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

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Detailed material and methods

Patients

Healthy controls (HC) and patients with newly diagnosed wAIHA referred to our center 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). In case DAT was strongly positive for C3d, a cold agglutinin test was performed. None of the patients included had cold agglutinins. 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 (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.

Whole blood from patients or HC was collected in lithium heparin tubes and processed within hour. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient medium (Lymphoprep, Eurobio) and split for immunophenotyping, cell culture and storage. Cell cultures were performed in RPMI 1640 medium (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Eurobio), and 1% penicillin, streptavidin and fungizone (Lonza). Part of the PBMC was stored in liquid nitrogen for later use.

Immunophenotyping

Immunophenotyping was performed on fresh PBMC, antibodies and reagents are detailed in **Supplemental Table S1**. Intracellular cytokine production was measured after stimulation with phorbol-12-myristate-13-acetate (PMA, 100 ng/mL, Sigma-Aldrich) and ionomycin (1µg/mL, Sigma-Aldrich), for 4 hours, in the presence of brefeldin A (GolgiPlug, 0.1%, BD Biosciences). Data were acquired on BD Biosciences LSRII cytometer and analyzed with FlowJo® v10 software (BD Biosciences).

T cell proliferation suppression assay

Teff ($CD4^+CD25^-$) and Treg ($CD4^+CD25^{hi}$) were purified from fresh PBMC by magnetic cell isolation ($CD4^+CD25^+$ Regulatory T Cell Isolation Kit, Miltenyi Biotec). Teff labeled with CellTrace violet (Invitrogen) were seeded at $5x10^4$ cells/wells in 96-wells plate. Cells were activated with anti-CD2/CD3/CD28 microbeads (Treg suppression inspector, Miltenyi Biotec) and cultured for 4 days with or without Treg (Teff/Treg ratio=2/1). Proliferation was measured by flow cytometry based on CellTrace dilution. The proliferation index (PI) was computed from ModFit LT software (Verity Software House).

Assessment of monocyte function during hemolysis

Monocytes were isolated by positive magnetic selection using CD14 microbeads (Miltenyi Biotec) and seeded at 10^6 cells/wells in 24-wells plate. Cells were cultured during 24h alone or stimulated with hemin (40 µM, Sigma-Aldrich) or allogenic RBC (5.10^6 cells/wells) labeled with CellTrace and coated with anti-glycophorin A (R&D systems) or in presence of isotype control (R&D systems). RBC collected from a patient with relapsing wAIHA were also used. The effect of R406 (2 µM, Invivogen), the active metabolite of fostamatinib was also investigated. Flow cytometry was used to quantify TNF-α production and RBC phagocytosis. Data were acquired on Attune NxT flow cytometer.

RNA sequencing

Frozen PBMC samples from 4 primary AIHA and 4 sex- and age-matched HC were used for RNA sequencing (RNAseq). Their characteristics are reported in **supplemental Table S2**. After dead cell removal (Dead Cell Removal Kit, Miltenyi Biotech), Fluorescent activated cell sorting (FACS) of Treg ($FVS780^{-}CD3^{+}CD4^{+}CD25^{hi}CD127^{-}$) was performed on FACSAria III sorter (BD Biosciences). Cells were recovered into lysis buffer (RLT Buffer, Qiagen) for total RNA extraction using the Maxwell RSC RNA FFPE kit (Promega). RNAseq was processed in one batch. Ribosomal RNA-depleted RNA was used for library preparation with the NEBNext Ultra II Directional RNA library prep kit for Illumina (New England BioLabs). Libraries were paired and sequenced (2x76 base pairs) on a NextSeq500 device (Illumina), with a read depth of 40 million. Kallisto software was used for quantifying transcript abundance from RNA-seq data against GRCh38 cDNA reference transcriptome from the Ensembl database (v96). Only protein-coding transcripts and genes were included in the downstream analysis. Differential expression analysis was performed using DEseq-2 R script and SARTools package in R environment (Galaxyeast, Galaxy instance). Genes with a P-value below 0.05 and normalized read count greater than 25 were considered as significantly differentially expressed. RNA-sequencing data are available in the Gene Expression Omnibus database (accession number GSE195791).

