patients (7.4 *vs*. 14.2 g/dL; *P*<0.0001) while total leukocyte and lymphocyte counts were similar. The flowchart of the study is depicted in Figure 1A.

Quantitative and functional alterations of circulating Treg in warm autoimmune hemolytic anemia

The frequency of circulating Treg (CD3$^+$CD4$^+$CD25^{hi}Foxp3$^+$) was lower in wAIHA (3.2% *vs*. 4.5% of CD4 T cells; *P*=0.01; Figure 1B). Moreover, the quantification of Treg subtypes[21] showed that CD4$^+$CD45RA$^-$Foxp3^{hi} effector Treg (Fr.II/eTreg), known to have the strongest suppressive capabilities, were twice less frequent in wAIHA (1.1% *vs*. 2.1%; *P*=0.0008; Figure 1C), while the proportions of naive Treg (CD45RA$^+$Foxp3^{lo}, Fr.I/nTreg) were similar. The frequency of CD25^{hi}Foxp3$^+$Helios$^+$ cells among total CD4 T cells was also lower in wAIHA patients (2.4 *vs*. 3.1%; *P*=0.007; Figure 1D). As lymphocyte count and CD4 T-cell proportions were similar between patients and controls, the decrease in Treg subsets was also observed when considering cell absolute numbers (*Online Supplementary Table S4*).

Associated with this quantitative reduction, the suppressive function of Treg was also altered in wAIHA, as shown by a decreased ability to inhibit Teff proliferation (51% *vs*. 73%; *P*=0.003; Figure 1E).

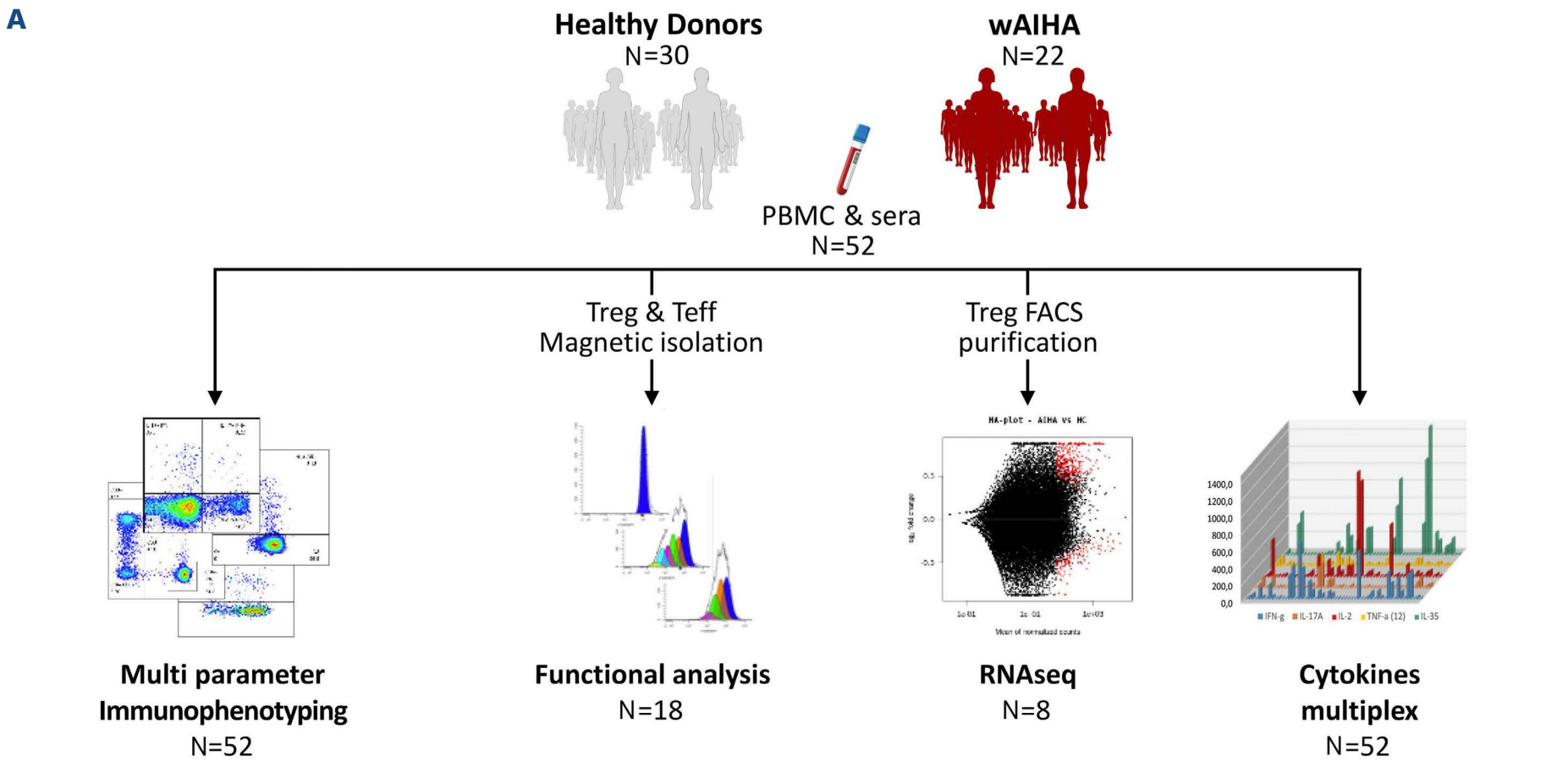
Warm autoimmune hemolytic anemia is associated with a Th17 polarization

Effector T-cell subpopulations were similar between HC and patients (*Online Supplementary Figure S1; Online Supplementary Table S4*). We then focused on Teff polarization determined by their cytokine production. Measurement of cytokines in culture supernatants of Teff activated for 4 days showed a higher production of IL-17 in wAIHA (25.5 pg/mL *vs*. 7.5 pg/mL; *P*=0.02; Figure 2A), whereas IFN-γ concentration was similar and IL-4 was not detected. Supporting this Th17 polarization, we observed an increase in serum IL-17 concentrations (0.41 pg/mL *vs*. 0.68 pg/mL; *P*=0.02; Figure 2B) and an imbalance in the Th17/Treg ratio (0.22 *vs*. 0.11; *P*=0.02; Figure 2C). Interestingly, the serum concentration of IL-17 positively correlated with markers of RBC destruction, namely lactate dehydrogenase (LDH) and bilirubin (R=0.58, *P*=0.03 and R=0.68, *P*=0.02, respectively; Figure 2D), but not with reticulocyte count, a marker of RBC production, nor with hemoglobin level, resulting from the production and destruction RBC.

Overall, these results reflected an imbalance between pro- and anti-inflammatory immune responses in wAIHA, with a quantitative and functional alteration of circulating Treg, associated with Th17 polarization, the latter being positively correlated with disease activity, notably markers of RBC destruction such as LDH and bilirubin.

Transcriptomic profiling showed Treg activation during warm autoimmune hemolytic anemia

In order to further characterize the mechanisms involved in Treg dysfunctions, a transcriptomic analysis was performed on sorted CD3$^+$CD4$^+$CD25^{hi}CD127^{lo} Treg obtained from four representative patients (*Online Supplementary Table S2*) and four HC. The isolated cells were first confirmed to be



Continued on following page.

Figure 1. Quantitative and functional alterations of circulating Treg in warm autoimmune hemolytic anemia. (A) Flowchart of the overall experimental approach. (B) Dot plot showing the gating strategy for the quantification of regulatory T cells (Treg), defined as $CD4^+CD25^{hi}Foxp3^+$ lymphocytes, by flow cytometry (left panel). Scatter dot plot (right panel) showing Treg frequencies among circulating CD4 T cells in healthy controls (HC) (N=24) and warm autoimmune hemolytic anemia (wAIHA) patients (N=20). (C) Dot plot (left panel) showing the gating strategy for the determination of naive Treg ($CD45RA^+Foxp3^{low}$: Fr. I/nTreg) and effector Treg ($CD45RA^-Foxp3^{hi}$: Fr. II/eTreg) by fluorescense-activated cell sorting (FACS) (HC N=20; wAIHA N=19). Scatter dot plots (right panel) showing the frequency of Treg subsets among circulating CD4 T cells. (D) Scatter dot plot showing the frequency of Treg expressing Helios among circulating CD4 T cells. (E) Effector T-cell proliferation assay. Representative dot plots (left panel) showing the proliferation of effector T cells (Teff) stimulated or not with anti-CD2/CD3/CD28 microbeads, in presence or not of Treg (Teff/Treg ratio: 2/1), assessed by CellTrace dilution by flow cytometry after 4 days of culture, for one representative HC and one representative wAIHA patient. Proliferation index (PI) is mentioned. Histogram with scatter dot plot (right panel) showing Treg function as determined by the inhibition of Teff proliferation for 9 HC and 9 wAIHA patients. The PI of stimulated Teff alone is used as reference. *P* values derived from Mann-Whitney test. Median with 1st and 3rd quartiles are depicted on graphs. PBMC: peripheral blood mononucelar cells; FACS: fluorescence-activated cell sorting; RNAseq: RNA sequencing; NS: not significant.

