

SUPPLEMENTAL MATERIAL

Supplementary text

Materials and Methods

Study population

Forty-one GCA patients and 28 healthy blood donors (HD) were recruited for this cross-sectional study. All patients fulfilled the American College of Rheumatology (ACR) 1990 classification criteria for GCA. In all cases addition, the diagnosis of GCA was additionally based on either positive temporal artery biopsy (n=3) or vascular imaging demonstrating changes compatible with large vessel vasculitis (n=38). Vascular imaging included magnetic resonance angiography (n=4) or positron emission tomography (n=31). GCA patients were classified into active (n=14) and cases in remission (n=27). In addition to the ACR 1990 classification criteria, the diagnosis of GCA was based on either positive temporal artery biopsy (TAB) or vascular imaging. Imaging changes confirming the diagnosis of GCA included increased arterial wall thickness, mural contrast enhancement or increased uptake of 18-fluorodeoxyglucose by the aorta and/or its main branches. Studied patients were classified based on disease activity: active cases (n=14) and in remission (n=27). Active disease was defined on the basis of symptoms and/or clinical signs including new-onset headache, jaw/tongue claudication, scalp tenderness, diplopia, symptoms of polymyalgia rheumatica, scotomas, amaurosis fugax, ischemic optic neuropathy and/or otherwise unexplained systemic symptoms such as fever and night sweat, with or without concomitant elevation of acute-phase reactants, i.e. erythrocyte sedimentation rate (ESR) and/or C-reactive protein (CRP). Disease remission was defined as the absence of symptoms/clinical signs of GCA and normal ESR (<20 mm/1st hour) and CRP (<5 mg/l) at time of blood sampling. Recruited patients were treated according to the guidelines of the German Society for Rheumatology.¹ Characteristics of studied subjects and the assays for which their samples were used are shown in Suppl. Table 1. Healthy blood donors (HD) included anonymously recruited blood donors from the blood donation service, Institute for Transfusion Medicine and Transplant Engineering, Hannover Medical School and donors from the Clinic of Rheumatology and Immunology, Hannover Medical School.

PBMC isolation, FACS sorting

Whole blood was collected in EDTA tubes. PBMC isolation was performed at the same day of blood sampling, by layering whole blood (diluted 1:3 v/v in sterile phosphate buffered saline (PBS), Lonza) over density gradient medium (Pancoll human 1.077 g/ml; PAN-Biotech), and centrifuged at room temperature. PBMCs were collected, washed once in complete RPMI medium (Roswell Park Memorial Institute 1640 medium (Thermo Fisher), supplemented with 10% v/v heat-inactivated fetal calf serum (FCS) (PAN-Biotech), 1% v/v Penicillin-Streptomycin (Gibco), 1% v/v L-glutamine (Gibco), 1% v/v sodium pyruvate (Gibco)) and cryopreserved in freezing medium (heat-inactivated FCS, 10% v/v dimethyl sulfoxide) until use. Upon cell thawing, PBMCs were rested overnight in complete RPMI medium (at 37°C, 5% CO₂) for recovery, and collected in the morning for further processing. After washing once in FACS buffer (PBS, 5% v/v FCS), the cells were stained with antibodies in the presence of Octagam 10% (Octapharma). PBMCs were stained with CD4-BV421 (clone RPA-T4, BD Biosciences), CD25-BV510 (clone 2A3, BD Biosciences-OptiBuild), CD127-PE (clone A019D5, BioLegend) antibodies, and Fixable Viability Dye eFluor780 (Thermo Fisher). Tregs were gated as live CD4⁺CD25^{hi}CD127^{lo} (Suppl. Figure 1) sorted under aseptic condition in BD FACSAria Fusion cell sorter (Becton-Dickinson). Cell sorting typically yielded high enrichment (>90%) of Tregs.