Gene Ontology Terms, Treg signature, and index computations

Gene set enrichment analysis (GSEA) was performed using DAVID Bioinformatics Resources website (v6.8) for all differentially expressed genes. Treg transcriptomic identity was assessed by referring to the Treg-signature genes.[19] To reduce noise, genes with an inter-replicate CV intra-replicate <0.7 and an expression level >20 in either comparison groups were selected. The overlap significance into our dataset was assessed by Chi square test. Tumor necrosis factor (TNF) index was calculated for each subject by averaging the normalized expression of all genes belonging to TNF pathway (normalized expression *versus* 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 (R&D systems). Data were acquired on Bio-Plex® 200 system and analyzed with Bio-Plex Manager software (Bio-Rad). High sensitivity ELISA was performed for serum IL-17A (Invitrogen).

Statistics

Statistical analyses and graphs were performed with Prism v9.3.0 (GraphPad Software), R Studio (1.4.1717) and Heatmaper. Unless specified, data are reported as the median with interquartile range, except for box plots that expressed the median with min and max values. Data were compared using non-parametric tests when either non-normal distribution or unequal variances were observed (Mann-Whitney test for independent data, Wilcoxon signed-rank test for paired conditions, and Spearman's rank correlation test for correlation analyses) or parametric tests otherwise (unpaired or paired t test as appropriate). P-value <0.05 was considered significant.

Supplemental Table S1

Antibodies and reagents used for flow cytometry.

Antibodies	Conjugated Fluorophore	Clone	Supplier	Catalogue number
CD3	PECy7	SK7	BD Biosciences	557851
CD3	BV510	OKT3	BioLegend	317332
CD4	PECy7	SK3	eBioscience	25-0047-42
CD4/CD25	PECy5/PE	nc.	BioLegend	320403
CD14	PECy7	61D3	eBioscience	25-0149-42
CD45RA	APC eFluor780	HI100	eBioscience	47-0458-42
CD127	BV510	A019D5	BioLegend	351332
CCR7	PE	3D12	eBioscience	12-1979-42
Foxp-3	AF488	259D	BioLegend	320211
Helios	PB	22F6	BioLegend	137220
IFN-γ	APC	4S.B3	eBioscience	17-7319-82
IL-17	PE	eBio64CAP17	eBioscience	12-7178-42
IL-4	AF488	8D4-8	eBioscience	53-7049-42
TNF-α	PE	MAb11	eBioscience	12-7349-82
Reagent			**Supplier**	**Catalogue number**
Fixable Viability Stain 780			BD Biosciences	565388
Intracellular Fixation & Permeabilization Buffer Set			eBioscience	88-8824-00
True-Nuclear™ Transcription Factor Buffer Set			BioLegend	424401

nc: not communicated; AF488: alexa fluor 488; APC: allophycocyanin; BV510: Brilliant Violet 510; Cy:cyanin; PB: pacific blue; PE: phycoerythrin.

Supplemental Table S2.

Characteristics of wAIHA patients whose samples were used for Treg transcriptomic analysis.

	wAIHA without RNAseq n=18	wAIHA with RNAseq n=4	P-value
Age. years - Median [25 and 75% percentile]	63 [55 - 80]	76 [38 - 75]	0.24
Sex ratio. Female/Male	12/6	3/1	>0.999
Primary AIHA. n (%)	14 (78)	3 (75)	na
Hemoglobin. g/dL - Median [25 and 75% percentile]	7.35 [6.15 - 9.20]	6.45 [5.80 - 7.63]	0.5
Reticulocytes. x109/L - Median [25 and 75% percentile]	164 [139 - 272]	330 [25 - 459]	0.65
Leukocytes. x109/L - Median [25 and 75% percentile]	6.4 [4.48 - 8.80]	5.4 [4.48 - 5.9]	0.37
Lymphocytes. x109/L - Median [25 and 75% percentile]	1845 [0.81 - 2.66]	790 [0.47 - 1.70]	0.13
Platelets. x109/L - Median [25 and 75% percentile]	222 [160 - 314]	193 [171 - 344]	0.86
Haptoglobin. g/L - Median [25 and 75% percentile]	0,00 [0.00 - 0.10]	0.08 [0.00 - 5.74]	0.44
Direct antiglobulin test. n (%)	18 (100)	4 (100)	na
Treg. % among CD4 T cells - Median [25 and 75% percentile]	3.21 [2.36 - 4.07]	2.66 [1.48 - 3.93]	0.62
nTreg. % among CD4 T cells - Median [25 and 75% percentile]	0.49 [0.39 - 1.25]	0.48 [0.08 - 1.60]	0.4
eTreg. % among CD4 T cells - Median [25 and 75% percentile]	1.18 [0.60 - 1.53]	0.81 [0.22 - 2.40]	0.68
Treg Helios+. % among CD4 T cells - Median [25 and 75% percentile]	2.35 [1.71 - 2.89]	2 [2.00 - 3.21]	0.88
TEM. % among CD4 T cells - Median [25 and 75% percentile]	28.1 [22.6 - 37.0]	48.9 [17.3 - 70.8]	0.42
TCM. % among CD4 T cells - Median [25 and 75% percentile]	15.3 [12.3 - 23.0]	9.99 [4.99 - 20.2]	0.22
TN. % among CD4 T cells - Median [25 and 75% percentile]	34.2 [20.7 - 49.4]	18.5 [1.94 - 56.6]	0.4
TEMRA. % among CD4 T cells - Median [25 and 75% percentile]	0.95 [0.66 - 1.61]	0.61 [0.44 - 12.0]	0.41
Th17. % among CD4 T cells - Median [25 and 75% percentile]	0.53 [0.32 - 0.80]	0.8 [0.37 - 1.25]	0.38
Th17/Treg. ratio - Median [25 and 75% percentile]	0.22 [0.11 - 0.29]	0.37 [0.10 - 0.87]	0.62
Serum IL-17A. pg/ml - Median [25 and 75% percentile]	0.87 [0.60 - 1.06]	0.44 [0.30 - 0.82]	0.12
Serum TNF-α. pg/ml - Median [25 and 75% percentile]	4.74 [3.32 - 6.14]	6.88 [5.21 - 22.7]	0.1
Foxp3. MFI among Treg - Median [25 and 75% percentile]	662 [440 - 1058]	809 [266 - 1207]	0.85