Treg as shown by the specific upregulation of 194 genes and downregulation of 192 genes (Figure 3A) consistent with Treg signature.[19] The transcriptomic profile of Treg from patients was distinct from the one of HC, as revealed by the differential expression of 455 genes, some of which being implicated in transcription or translation processes or reflecting the activation of the T-cell receptor (TCR) and TNF pathways (Figure 3B).

The activation of the transcription and translational processes (Figure 3B) were revealed by increased transcription of RNA splicing proteins (*DHX38, U2AF1, PNN*), transcription elongation or termination factors (*SUPT6H, XRN2*), ribosomal proteins (*RPL, RPS*) and translation initiation factor (*EIF3B*). The engagement of the TCR pathway in wAIHA Treg (Figure 3C) was supported by the overexpression of 16 genes such as *CD247* (TCR ζ-chain), *ZAP70* (ζ-chain associated protein 70), *CD4*, and Janus kinase 3 (*JAK3*).

Upon activation, multiple mechanisms are involved in the suppressive functions of Treg.[22,23] Although our functional analysis showed an impairment of Treg function, a slight but significant increase in the transcripts of CTLA-4 (*CTLA4*), CD25 (*IL2RA*), TIGIT (*TIGIT*), and GARP (*LRRC32*), the protein binding latent TGF-β at cell surface, was observed, while others were expressed at similar rates. The expression of GPA33 a marker that allows the identification of a subset of naive Treg of thymic origin, expressing Foxp3 and Helios, with immunosuppressive functions and not secreting pro-inflammatory cytokines such as IL-17[24] was not different between patients and controls (Figure 3D).

The *RORC* (ROR-γt), *CCR6* and *IL17A* transcripts were compared between patients and controls and found similar (Figure 3E), arguing against the hypothesis of a loss of inhibitory functions of Treg due to their differentiation into IL-17 producing cells in a Th17 environment.[23,25]

TNF-α signaling pathway is engaged in Treg and is correlated with the decreased Foxp3 expression

Transcriptomic analyses of Treg showed a strong engagement of TNF-α signaling pathway in wAIHA, as supported by the overexpression of ten genes such as *MADD* (MAP kinase-activating death domain), *CASP8* (caspase 8), *TAB2* and *TRAF1* ($P<0.0001$; Figure 4A). In order to better understand these results, TNF-α was measured in sera and found to be almost twice as high in wAIHA patients (5.7 pg/mL *vs*. 3.0 pg/mL; $P<0.0001$; Figure 4B).

The expression of the transcripts of the two TNF receptors were investigated in Treg. *TNFR2* transcripts were more abundant than the ones of *TNFR1*, both in HC and patients (Table 2), and were higher in patients (average expression: 138 *vs*. 103; $P=0.02$; Table 2). The activation of TNFR2 can promote the inhibitory functions of Treg through NF-κB pathway, with the transcription of *NFLB2* and *RELB*.[26] These transcripts were increased by 1.4-fold in wAIHA Treg ($P=0.04$ and $P=0.07$, respectively; Table 2). However, repressive effects have also been reported after TNFR2 ligation,[27] involving the NF-κB[28] pathway or molecules such as DBC1 (deleted breast cancer 1)[29] and PP1 (protein phosphatase 1).[30] Their transcripts were also increased in wAIHA patients (Table 2), thus arguing for an engagement of both activating and inhibiting pathways in Treg during wAIHA. In order to assess the effect of TNF-α stimulation on Treg, the level of TNF engagement was quantified using a TNF index, as previously described for Treg signature.[19,20] This TNF index inversely correlated with the frequency of circulating Treg ($R=-0.89$; $P=0.01$; Figure 4C), notably with the one of eTreg ($R=-0.79$; $P=0.05$; Figure 4C), thus supporting an inhibitory effect of TNF-α on Treg. Considering the major role of Foxp3 in the maintenance of Treg phenotype and functions[31] and that TNF-α could participate to its downregulation,[32] the expression of Foxp3 protein was measured and found to be lower in wAIHA patient Treg (mean fluorescence intensity [MFI]: 662 *vs*. 1,149; $P=0.012$; Figure 4D). Consistently, the TNF index inversely correlated with Foxp3 protein level ($R=-0.89$; $P=0.01$; Figure 4E), although *FOXP3* transcripts were increased (Figure 4F). Of note, neither Treg frequencies nor TNF-α concentrations correlated with disease activity markers such as hemoglobin level, reticulocyte count, bilirubin and LDH concentrations (*Online Supplementary Figure S2*). As Treg functions are strengthened by the interaction of Foxp3 with Helios,[33,34] its expression was also determined and found to be decreased in wAIHA (MFI: 291 *vs*. 1,346; $P=0.002$). In contrast to Foxp3, the reduction of Helios protein expression was not correlated with the TNF index (*Online Supplementary Figure S3*).