RNA-Sequencing and raw data processing

PBMC isolation and Treg sorting were performed at the same day of blood sampling as described above. Sorted Tregs were washed once in sterile, ice-cold PBS. Total RNA was extracted using RNeasy Micro Kit (Qiagen), according to the protocol. A minimum of 350 pg of total RNA were used for library preparation with the 'SMARTer Stranded Total RNA-Seq Kit v2 – Pico Input Mammalian' (#634413; Takara/Clontech) according to conditions recommended in user manual #063017. Generated libraries were barcoded by dual indexing approach and were finally amplified with 13 cycles of PCR. Sequencing was performed on an Illumina NextSeq 550 sequencer using a High Output Flowcell for single reads (20024906; Illumina). Generated libraries were amplified with 13 cycles of PCR. Fragment length distribution was monitored using

'Bioanalyzer High Sensitivity DNA Assay' (5067-4626; Agilent Technologies). Quantification of libraries was performed by use of the 'Qubit® dsDNA HS Assay Kit' (Q32854; ThermoFisher Scientific). Equal molar amounts of the libraries were pooled for a common sequencing run. The library pool was denatured with NaOH and was finally diluted to 2pM according to the Denature and Dilute Libraries Guide (Document # 15048776 v02; Illumina). 1.3 ml of denatured pool was loaded on an Illumina NextSeq 550 sequencer using a High Output Flowcell for single reads (20024906; Illumina). Sequencing was performed with the following settings: Sequence reads 1 and 2 with 38 bases each; Index reads 1 and 2 with 8 bases each.

BCL files were converted to FASTQ files using bcl2fastq Conversion Software version v2.20.0.422 (Illumina). Raw data processing was conducted by use of nfcorn/rnaseq (version 1.4.2) which is a bioinformatics best-practice analysis pipeline used for RNA sequencing data at the National Genomics Infrastructure at SciLifeLab Stockholm, Sweden. The pipeline uses Nextflow, a bioinformatics workflow tool. It pre-processes raw data from FastQ inputs, aligns the reads and performs extensive quality-control on the results. The genome reference and annotation data were taken from GENCODE.org (Homo sapiens; GRCh38.p13; release 34). Normalization and differential expression analysis was performed with DESeq2 (Galaxy Tool Version 2.11.40.2) with default settings except for "Output normalized counts table", "Turn off outliers filtering", and "Turn off independent filtering", all of which were set to "True". Volcano plot was prepared with ggplot2 package on R (v.4.0.5). Heatmaps of selected genes were prepared in Excel file. Output from Galaxy was log₂-transformed, median of log₂-transformed values was calculated for each row, and then baseline normalization to the median was performed. Coloring was applied with conditional formatting tool, with red and blue colors indicating highest and lowest expression for each gene, respectively.

Flow cytometry - Phenotypic characterization of Tregs

Surface staining was performed in the presence of Octagam 10% (Octapharma) for Fc receptor blocking. GARP-PerCP-Cy5.5 (BioLegend, clone 7B11), CD45RA-APC (BioLegend, clone HI100) and TIGIT-Alexa Fluor 647 (BioLegend, clone A15153G) were used for surface staining at 4°C, in the dark, for 30 minutes. For

intracellular staining, True-Nuclear Transcription Factor staining buffer set (BioLegend) was used according to the protocol. FOXP3-PerCP-Cy5.5 (BD Biosciences, clone 236A/E), FOXP3-Alexa Fluor 488 (BioLegend, clone 150D), human IL17A-PE/Cy7 (BioLegend, clone BL168) and IRF4-PE-Cy7 (BioLegend, clone IRF4.3E4) were used. Acquisition of the data by FACSCanto II (Becton-Dickinson). Analysis by FlowJo v.X.0.7 (Tree Star). Median fluorescence intensity (MFI) was determined in the sorted Tregs population, or cell frequencies (%parent) were determined, using fluorescence-minus-one (FMO) controls as appropriate.

For phenotypic characterization of Tregs, sorted cells were washed once in ice-cold complete RPMI medium, and plated in U-bottom, 96-well plates (Corning) in complete RPMI medium at 37°C, 5% CO₂. For TCR stimulation, Dynabeads Human T-activator CD3/CD28 (Gibco) was used (bead-to-cell ratio of 1:1). For *in vitro* glycolysis inhibition, 2-deoxyglucose (Sigma) was prepared in cell culture grade water, sterile filtered (0.2 µm) and resuspended in complete RPMI medium to the final concentration of 2 mM. Glycolysis inhibition was performed for 48 hours (37°C, 5% CO₂) prior to downstream assays (with or without TCR stimulation), except otherwise indicated.