na: not applicable; nTreg: naive Treg ($CD45RA^{+}Foxp3^{lo}$); eTreg: effector Treg ($CD45RA^{-}Foxp3^{hi}$); TEM: effector memory T cells; TCM: central memory T cells; TN: naïve T cells; TEMRA: terminally differentiated effector memory.

Supplemental Table S3.

Comparison of primary and secondary wAIHA.

	Primary wAIHA n=17	Secondary wAIHA n=5	P-value
Age. years - Median [25 and 75% percentile]	66 [55 - 80]	58 [38 - 75]	0.54
Sex ratio. Female/Male	11/6	4/1	>0.999
Hemoglobin. g/dL - Median [25 and 75% percentile]	7.4 [5.7 - 9.2]	6.8 [6.3 - 7.7]	0.64
Reticulocytes. x109/L - Median [25 and 75% percentile]	176 [124 - 343]	218 [157 - 316]	0.51
Leukocytes. x109/L - Median [25 and 75% percentile]	6.2 [5.0 - 8.8]	3.8 [2.4 - 5.9]	0.1
Lymphocytes. x109/L - Median [25 and 75% percentile]	1.7 [0.8 - 2.6]	0.8 [0.5 - 2.0]	0.3
Platelets. x109/L - Median [25 and 75% percentile]	197 [162 - 325]	189 [117 - 295]	0.56
Haptoglobin. g/L - Median [25 and 75% percentile]	0,0 [0.0 - 0.4]	0,0 [0.0 - 0.0]	0.24
Treg. % among CD4 T cells - Median [25 and 75% percentile]	3.04 [1.77 - 3.75]	3.60 [2.59 - 5.12]	0.27
nTreg. % among CD4 T cells - Median [25 and 75% percentile]	0.44 [0.18 - 1.10]	1.02 [0.68 - 1.80]	0.10
eTreg. % among CD4 T cells - Median [25 and 75% percentile]	0.77 [0.13 - 1.44]	1.52 [1.10 - 2.20]	0.13
Treg Helios+. % among CD4 T cells - Median [25 and 75% percentile]	2.01 [0.95 - 2.8]	2.90 [2.20 - 4.11]	0.06
TEM. % among CD4 T cells - Median [25 and 75% percentile]	32.3 [25.4 - 47.3]	27.0 [12.2 - 30.7]	0.15
TCM. % among CD4 T cells - Median [25 and 75% percentile]	13.8 [7.8 - 23.15]	18.7 [13.35 - 22.2]	0.43
TN. % among CD4 T cells - Median [25 and 75% percentile]	25.10 [15.3 - 48.3]	41.50 [34.6 - 66.9]	0.07
TEMRA. % among CD4 T cells - Median [25 and 75% percentile]	1.05 [0.55 - 1.89]	0.71 [0.57- 0.85]	0.19
Th17. % among CD4 T cells - Median [25 and 75% percentile]	0.59 [0.33 - 0.99]	0.41 [0.27 - 0.62]	0.22
Th17/Treg. ratio - Median [25 and 75% percentile]	0.24 [0.13 - 0.57]	0.15 [0.06 - 0.21]	0.10
Serum IL-17A. pg/ml - Median [25 and 75% percentile]	0.66 [0.46 - 0.98]	0.82 [0.45- 2.19]	0.39
Serum TNF-α. pg/ml - Median [25 and 75% percentile]	5.37 [3.94 - 9.76]	5.70 [3.54 - 8.96]	0.96
Foxp3. MFI among Treg - Median [25 and 75% percentile]	536 [407 - 1102]	1090 [953 - 1227]	0.23