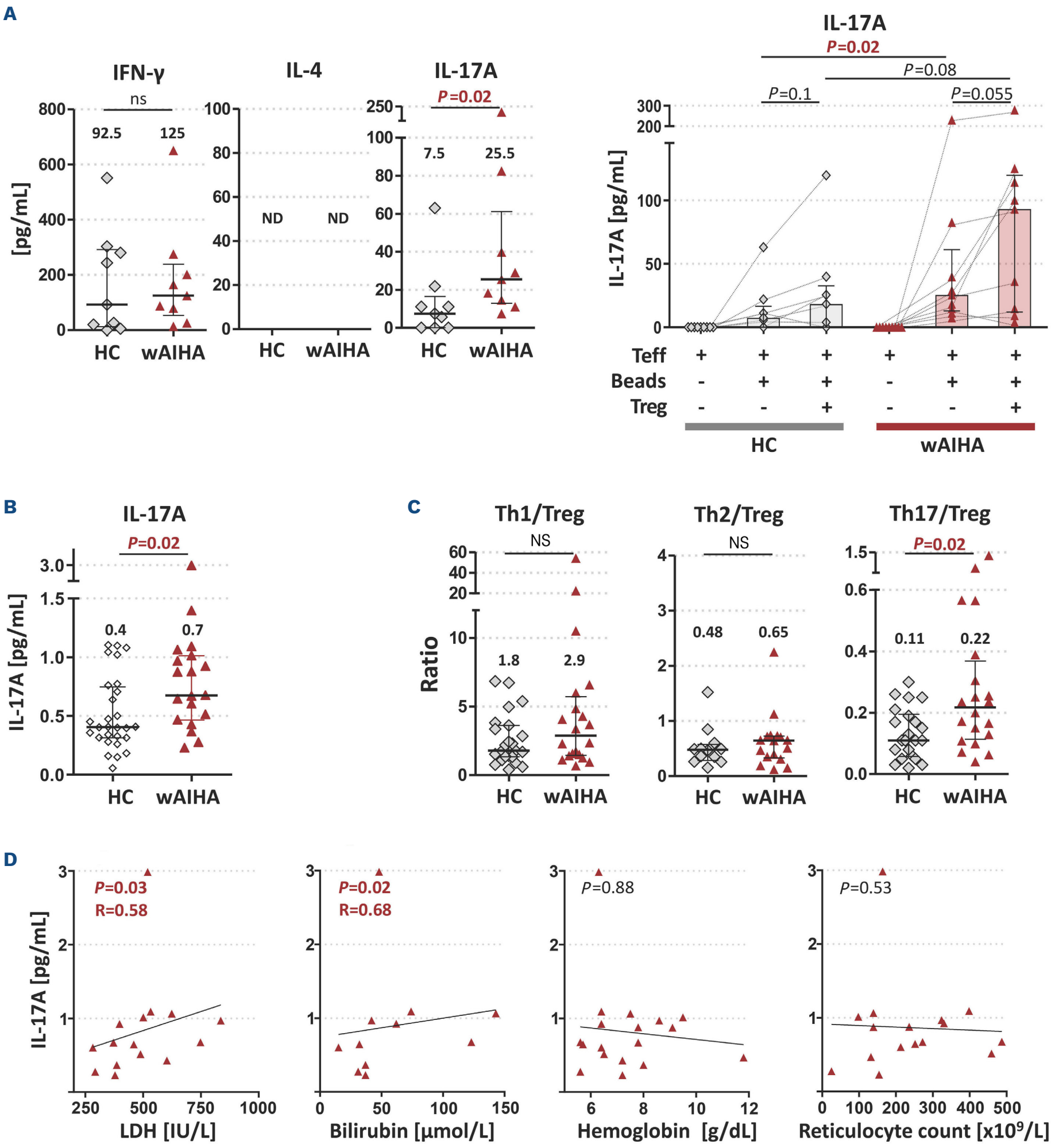


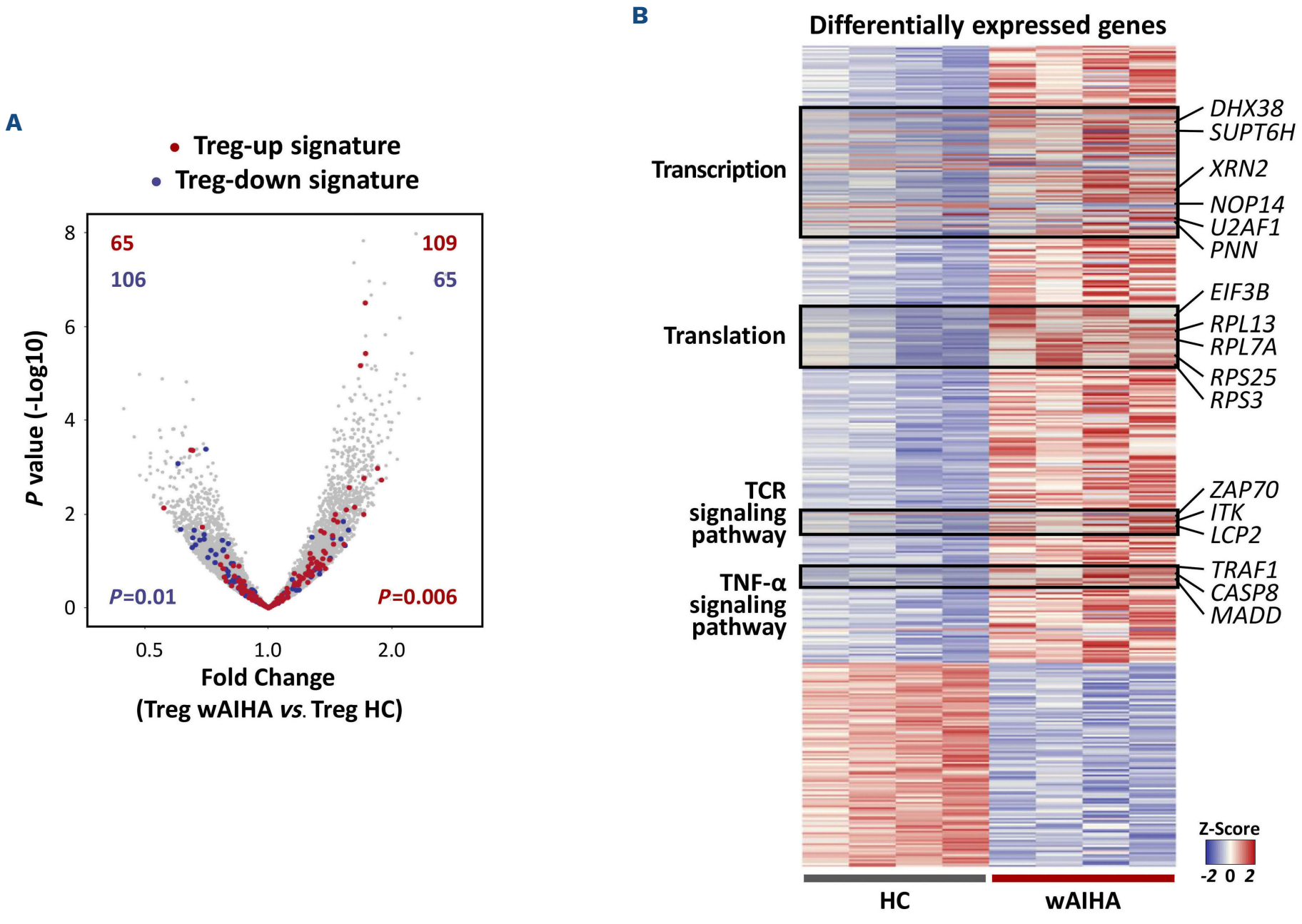
Figure 2. Warm autoimmune hemolytic anemia is associated with a Th17 polarization. (A) Scatter dot plots of interferon (IFN)-γ, interleukin (IL)-4 and IL-17 concentrations measured in supernatants of 4-day culture of CD4 T cells stimulated by anti-CD2/CD3/CD28 microbeads (healthy controls [HC] N=9; warm autoimmune hemolytic anemia [wAIHA] N=9; upper panel). Line plots with histograms showing the concentration of IL-17 measured in culture supernatants of effector T cells (Teff) activated or not with microbeads, with or without regulatory T cells (Treg) (Teff/Treg ratio:2/1; lower panel). (B) Scatter dot plots of serum IL-17A concentrations measured in 20 wAIHA patients and 26 HC. (C) Scatter dot plots showing the balance between pro- and anti-inflammatory immune responses as represented by Th1/Treg, Th2/Treg and Th17/Treg ratios (HC N=23; wAIHA N=20). *P* values derived from Mann-Whitney test, Wilcoxon signed-rank as appropriate. (D) Correlation between serum IL-17 concentration and red blood cell (RBC) destruction markers (lactate dehydrogenase [LDH] and bilirubin), hemoglobin and reticulocyte count. Spearman's rank correlation coefficient (R) and *P* value are depicted. Line represents linear regression. ND: not detected; NS: not significant.

Post-translational mechanisms may downregulate Foxp3 expression in Treg

The maintenance of Foxp3 protein expression is crucial to ensure a stable pool of functional Treg and depends on multiple mechanisms such as transcriptional, translational and post-translational regulation.[35] The fact that *FOXP3* transcripts were increased during wAIHA while Foxp3 protein was diminished suggested translational or post-translational regulatory dysfunctions rather than transcriptional alterations. We thus investigated post-translational mechanisms known to regulate Foxp3 protein expression or function. We observed a higher transcription of genes coding for Pim-2, a protein involved in Foxp3 phosphorylation, for HDAC7, responsible for Foxp3 deacetylation, but also an increase in DBC1 and caspase 8, previously reported to degrade Foxp3 in an inflammatory environment (*Online Supplementary Table S5*).[35] Suggesting a role for TNF-α in these post-translational regulatory mechanisms of Foxp3, TNF index positively correlated with these different transcripts, while negatively associated with Foxp3 protein expression and circulating Treg frequency (*Online Supplementary Figure S4*).