Calcium flux assay

For clinical samples (GCA patients and HD), the whole PBMCs were stained with CD4-BV421, CD25-BV510, CD127-PE and loaded with Fluo-3 (10 µM) and Fura-Red (25 µM) as calcium indicators. For glycolysis inhibition experiment (with less constraint on sample availability), healthy Tregs were FACS-sorted (described above) before Fluo-3/Fura-Red loading. After Fluo-3/Fura-Red loading, cells were washed and resuspended in calcium-free Hank Balanced Salt Solution (HBSS) medium (supplemented with 0.5 mM EGTA). Data were acquired with FACSCanto II (Becton-Dickinson). 100 µl of loaded cells were diluted with 100 µl calcium-free HBSS in a 5 ml FACS tube, and then the baseline calcium flux was recorded for the first 60 seconds. Afterwards, anti-CD3 antibody (clone OKT3) was added to the final concentration of 5 µg/ml. Calcium flux was recorded for further 120 seconds. Then, calcium chloride (CaCl₂) was added to the final concentration of 7 mM, and the flux was recorded for 150 seconds after calcium addition (so-called "SOCE

period"). Lastly, ionomycin (14 $\mu\text{g/ml}$) was added and the maximal calcium flux was recorded for 60 seconds. Each sample was measured twice. Analysis was performed with FlowJo v.X.0.7 (Tree Star). Time series were extracted, and Fluo-3 to Fura-Red ratios were calculated for the recorded timepoints. The values for calcium signaling quantification were based on median of the values during SOCE period (after CaCl_2 addition), normalized to the baseline Fluo-3/Fura-Red ratios. For glycolysis inhibition experiment (48 hours of 2-deoxyglucose), calcium flux was measured in the presence of 2-deoxyglucose in the HBSS medium (final concentration 2 mM).

Treg suppression assay

Tregs (live $\text{CD4}^+\text{CD25}^{\text{hi}}\text{CD127}^{\text{lo}}$) and Tconvs (live $\text{CD4}^+\text{CD25}^{\text{lo}}\text{CD127}^{\text{hi}}$) were sorted aseptically with FACSAria Fusion as described above. Both cell subsets were resuspended in complete RPMI medium, rested in a 37°C , 5% CO_2 incubator for 2 hours to allow recovery. Tregs were plated in a round-bottom 96-well plate (Greiner Bio One), whereas Tconvs were loaded with CFSE (BD Biosciences) at final concentration of 1 μM as per the company's instruction, then washed and plated in the presence or absence of autologous Tregs at 1:1 ratio. The cells were stimulated with Dynabeads CD3/CD28 (Thermo Fisher) at 1:10 ratio (bead:total cell) and were left in a 37°C , 5% CO_2 incubator for 72 hours before harvesting. Harvested cells were washed with FACS buffer, stained with eFluor 506 Fixable Viability Dye (Invitrogen), and the CFSE dilution was acquired with FACSCanto II. Gating of proliferating cells was determined by the negative control (without Dynabeads stimulation) from each individual. The percentage of proliferating cells has been calculated as the ratio of %-proliferating cells in presence of Tregs (experimental condition) to %-proliferating cells in absence of Tregs (positive control).

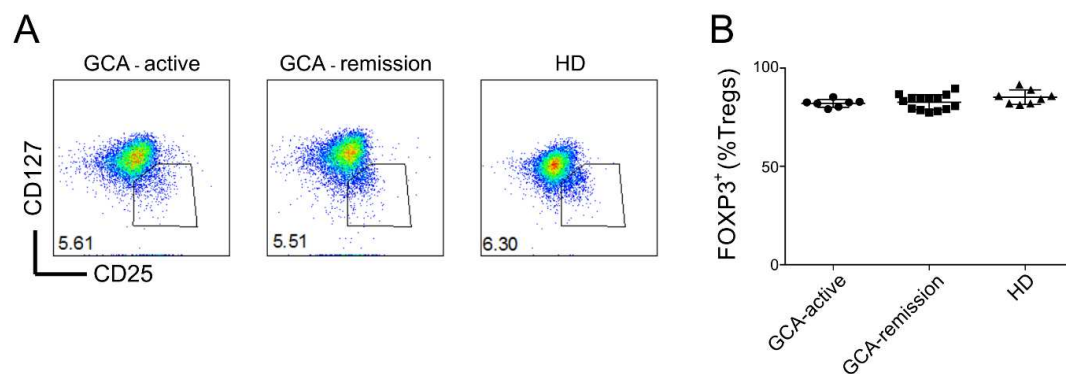
Statistical analysis

Data were tested for normal distribution, using D'Agostino-Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests. When normality was reached, two-group comparison was done with unpaired Student's t-

test. For glycolysis inhibition experiments, paired Student's t-test was performed (uninhibited vs. 2-deoxyglucose treatment for the same biological replicates). When normality was not reached, Mann-Whitney test was performed. Comparison of more than two groups was performed with the Kruskal-Wallis test. Categorical variables were compared by the Fisher's exact test. A value of 0.05 was set as the threshold for statistical significance, with 95% confidence level, two-sided (*); ** indicates p-value < 0.01, *** p-value < 0.001. All statistical tests were done with GraphPad Prism 6 (GraphPad Software Inc.).