nTreg: naive Treg ($CD45RA^{+}Foxp3^{lo}$); eTreg: effector Treg ($CD45RA^{-}Foxp3^{hi}$); TEM: effector memory T cells; TCM: central memory T cells; TN: naïve T cells; TEMRA: terminally differentiated effector memory.

Supplemental Table S4.

Comparison of absolute values of circulating CD4 T cell populations between wAIHA and HC.

	HC	wAIHA	P-value
CD4 T cells, x10^9/L - Median [25 and 75% percentile]	0.86 [0.62 - 1.28]	0.59 [0.36 - 1.46]	0.14
Treg, x10^9/L - Median [25 and 75% percentile]	0.038 [0.026 - 0.048]	0.011 [0.007 – 0.042]	**0.008**
nTreg, x10^9/L - Median [25 and 75% percentile]	0.005 [0.002 - 0.009]	0.005 [0.001 - 0.008]	0.76
eTreg, x10^9/L - Median [25 and 75% percentile]	0.018 [0.014 - 0.024]	0.004 [0.001 - 0.010]	**<0.0001**
Treg Helios$^+$, x10^9/L - Median [25 and 75% percentile]	0.028 [0.020 - 0.035]	0.008 [0.004 - 0.034]	**0.02**
TEM, 10^9/L - Median [25 and 75% percentile]	0.23 [0.15 - 0.36]	0.26 [0.08 - 0.40]	0.87
TCM, x10^9/L - Median [25 and 75% percentile]	0.15 [0.10 - 0.20]	0.10 [0.05 - 0.18]	0.14
TN, x10^9/L - Median [25 and 75% percentile]	0.31 [0.18 - 0.55]	0.21 [0.05 - 0.51]	0.26
TEMRA, x10^9/L - Median [25 and 75% percentile]	0.010 [0.006 - 0.018]	0.010 [0.004 - 0.019]	0.77
Th1, x10^9/L - Median [25 and 75% percentile]	0.094 [0.055 - 0.130]	0.081 [0.017 - 0.116]	0.47
Th2, x10^9/L - Median [25 and 75% percentile]	0.018 [0.010 - 0.023]	0.01 [0.006 - 0.021]	0.25
Th17, x10^9/L - Median [25 and 75% percentile]	0.004 [0.002 - 0.007]	0.003 [0.002 - 0.005]	0.43

nTreg: naive Treg (CD45RA$^+$Foxp3^{lo}); eTreg: effector Treg (CD45RA$^-$Foxp3^{hi}); TEM: effector memory T cells; TCM: central memory T cells; TN: naive T cells; TEMRA: terminally differentiated effector memory.

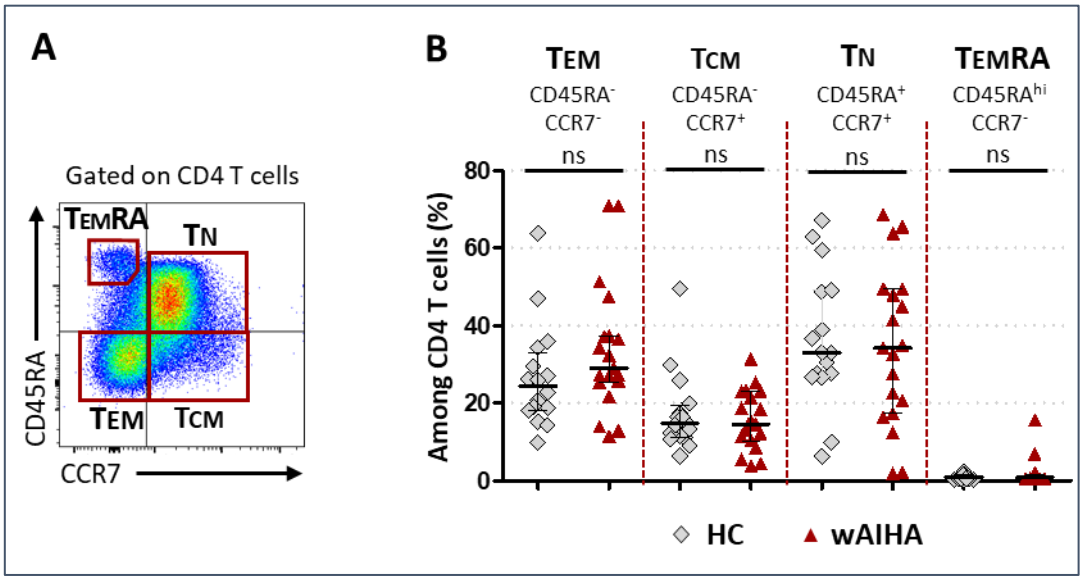
Supplemental Table S5.