In vitro, the Syk inhibitor fostamatinib decreased both the phagocytosis of red blood cells and the production of TNF-α by monocytes

TNF-α has been targeted in clinical practice for years, notably in rheumatoid arthritis, in which anti-TNF monoclonal antibodies were shown to restore Treg functions.[36] Considering that during wAIHA monocytes and macrophages that are activated after phagocytosis of RBC represent an important source of TNF-α,[37,38] what we confirmed *in vitro* (*Online Supplementary Figure S5*), and that the spleen tyrosine kinase SYK participates to the downstream signaling of the Fc portion of immunoglobulin G receptor (FcγR), we investigated the effects of fostamatinib, on monocyte functions *in vitro*, as it showed promising results in wAIHA.[39] As SYK also participates to the production of TNF-α by monocytes,[40,41] its inhibition could have a dual interest in wAIHA. As expected, R406, the active metabolite of fostamatinib, dramatically decreased phagocytosis of RBC coated with anti-GPA antibody (mean of 58.8% *vs*. 5.6% of monocytes; *P*=0.03; Figure 5A). Interestingly, the production of TNF-α induced by heme and either by anti-GPA-coated RBC or RBC obtained from a patient with active wAIHA were profoundly



Continued on following page.

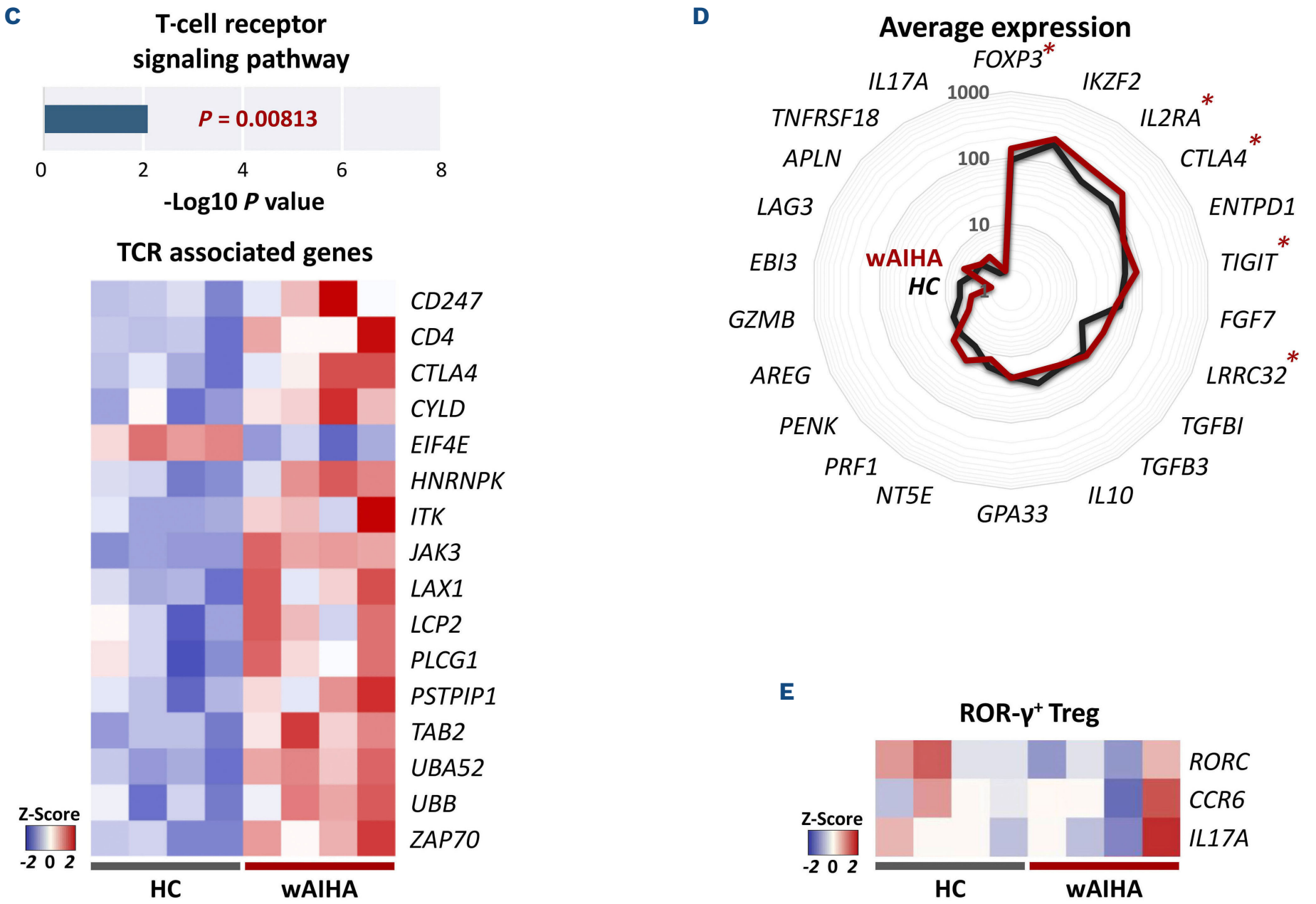


Figure 3. Transcriptomic profiling of Treg showing T-cell receptor activation and the engagement of transcriptional and translational processes. (A) Volcano plot (fold change *vs*. *P* value) displaying the transcriptomes of regulatory T cells (Treg) from patients with warm autoimmune hemolytic anemia (wAIHA) compared to healthy controls (HC). Genes that are part of the Treg signature are highlighted in red and blue when their expression is increased or decreased in wAIHA, respectively. *P* values derived from X^2 test. (B) Heat map showing Treg genes that are differentially expressed between HC (N=4) and wAIHA patients (N=4). The relative gene expression (ζ-score) is represented by color gradient (decreased expression in blue and increased in red). Four of the key terms predicted by gene ontology analysis are annotated along with the main genes implicated. (C) T-cell receptor (TCR) signaling pathway evaluated by gene ontology analysis. *P* value is shown (upper panel) with the heat map of differentially expressed genes (lower panel). (D) Radar chart showing the average expression of transcripts of Treg effector molecules (HC N=4; wAIHA N=4). (E) Heat map showing the relative expression of transcripts used for the identification of ROR-γ^+ Treg between HC (N=4) and wAIHA patients (N=4). The relative gene expression (ζ-score) is depicted by color gradient of blue (downregulated) and red (upregulated). *P<0.05.

Table 2. Expression of genes coding for TNFR pathway effectors involved in Treg function.

Effectors	Genes	Average expression			
		HC	wAIHA	Fold change	*P*
TNFR1	*TNFRSF1A*	49	37	0.78	0.117
TNFR2	*TNFRSF1B*	103	138	1.34	0.023
NF-kB	*NFKB2*	66	97	1.34	0.041
RelB	*RELB*	26	50	1.47	0.074
DBC1	*CCAR2*	61	81	1.33	0.030
PP1	*PPP1CA*	24	40	1.44	0.032

TNFR: tumor necrosis factor receptor; Treg: regulatory T cell; wAIHA: warm autoimmune hemolytic anemia; HC: healthy controls.