Supplemental Figures and Tables

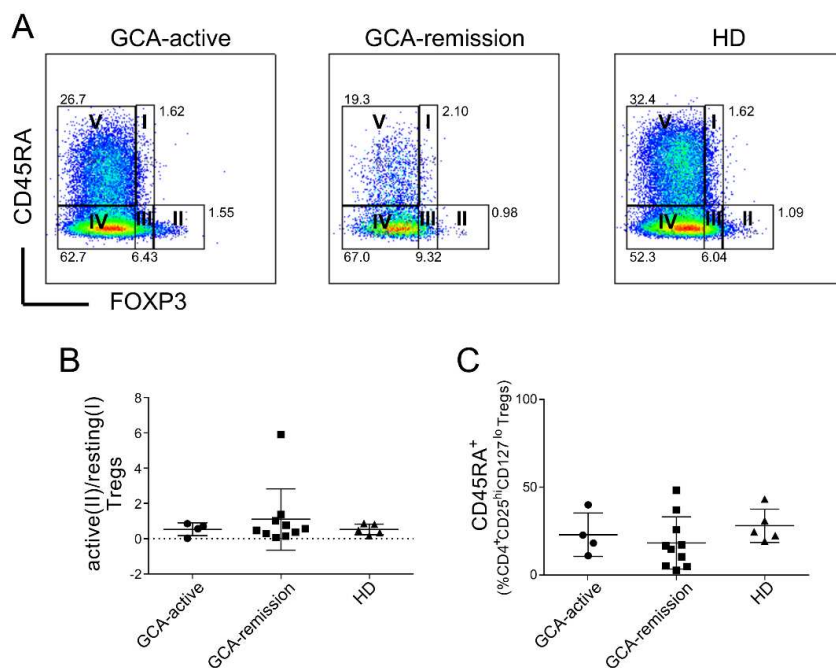
Supplemental Figure 1:



Gating strategy for defining Tregs. Viable CD4⁺ cells were gated as Tregs based on their expression of CD25 and CD127. (A) Representative plots from patients with GCA (active and in remission) and a blood donor (HD) are depicted. (B) Percentage of FOXP3⁺ cells among Tregs sorted as CD4⁺CD25^{hi}CD127^{lo} cells.

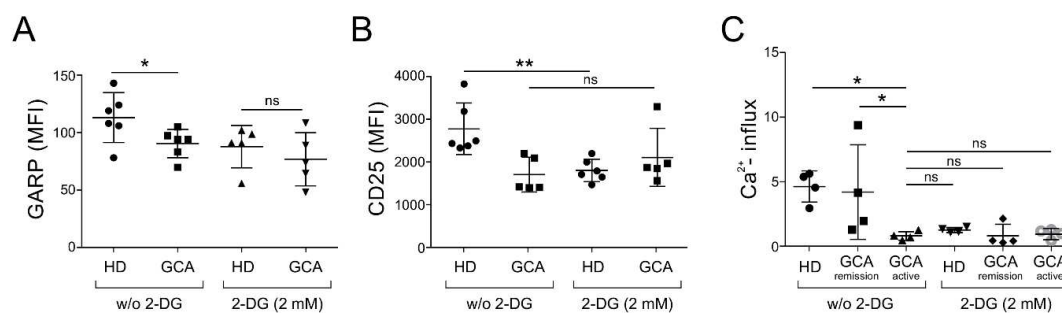
Supplemental Figure 2:

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Comparable differentiation-activation status of Tregs in GCA patients and HD. (A) Gating strategy to define the differentiation states of Tregs as defined by Miyara et al. (see reference 33 of main text). (B) Ratios of active to resting Tregs as defined by Miyara et al. (see reference 33 of main text). (C) Percentage of CD45RA-expressing cells in CD4⁺CD25^{hi}CD127^{lo}-gated Tregs.