Expression of genes coding for proteins involved in post-translational regulation of Foxp3.

				Average expression			
Effectors	**Impact on Foxp3**	**Effects on Treg**	**Genes**	**HC**	**wAIHA**	**Fold-change**	**P-value**
	Acetylation						
Sirtuin 1	-	↘ Foxp3, ↘ Treg function	*SIRT1*	57	60	1.11	0.549
MST1	+	↗ Foxp3, ↗ Treg function	*MST1*	23	24	1.02	0.930
HDAC7	-	↘ Foxp3	*HDAC7*	46	68	1.40	**0.022**
TIP60	+	↗ Foxp3 stability	*KAT5*	20	22	1.02	0.917
TAZ	-	↘ Foxp3 stability	*TAZ*	16	18	1.11	0.591
p300	+	↗ Foxp3 stability	*P300*	198	254	1.24	nc
	Phosphorylation						
Pim-1	+	↘ Foxp3, ↘ Treg function	*PIM1*	65	85	1.32	0.121
Pim-2	+	↘ Treg stability, ↘ Treg function	*PIM2*	50	118	2.04	**0.000013**
Cdk2	+	↘ Foxp3, ↘ Treg function	*CDK2*	14	24	1.42	0.100
NLK	+	↗ Foxp3	*NLK*	21	23	1.16	0.484
	Ubiquitination						
Stub1	+	↘ Foxp3, ↘ Treg function	*STUB1*	14	32	1.71	**0.008**
USP7	-	↗ Foxp3 expression	*USP7*	142	212	1.42	**0.009**
HIF-1α	+	↘ Foxp3, ↗ Th17/Treg ratio	*HIF1A*	114	125	1.03	0.837
Traf6	+	↘ Treg function	*TRAF6*	27	30	0.98	0.929
	Other mechanism						
DBC1		↘ Foxp3 upon TNF-α stimulation, ↘ Treg function	*DBC1*	61	81	1.33	**0.030**
Caspase 8			*CASP8*	200	288	1.39	**0.0002**