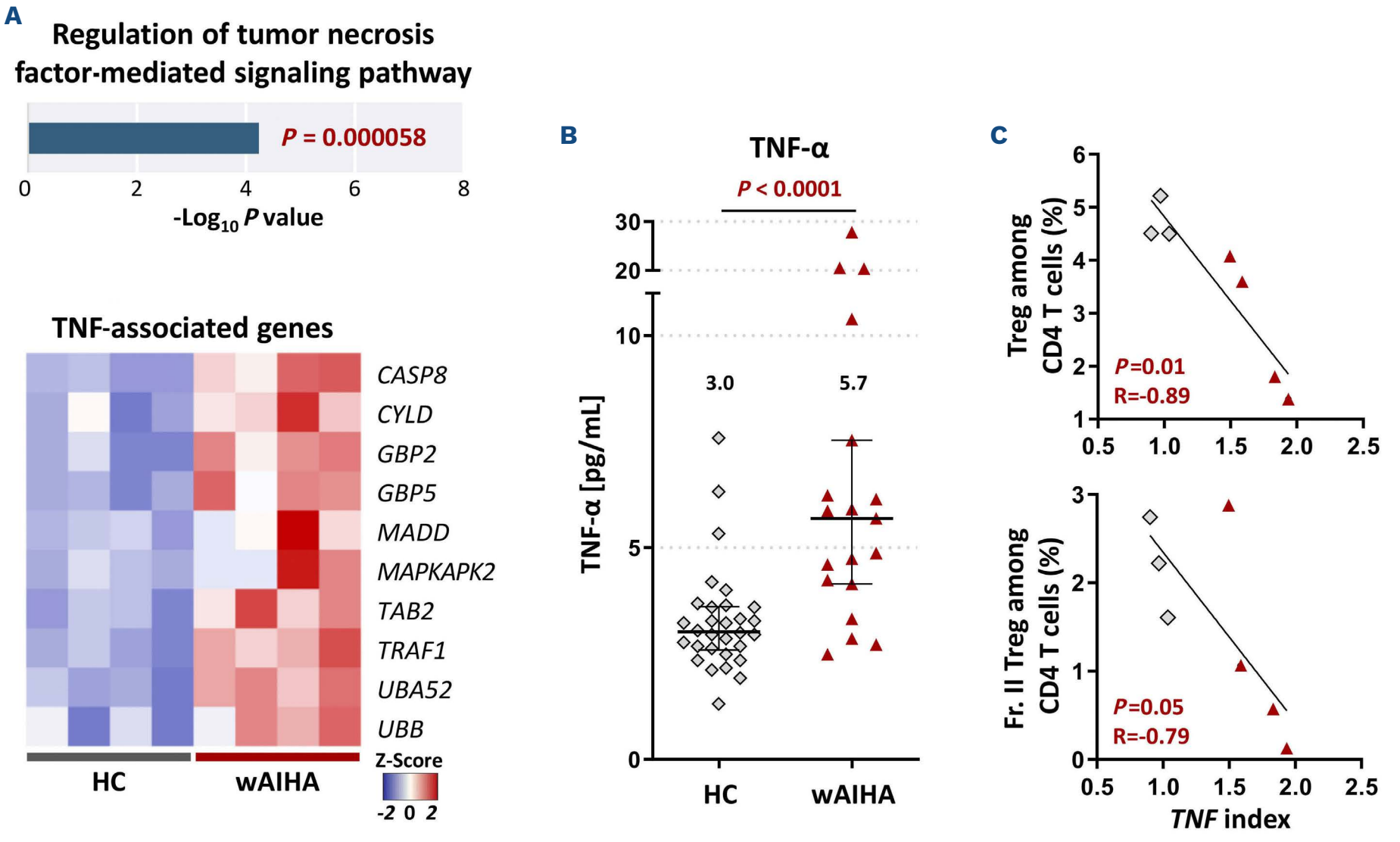


Figure 4. TNF-α signaling engagement correlates with Treg dysfunctions. (A) Tumor necrosis factor (TNF)-mediated signaling pathway was evaluated by gene ontology analysis. *P* value is shown (upper panel) with heat map (lower panel) showing the relative expression of genes (ζ-score) between healthy controls (HC) (N=4) and warm autoimmune hemolytic anemia (wAIHA) patients (N=4), depicted by color gradient of blue (downregulated) and red (upregulated). (B) Scatter dot plots of TNF-α concentration measured in sera from HC (N=26) and wAIHA patients (N=20). *P* values derived from Mann-Whitney test. (C) The activation of the TNF pathway is represented as TNF index, calculated by averaging the normalized expression of TNF-associated genes differentially expressed in regulatory T cells (Treg). Correlation of the TNF index with Treg frequency (upper panel) and Fr.II/effector Treg (eTreg) frequency (lower panel) among circulating CD4 T cells from HC (grey diamonds, N=3) and wAIHA patients (red triangles, N=4). *P* and R values derived from Spearman correlation analysis. (D) Nuclear Foxp3 protein expression assessed by the mean fluorescence intensity (MFI) measured by flow cytometry in circulating Treg from HC (N=24) and wAIHA patients (N=15). Representative histogram of nuclear expression (left panel). Scatter dot plots of Foxp3 MFI among circulating Treg (right panel). (E) Correlation between TNF index and Foxp3 MFI in circulating Treg (HC N=3; wAIHA N=4). *P* and R values derived from Spearman correlation analysis. (F) Box-and-whiskers plots of normalized expression of *FOXP3* transcripts in Treg (HC N=4; wAIHA N=4). *P* values derived from Mann-Whitney test. NS: not significant.

inhibited (mean of 60.3 *vs*. 3.1-fold change compared to unstimulated monocytes; *P*=0.03; 90 *vs*. 19.7-fold change; *P*=0.005; and 35.6 *vs*. 3.3-fold changes; *P*=0.01 respectively; Figure 5B).