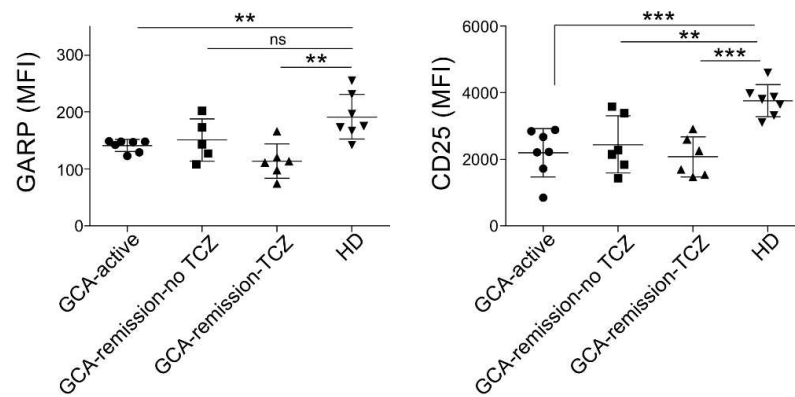
Supplemental Figure 3:



Glycolysis inhibition with 2-deoxyglucose (2-DG), comparative analysis of Tregs from HDs and GCA patients. (A) Protein expression of GARP after 18 hours of CD3/CD28 stimulation, following 48 hours of pre-incubation with or without (w/o) 2-DG. (B) Protein expression of CD25 after 18 hours of CD3/CD28

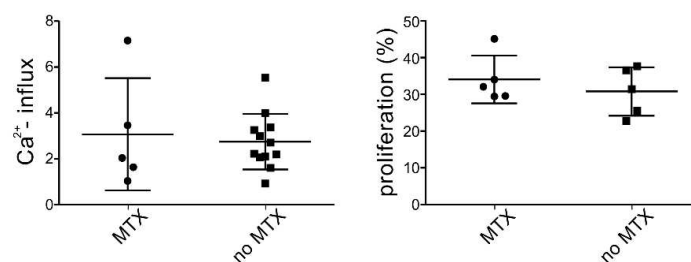
stimulation, following 48 hours of pre-incubation with or without (w/o) 2-DG. Unstimulated cells from HDs and GCA patients displayed similar levels of CD25 expression after pre-incubation with or without 2-DG (not shown). (C) Anti-CD3 activated calcium flux in Tregs (normalized to baseline) after CaCl_2 addition in cells pre-incubated with or without 2-DG.

Supplemental Figure 4:



Tocilizumab did not normalize CD25 and GARP induction after CD3/CD28 stimulation. Protein expression of GARP (left) and CD25 (right), both after 18 hours of CD3/CD28 stimulation, expressed as median fluorescence intensity (MFI). Statistics: unpaired Student's t-tests (except GARP GCA active vs. HD: Mann-Whitney test).

Suppl. Figure 5



Subanalysis of GCA patients in remission with and without methotrexate (MTX)-containing regimens. Anti-CD3 activated calcium flux in Tregs (normalized to baseline) after CaCl_2 addition (left panel) and Treg suppression assay results, shown as %proliferation of conventional T cells in the presence of Treg ($\text{CD4}^+\text{CD25}^{\text{hi}}\text{CD127}^{\text{lo}}$) normalized to the positive control of each sample (without Tregs).

Suppl. Table 1: Characteristics of studied patients with GCA and sample recruitment for different assays