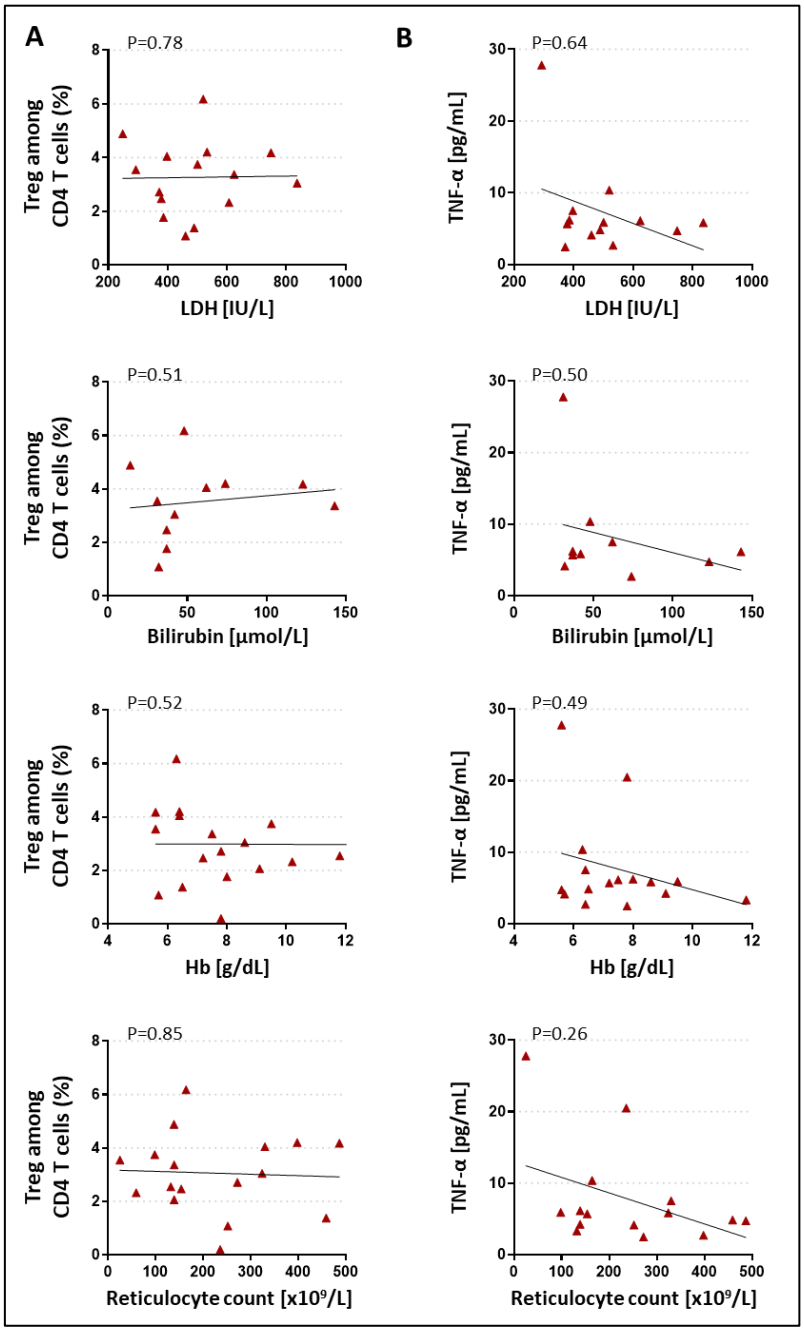
nc: not calculated due to outlier

Supplemental Figure 1.



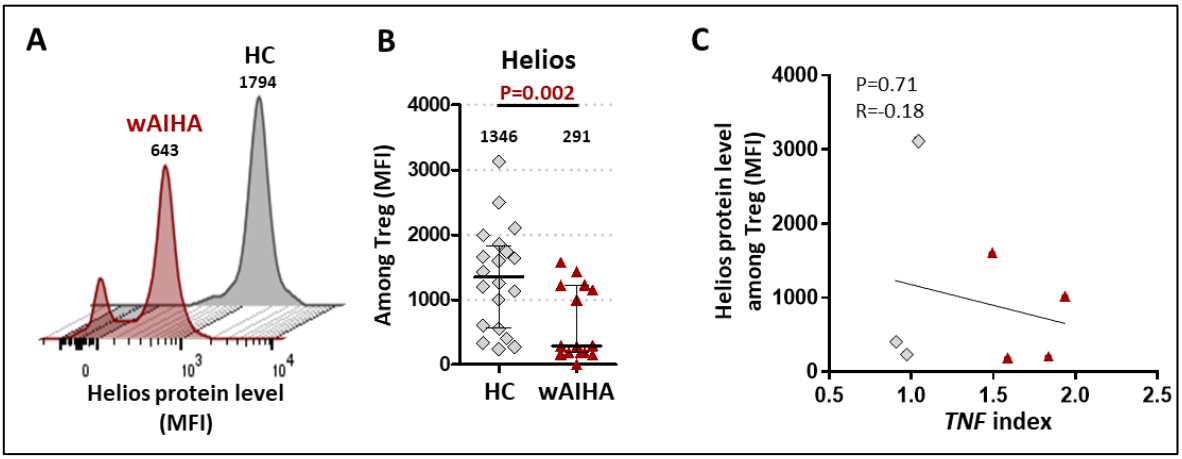
Quantification of circulating effector T cell subpopulations. (**A**) Dot plot showing the gating strategy for the identification of CD4 T cell subsets among peripheral blood mononuclear cell (PBMC) by flow cytometry: effector memory (Tem), central memory (Tcm), naive (Tn) and terminally differentiated effector memory (TemRA) were distinguished according to CD45RA and CCR7 expression within CD4 T cells. (**B**) Frequencies of the different CD4 T cell subsets from HC (n=17), and wAIHA patients (n=19) are reported in scatter dot plots.