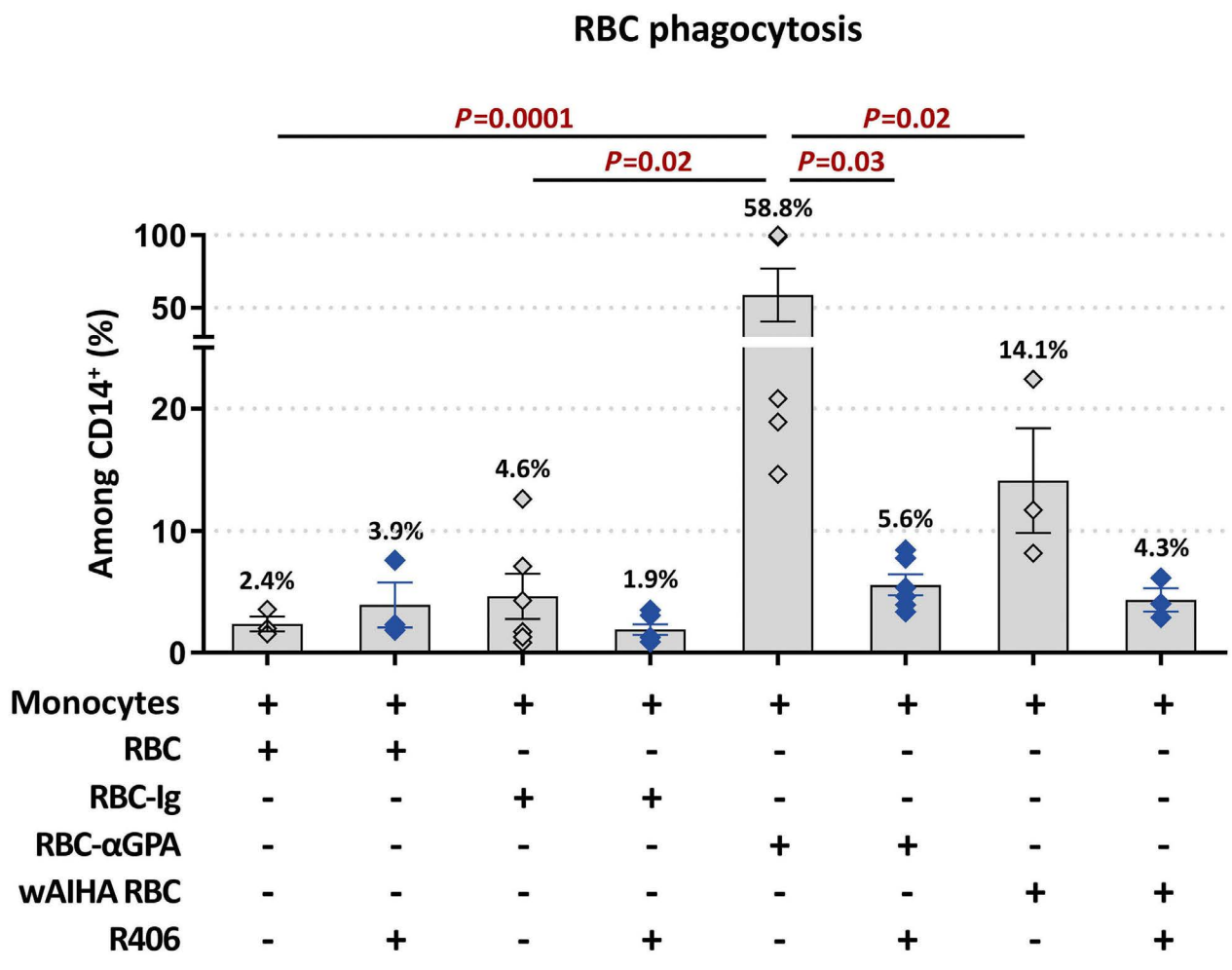
Discussion

Conversely to ITP, the most frequent autoimmune cytopenia, in which a decrease in Treg has been clearly demonstrated,[11,12] the literature is scarce in human wAIHA. We here confirmed that the frequency of circulating Treg in human wAIHA was reduced,[16] and specified that eTreg, the subset with the most intense inhibitory activity,[21] was mostly affected as represented by its two-times lower frequency. We also provide the first functional evaluation of Treg in wAIHA and observed altered functions characterized by a reduced inhibition of Teff proliferation and by the promotion of IL-17 secretion. A dysfunction of Treg has been implicated in the occurrence and the maintenance of multiple autoimmune diseases,[10,13] although the underlying mechanisms are still puzzling. In wAIHA, we observed that Treg dysfunctions were associated with a decreased expression of the major transcription factor Foxp3, that is critical for Treg survival and the maintenance of their inhibitory functions,[31,42] but also to prevent their conversion toward Teff.[43] The lower level of the transcription factor Helios, which associates with Foxp3 and enhances Treg functions, might also participate in these Treg dysfunctions. In order to clarify the processes involved in the diminution of Foxp3 during wAIHA, we used transcriptomic analysis to assess the mechanisms that have been described so far as key regulators of Foxp3,[35] and observed higher transcription of genes involved in Foxp3 phosphorylation or deacetylation, such as Pim2 and HDAC7. The transcripts of DBC1 and caspase 8, previously reported to degrade Foxp3 in an inflammatory environment were also increased.[29] In order to formally demonstrate their involvement in the downregulation of Foxp3 during wAIHA, a specific assessment of Foxp3 phosphorylation and acetylation would be of interest.

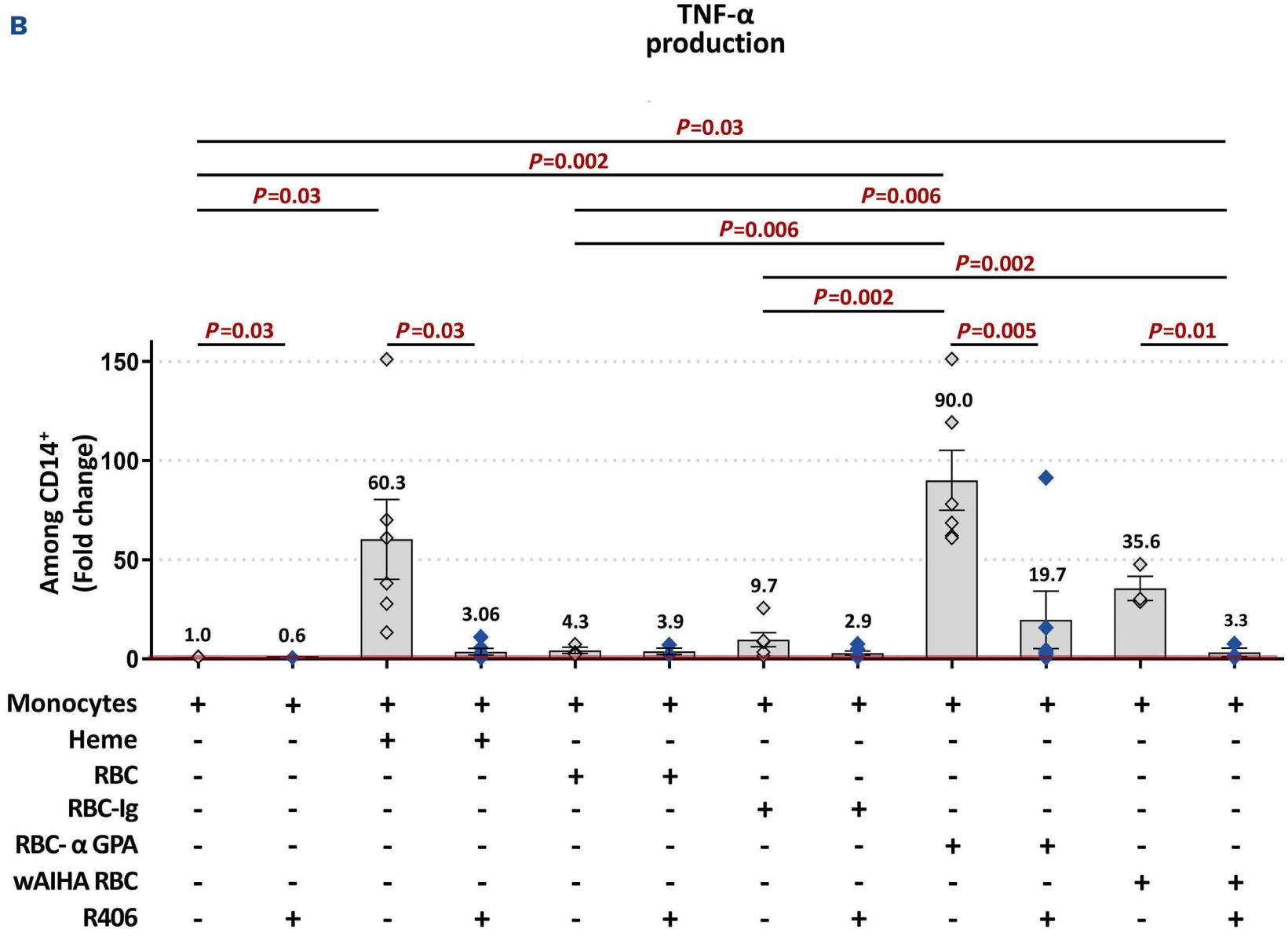
Until recently, TNF-α was not known to be increased in wAIHA and had even been found diminished.[44] However, when measured in the active phase of the disease and prior to the initiation of any treatment, as done in our study and in a recent report,[45] the concentration of TNF-α is twice as high than in controls. *In vitro*, we observed that in conditions mimicking wAIHA, monocytes were the main cells producing TNF-α. As previous studies showed that TNF-α could alter Treg functions[28,36,46,47] and downmodulate Foxp3 expression,[32] we investigated the potential link between TNF-α and Foxp3. Indeed, the more the TNF-α signaling pathway was engaged in Treg, the lower the number of circulating Treg was and the more the level of Foxp3 protein expression was reduced, highly suggesting a pivotal role of TNF-α in Treg dysregulation in wAIHA. However, these results appeared controversial, as it is increasingly recognized that TNF-α, by its binding to TNFR2, has a positive effect on Treg, as represented by the increased expression of CD25 and Foxp3, their expansion and the fostering of their inhibitory properties.[27,48] However, all these conclusions were drawn from studies using Treg from healthy subjects. Moreover, the presence of other cytokines such as IL-2 were required to allow TFN-α to increase Foxp3 expression, the generation of Treg and to foster their inhibiting functions.[48] Conversely, a study performed on Treg obtained under inflammatory conditions from synovial fluid during juvenile arthritis showed an increase in TNFR2 expression and a decrease in Treg immunosuppressive functions, similarly[21] to our results. In the same way, in the presence of TNF-α, a reduction of suppressive functions from Treg obtained from rheumatoid arthritis patients was also observed.[46] On the other hand, TNF blockade restores Treg suppressive functions[28] in rheumatoid arthritis[36,46] and enables the expansion of Treg in ITP.[47] In wAIHA, although we observed an overexpression of *TNFRII* transcripts and an activation of the NF-κB pathway, as reflected by the increase in *RELB* and *NFKB2* transcripts, in accordance with previous reports,[26,28] our functional assay clearly demonstrated a Treg dysfunction. Thus, while TNF-α promotes the functions of Treg from healthy donors *in vitro*,[48] during wAIHA, there is both a functional and quantitative deficit of Treg. Moreover, this deficit is associated with a decrease in Foxp3 expression which could result from post-translational regulation mechanisms that correlate with TNF-α pathway engagement. Notably, our results are supported by a previous study showing that the regulation by DBC1, a protein binding Foxp3 and leading to its degradation in a caspase 8-dependent mechanism can be initiated in an inflammatory environment, i.e., in the presence of IL-6 or TNF-α.[29]

In a mouse model of AIHA, Treg deficiency was not sufficient for disease initiation.[15] In humans, it is impossible to determine whether Treg dysfunction precedes the onset of the disease and promotes it, or whether this deficit merely sustains the disease. However, our data suggest that during wAIHA, the Treg deficit at least perpetuates the autoimmune response, as suggested by the trend to an increased production of IL-17 when Teff are cocultured with Treg. Moreover, hemolysis by itself could indirectly maintain Treg deficiency, as supported by the negative correlation between Foxp3 expression or Treg frequency and TNF-index, while the production of TNF-α by monocytes is increased *in vitro* in the presence of either IgG-opsonized RBC or heme. As the release of heme from RBC can activate Toll-like receptor (TLR)-4 and induces TNF-α secretion by macrophages through a mechanism involving the adaptor protein SYK,[40,41] we investigated the effect of the SYK inhibitor fostamatinib, on monocytes in conditions mimicking wAIHA. The primary mechanism of action of fostamatinib is the inhibition of FcγR signaling pathway and thus phagocytosis of autoantibody-recognized RBC,[39] what we confirmed *in vitro*. We

A



B



Continued on following page.

Figure 5. Fostamatinib reduced red blood cell phagocytosis and TNF-α production by monocytes *in vitro*. Monocytes from 7 healthy controls were cultured 24 hours alone or in conditions mimicking hemolysis, i.e., in the presence of heme or red blood cells (RBC) labeled with CellTrace and either coated with immunoglobulin (Ig)G anti-glycophorin A (RBC/monocytes ratio: 5/1) or in the presence of isotype control (RBC-Ig). RBC obtained from a patient with active warm autoimmune hemolytic anemia (wAIHA) were also used (wAIHA RBC). R406 (2 μM), the active metabolite of fostamatinib was added in the different conditions. In order to exclude RBC attached to monocyte membrane during phagocytosis assay, RBC lysis was performed before flow cytometry. (A) Histograms showing the mean percentage of RBC phagocytosis by monocytes with standard of the mean. (B) Histograms representing the mean fold changes of tumor necrosis factor (TNF)-α production in hemolysis conditions, with or without R406 (lower panel, N=7). The production of TNF-α by unstimulated monocytes was used as reference. *P* values derived from paired *t* test. AGPA: anti-glycophorin A antibody.

also observed a profound diminution of TNF-α production by monocytes in the presence of fostamatinib. Whether neutrophils, that are stimulated by TNF-α and are involved in RBC phagocytosis during wAIHA,[38] also participate in the increase in TNF-α remains to be determined. However, previous publications have reported that neutrophils produce only little amount of TNF-α in humans.[49,50] Finally, fostamatinib, with a response rate observed in nearly half of wAIHA patients in a phase I/II clinical trial, may play a broader action than previously thought, by acting on multiple pathways of wAIHA pathogenesis. These multiple effects could confer to fostamatinib both a short-term effect by reducing RBC phagocytosis and possibly the production of anti-RBC antibodies,[39,51] but also a long-term effect, by restoring the immune tolerance mediated by Treg, which could allow its discontinuation overtime.

The interest of our study also relies on the concomitant evaluation of the anti-inflammatory and the pro-inflammatory responses, as an imbalance is frequent in autoimmune diseases. T-cell polarization in wAIHA was not formally established in humans and was successively related to Th2 polarization,[6] then Th1[7] and finally Th17.[8,9] Our results firmly support the latter as evidenced by the increase in IL-17 concentration in serum and the imbalance of the Th17/Treg ratio, while Th1/Treg and Th2/Treg ratios were unaffected. However, although blood samples were taken before any treatment was started, serum IL-17 concentrations or its production by Teff was highly variable from one patient to another, resulting in overlapping results with controls. Thus, the involvement of pathophysiological mechanisms differing from one patient to another, as is the case in ITP notably,[52] cannot be ruled out. However, the positive correlation between IL-17 concentration and hemolysis parameters such as LDH and bilirubin strongly supports a link between RBC destruction and Th17 polarization. There was also a trend to an increase in IL-17 secretion in co-culture of Teff and Treg from wAIHA conversely to healthy donors, suggesting that in addition to the loss of their inhibitory functions, Treg could promote Th17 polarization.

Taken altogether, our results show that in wAIHA, Treg harbor both quantitative and functional dysfunctions that cannot counteract the pro-inflammatory Th17 polarization of Teff, with a production of IL-17 that correlates with the intensity of RBC destruction. These Treg defects are associated with a downmodulation of Foxp3 expression that could be driven by post-translational mechanisms such as deacetylation and phosphorylation of Foxp3, and also involved DBC1 and caspase 8. The activation of these mechanisms positively correlates with the engagement of the TNF-α pathway in Treg, whose serum concentration is increased. Targeting TNF-α could be a novel approach complementary to current treatments to restore immune tolerance in wAIHA. Along this line, fostamatinib could be a promising treatment as it reduces both the production of TNF-α and the phagocytosis of RBC by monocytes.

Disclosures

SA served on advisory committee boards for Novartis and SOBI; received consultancy fees from Amgen, Argenx and Novartis; received lecture fee/congress support from Amgen, Grifols and Novartis; and received a research grant from Novartis. BB served on advisory committee boards for Novartis; received consultancy fees from Amgen and Novartis; received lecture fees/congress support from Amgen and Novartis. MS received consultancy fees from Abvvie, Boehringer Ingelheim, Novartis, Roche Chugai and Vifor. All other authors have no conflicts of interest to disclose.

Contributions

SA, BB, MC and ASF designed the study. SA, PS and BB funded the research. SA, BB, TM, VP, MS and FM recruited patients and provided clinical information. SA and MC designed the experiments. MC, SO, CC, TG, MN, MT, HG, CG, AR and MT performed experiments. RB, SC, and CR performed RNA sequencing. MC, BL and SA analyzed data. MC, BL and SA drew the figures. SA and MC wrote the manuscript. SA, MC, MS, BL, PS and BB edited the paper.

Acknowledgments

We thank Y. Duffourd and Dr. H. Begue for their valuable advice for RNA-sequencing analysis. We thank Dr. A. Legrand, N. Pernet and S. Monier, from the flow cytometry core facility (INSERM UMR1231, Université de Bourgogne Franche-Comté), for their technical support for flow cytometry and cell sorting.

Funding

This investigation was supported by a research grant (2012-A001154-39) from the GIRCI Est (Groupement Interrégional pour la Recherche Clinique et l'Innovation), by the French Ministry of Health and Solidarity (Referral Centers for Rare

Diseases), and by the French National Institute of Health and Medical Research (INSERM). The flow cytometry core facility is supported by the Burgundy Regional Council.

Data-sharing statement

RNA-sequencing data are available in the Gene Expression Omnibus database (accession number GSE195791).