Supplemental Figure 2



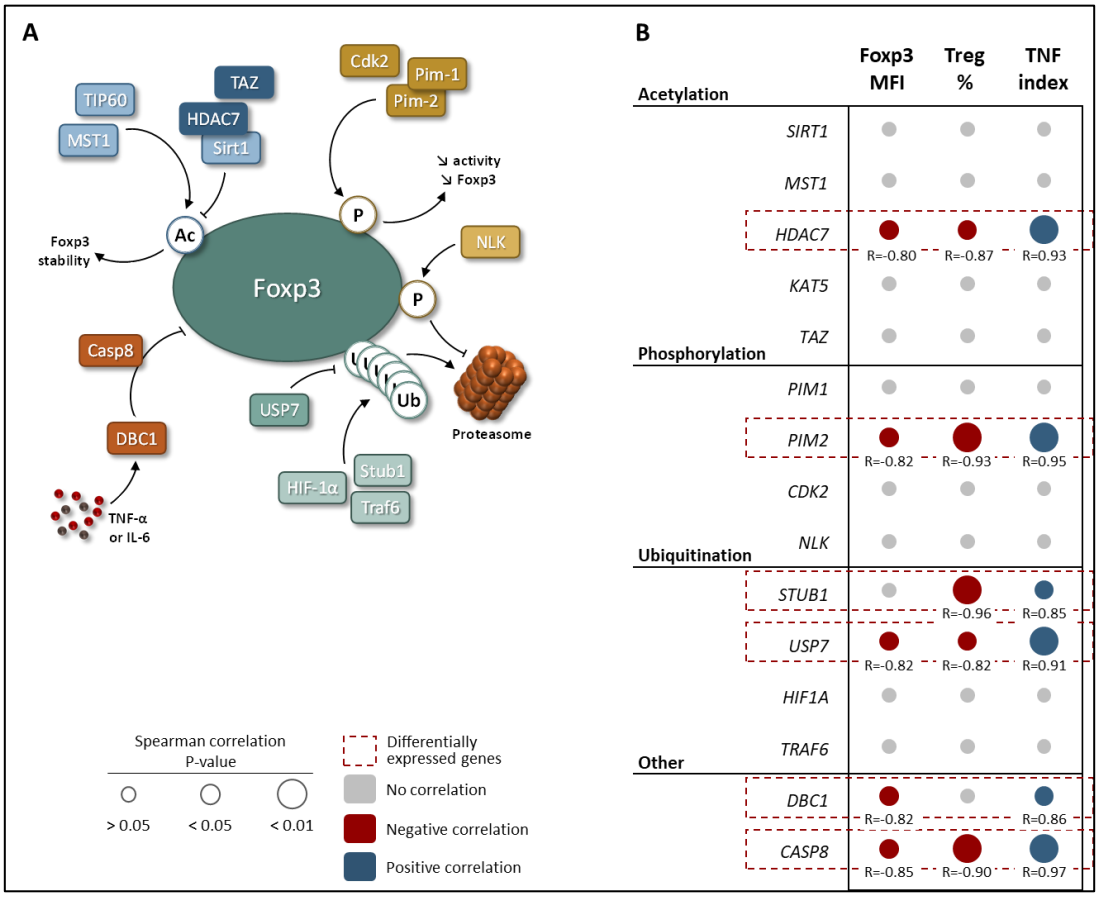
Correlation between circulating Treg frequencies or serum TNF-α concentrations and markers of RBC destruction or production. Neither (**A**) the frequency of circulating T reg, nor (**B**) the concentration of serum TNF-α correlated with RBC destruction markers (LDH and bilirubin), hemoglobin and reticulocyte count. P-value derived from Spearman's correlation test. Line represents linear regression.

Supplemental Figure 3



Expression of Helios in circulating Treg. (**A**) Nuclear Helios protein expression assessed by the mean fluorescence intensity (MFI) measured in circulating Treg from HC (n=24) and wAIHA patients (n=15). Representative histogram of its nuclear expression. (**B**) Scatter dot plots of Helios MFI among circulating Treg. P-values derived from Mann-Whitney test. (**C**) Correlation between TNF index and Helios MFI in circulating Treg from HC and wAIHA patients (HC n=3; wAIHA n=4). P- and R-values derived from Spearman correlation analysis.