References

1. Michel M. Warm autoimmune hemolytic anemia: advances in pathophysiology and treatment. Presse Med. 2014;43(4 Pt 2):e97-e104.
2. Barcellini W, Zaninoni A, Giannotta JA, Fattizzo B. New insights in autoimmune hemolytic anemia: from pathogenesis to therapy stage 1. J Clin Med. 2020;9(12):3859.
3. Mahévas M, Michel M, Vingert B, et al. Emergence of long-lived autoreactive plasma cells in the spleen of primary warm autoimmune hemolytic anemia patients treated with rituximab. J Autoimmun. 2015;62:22-30.
4. Shen CR, Mazza G, Perry FE, et al. T-helper 1 dominated responses to erythrocyte Band 3 in NZB mice. Immunology. 1996;89(2):195-199.
5. Youssef AR, Shen CR, Lin CL, Barker RN, Elson CJ. IL-4 and IL-10 modulate autoimmune haemolytic anaemia in NZB mice. Clin Exp Immunol. 2005;139(1):84-89.
6. Fagiolo E, Toriani-Terenzi C. Th1 and Th2 cytokine modulation by IL-10/IL-12 imbalance in autoimmune haemolytic anaemia (AIHA). Autoimmunity. 2002;35(1):39-44.
7. Hall AM, Ward FJ, Vickers MA, Stott LM, Urbaniak SJ, Barker RN. Interleukin-10-mediated regulatory T-cell responses to epitopes on a human red blood cell autoantigen. Blood. 2002;100(13):4529-4536.
8. Hall AM, Zamzami OM, Whibley N, et al. Production of the effector cytokine interleukin-17, rather than interferon-gamma, is more strongly associated with autoimmune hemolytic anemia. Haematologica. 2012;97(10):1494-1500.
9. Xu L, Zhang T, Liu Z, Li Q, Xu Z, Ren T. Critical role of Th17 cells in development of autoimmune hemolytic anemia. Exp Hematol. 2012;40(12):994-1004.
10. Grant CR, Liberal R, Mieli-Vergani G, Vergani D, Longhi MS. Regulatory T-cells in autoimmune diseases: challenges, controversies and - yet - unanswered questions. Autoimmun Rev. 2015;14(2):105-116.
11. Stasi R, Cooper N, Del Poeta G, et al. Analysis of regulatory T-cell changes in patients with idiopathic thrombocytopenic purpura receiving B cell-depleting therapy with rituximab. Blood. 2008;112(4):1147-1150.
12. Yu J, Heck S, Patel V, et al. Defective circulating CD25 regulatory T cells in patients with chronic immune thrombocytopenic purpura. Blood. 2008;112(4):1325-1328.
13. Zufferey A, Kapur R, Semple JW. Pathogenesis and therapeutic mechanisms in immune thrombocytopenia (ITP). J Clin Med. 2017;6(2):16.
14. Mqadmi A, Zheng X, Yazdanbakhsh K. CD4+CD25+ regulatory T cells control induction of autoimmune hemolytic anemia. Blood. 2005;105(9):3746-3748.
15. Richards AL, Kapp LM, Wang X, Howie HL, Hudson KE. Regulatory T cells are dispensable for tolerance to RBC antigens. Front Immunol. 2016;7:348.
16. Ahmad E, Elgohary T, Ibrahim H. Naturally occurring regulatory T cells and interleukins 10 and 12 in the pathogenesis of idiopathic warm autoimmune hemolytic anemia. J Investig Allergol Clin Immunol. 2011;21(4):297-304.
17. Barcellini W, Fattizzo B. The changing landscape of autoimmune hemolytic anemia. Front Immunol. 2020;11:946.
18. Jager U, Barcellini W, Broome CM, et al. Diagnosis and treatment of autoimmune hemolytic anemia in adults: recommendations from the First International Consensus Meeting. Blood Rev. 2019;41:100648.
19. Ferraro A, D'Alise AM, Raj T, et al. Interindividual variation in human T regulatory cells. Proc Natl Acad Sci U S A. 2014;111(12):E1111-1120.
20. Galvan-Pena S, Leon J, Chowdhary K, et al. Profound Treg perturbations correlate with COVID-19 severity. Proc Natl Acad Sci U S A. 2021;118(37):e2111315118.
21. Miyara M, Yoshioka Y, Kitoh A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity. 2009;30(6):899-911.
22. Grover P, Goel PN, Greene MI. Regulatory T cells: regulation of identity and function. Front Immunol. 2021;12:750542.
23. Sakaguchi S, Mikami N, Wing JB, Tanaka A, Ichiyama K, Ohkura N. Regulatory T cells and human disease. Annu Rev Immunol. 2020;38:541-566.
24. Opstelten R, de Kivit S, Slot MC, et al. GPA33: a marker to identify stable human regulatory T cells. J Immunol. 2020;204(12):3139-3148.
25. Komatsu N, Okamoto K, Sawa S, et al. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. Nat Med. 2014;20(1):62-68.
26. Wang J, Ferreira R, Lu W, et al. TNFR2 ligation in human T regulatory cells enhances IL2-induced cell proliferation through the non-canonical NF-κB pathway. Sci Rep. 2018;8(1):12079.
27. Salomon BL. Insights into the biology and therapeutic implications of TNF and regulatory T cells. Nat Rev Rheumatol. 2021;17(8):487-504.
28. Nagar M, Jacob-Hirsch J, Vernitsky H, et al. TNF activates a NF-kappaB-regulated cellular program in human CD45RA- regulatory T cells that modulates their suppressive function. J Immunol. 2010;184(7):3570-3581.
29. Gao Y, Tang J, Chen W, et al. Inflammation negatively regulates FOXP3 and regulatory T-cell function via DBC1. Proc Natl Acad Sci U S A. 2015;112(25):E3246-3254.
30. Nie H, Zheng Y, Li R, et al. Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-α in rheumatoid arthritis. Nat Med. 2013;19(3):322-328.
31. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol. 2007;8(3):277-284.
32. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood. 2006;108(1):253-261.
33. Seng A, Krausz KL, Pei D, et al. Coexpression of FOXP3 and a Helios isoform enhances the effectiveness of human engineered regulatory T cells. Blood Adv. 2020;4(7):1325-1339.
34. Takatori H, Kawashima H, Matsuki A, et al. Helios enhances Treg cell function in cooperation with FoxP3. Arthritis Rheumatol.

2015;67(6):1491-1502.
35. Colamatteo A, Carbone F, Bruzzaniti S, et al. Molecular mechanisms controlling Foxp3 expression in health and autoimmunity: from epigenetic to post-translational regulation. Front Immunol. 2019;10:3136.
36. Ehrenstein MR, Evans JG, Singh A, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J Exp Med. 2004;200(3):277-285.
37. Gallagher MT, Branch DR, Mison A, Petz LD. Evaluation of reticuloendothelial function in autoimmune hemolytic anemia using an in vitro assay of monocyte-macrophage interaction with erythrocytes. Exp Hematol. 1983;11(1):82-89.
38. Meinderts SM, Oldenborg PA, Beuger BM, et al. Human and murine splenic neutrophils are potent phagocytes of IgG-opsonized red blood cells. Blood Adv. 2017;1(14):875-886.
39. Kuter DJ, Rogers KA, Boxer MA, et al. Fostamatinib for the treatment of warm antibody autoimmune hemolytic anemia: phase 2, multicenter, open-label study. Am J Hematol. 2022;97(6):691-699.
40. Fortes GB, Alves LS, de Oliveira R, et al. Heme induces programmed necrosis on macrophages through autocrine TNF and ROS production. Blood. 2012;119(10):2368-2375.
41. Prestes EB, Alves LS, Rodrigues DAS, et al. Mitochondrial reactive oxygen species participate in signaling triggered by heme in macrophages and upon hemolysis. J Immunol. 2020;205(10):2795-2805.
42. Zhou X, Bailey-Bucktrout S, Jeker LT, Bluestone JA. Plasticity of CD4(+) FoxP3(+) T cells. Curr Opin Immunol. 2009;21(3):281-285.
43. Bailey-Bucktrout SL, Martinez-Llordella M, Zhou X, et al. Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. Immunity. 2013;39(5):949-962.
44. Barcellini W, Zaja F, Zaninoni A, et al. Low-dose rituximab in adult patients with idiopathic autoimmune hemolytic anemia: clinical efficacy and biologic studies. Blood. 2012;119(16):3691-3697.
45. Branch DR, Leger RM, Sakac D, et al. Chemokines IP-10/CXCL10 and IL-8/CXCL8 are potential novel biomarkers of warm autoimmune hemolytic anemia. Blood Adv. 2022;7(10):2166-2170.
46. Zanin-Zhorov A, Ding Y, Kumari S, et al. Protein kinase C-theta mediates negative feedback on regulatory T cell function. Science. 2010;328(5976):372-376.
47. Zhong H, Bussel J, Yazdanbakhsh K. In vitro TNF blockade enhances ex vivo expansion of regulatory T cells in patients with immune thrombocytopenia. Br J Haematol. 2015;168(2):274-283.
48. Zaragoza B, Chen X, Oppenheim JJ, et al. Suppressive activity of human regulatory T cells is maintained in the presence of TNF. Nat Med. 2016;22(1):16-17.
49. Cassatella MA, Meda L, Bonora S, Ceska M, Constantin G. Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta in mediating the production of IL-8 triggered by lipopolysaccharide. J Exp Med. 1993;178(6):2207-2211.
50. Tecchio C, Micheletti A, Cassatella MA. Neutrophil-derived cytokines: facts beyond expression. Front Immunol. 2014;5:508.
51. Roders N, Herr F, Ambroise G, et al. SYK inhibition induces apoptosis in germinal center-like B cells by modulating the antiapoptotic protein myeloid cell leukemia-1, affecting B-cell activation and antibody production. Front Immunol. 2018;9:787.
52. Audia S, Mahevas M, Nivet M, Ouandji S, Ciudad M, Bonnotte B. Immune thrombocytopenia: recent advances in pathogenesis and treatments. Hemasphere. 2021;5(6):e574.