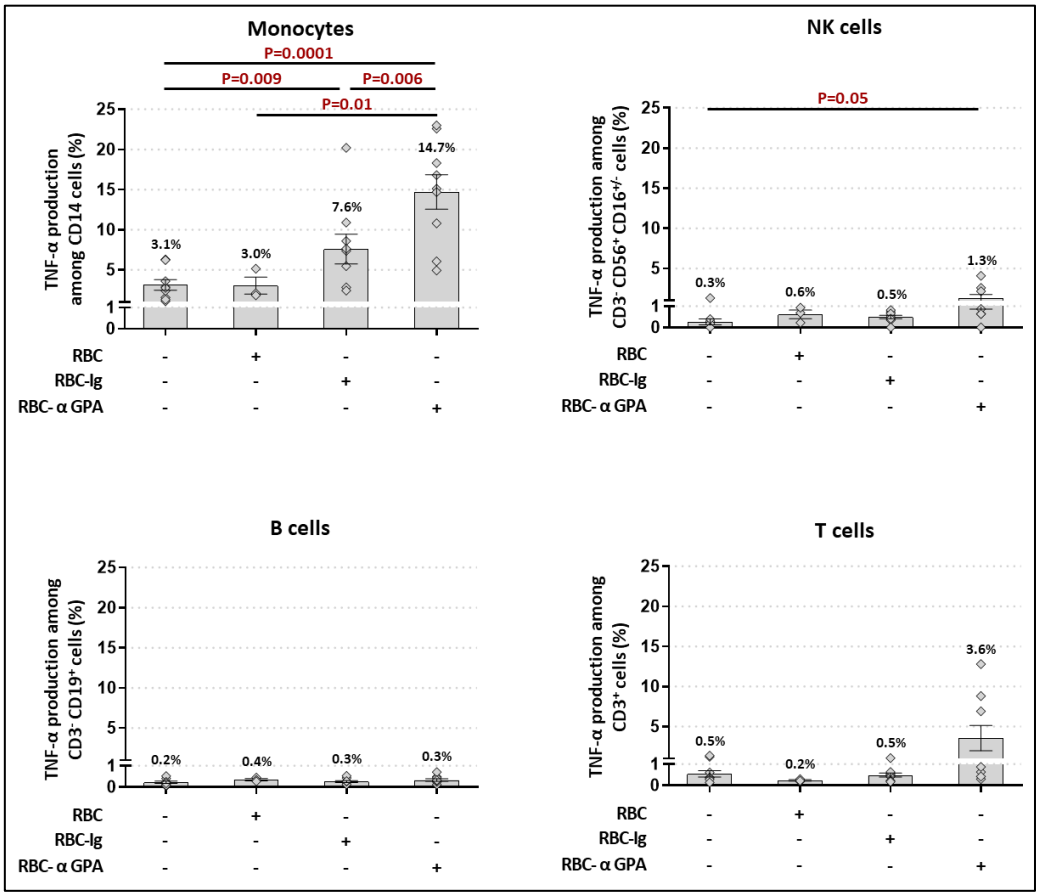
Supplemental Figure 4



***STUB1, USP7, DBC1,* and *CASP8* transcript overexpression is correlated with the frequency of circulating Treg, with Foxp3 protein level in Treg, and with the activation of TNF pathway.**

(**A**) Representation of the various post-transcriptional mechanisms involved in Foxp3 regulation (adapted from Colamatteo *et al*.): ubiquitination (Ub) mediated by ubiquitin-specific protease (USP)7, STIP1 homology and U-Box containing protein (Stub)1, TNF receptor associated factor (Traf)6, and hypoxia-inducible factor (HIF)-1α; acetylation (Ac) mediated by tat-interactive protein 60 kDa (TIP60), or Mammalian Sterile 20-like Kinase 1 (MST1) and deacetylation performed by Histone deacetylase (HDAC)7, sirtuin (Sirt)1, transcriptional co-activator with PDZ-binding motif (TAZ); phosphorylation (P) supported by cyclin-dependent kinase (Cdk)2, proto-oncogene serine/threonine-protein kinase (Pim)-1, Pim-2, and Nemo-like kinase (NLK); and degradation of Foxp3 involving deleted breast cancer (DBC)1 and caspase (Casp)8. (**B**) Dot plots showing the correlation between the expression of Foxp3 post-translational regulator transcripts and Foxp3 MFI in circulating Treg, circulating Treg frequency, and TNF index. Only R-values of significant correlations are shown. Positive correlations are depicted in blue, and negative correlations in red. P- and R-values derived from Spearman correlation analysis.

Supplemental Figure 5



Determination of circulating TNF-α producing cells under hemolysis conditions. PBMC obtained from healthy donors were cultured in various conditions to determine the cells producing TNF-α during hemolysis. Percentages of cells producing TNF-α was determined by flow cytometry after 1 day of culture. Histograms representing the mean value of percentage of cells producing TNF-α with bars representing the standard error of the mean. The production of TNF-α by unstimulated cells was used as reference. P-values derived from paired t test. NS, non-stimulated; RBC: red blood cells; RBC-Ig: red blood cells with isotype control; RBC-αGPA: red blood cells coated with anti-glycophorin A antibody.