ID	disease status	gender	diagnosis confirmation	CRP (mg/l)	ESR 1 hour (mm)	Age	disease duration	prednisolon equivalent dose (mg) at test	no treatment	TCZ	csDMARD	Calcium Influx assay	RNA-Seq.	FC Fig. 2&3*	FC (Treg differentiation & IL-17 expression)	FC 2-DG assays	Calcium influx assay with 2-DG	Suppression assay
3	R	F	MRI	3.6	26	64.6	4.1	0	0	0	AZA	1						
4	R	F	PET	1.7	29	71	0.3	5	0	0	0	1	1					
5	R	M	PET	0.5	18	81.3	9.8	2.5	0	0	AZA	1						
6	R	F	PET	2.8	26	55.3	3.9	0	0	0	MTX	1						
8	R	M	PET	0.0	0.1	55.8	1.4	2	0	1	0	1			1		1	
9	R	F	PET	1.0	26	79.7	14.6	5	0	1	0	1		1	1			
10	R	F	PET	0.3	11	66.5	1	0	0	1	MTX	1		1	1		1	1
11	R	M	PET	1.7	16	76.7	9.9	5	0	0	0	1		1	1			
14	R	F	PET	0.5	8	63.4	6.7	0	1	0	0	1	1	1		1	1	1
15	R	M	TAB	0.5	1	79.2	3.5	0	0	1	0	1		1				
16	R	M	PET	1.9	5	69.3	4.7	5	0	0	0	1						
17	R	M	MRI	1	4	49.1	0.2	5	0	0	0	1	1	1				
18	R	F	PET	1.4	25	78	0.2	2.5	0	0	0	1	1	1	1			
22	R	F	TAB	0.6	5	81.1	0.3	15	0	1	0		1	1				
23	R	M	PET	1.2	2	60.7	1	5	0	1	MTX	1	1	1	1			
25	R	F	PET	0.5	5	71.1	10.8	10	0	1	0	1		1	1			
27	R	F	PET	2.4	14	61.1	6.2	0	0	0	MTX	1		1				
29	R	M	PET	2.6	6	65.1	3.5	0	0	0	MTX	1		1				1
30	R	M	PET	4.4	7.7	77.1	1.4	5	0	0	0			1				1
33	R	M	PET	0.8	1	68.7	6.7	0	0	1	0			1				
34	R	F	PET	8	22	72.2	3.6	0	0	0	MTX				1		1	1
35	R	F	PET	0.5	2	77.4	0.6	0	0	1	0							1

36	R	F	PET	0.5	1	60.3	4.8	2.5	0	1	0				1	1		1
37	R	F	PET	5	11	76.2	3.1	0	0	0	MTX							1
38	R	M	PET	0.6	13	76.4	0.6	5	0	0	0				1			
39	R	F	PET	1	5	61.5	1.2	7.5	0	1	LFN							1
40	R	F	PET	0.6	6	67.4	0.8	5	0	1	0							1
1	A	F	MRI	26.9	33	67.8	11.5	0	1	0	0	1						
2	A	M	PET	5.5	16	52.7	3.1	0	1	0	0	1						
7	A	F	PET	10	2	61.9	5.3	0	1	0	0	1	1					
12	A	F	MRI	14.9	47	68.7	8.7	10	0	0	LFN	1	1	1				
13	A	F	PET	59.4	33	55.1	1.4	15	0	0	MTX	1	1	1				
19	A	M	TAB	54.4	81	86.8	0	0	1	0	0	1	1					
20	A	M	US	11.2	45	80	1	5	0	0	0				1			
21	A	M	PET	102		81.4	0.2	0	1	0	0	1	1	1				
24	A	F	PET	44.8	86	64.1	0.1	0	1	0	0	1		1	1		1	1
26	A	M	PET	64	80	76.9	0.1	0	1	0	0	1	1	1	1		1	
28	A	F	PET	10.8	33	63.7	3.7	5	0	0	0	1					1	1
31	A	M	PET	14.1	50	64	0.1	0	1	0	0			1	1		1	1
32	A	F	PET	79.4	112	72.1	1.1	0	1	0	0		1	1			1	1
41	A	F	PET	34.9	39	74.3	0	0	1	0	0						1	1

2-DG, 2-deoxyglucose; A, active; AZA, azathioprine; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; F, female; FC, flow cytometry; Fig., figure; M, male; LFN, leflunomide; MTX, methotrexate; PET, positron emission tomography; R, in remission; Seq., sequencing; TAB, temporal artery biopsy; TCZ, tocilizumab; US, ultrasound; *except for the IL-17 expression analysis

Suppl. Table 2: Characteristics of subjects included in the transcriptome analysis at blood sampling

	HD (n=6)	Active GCA (n=6)	Inactive GCA (n=6)
Age (years) – median (IQR)	69.3 (63.5–73.5)	72.8 (60.2–82.8)	67.1 (58.1–78.8)
Sex, female – no (%)	3 (50)	3 (50)	2 (33.3)
N. European ethnicity – no (%)	6 (100)	5 (83.3)	6 (100)
Disease duration (years) – median (IQR)	-	0.8 (0–6.1)	0.7 (0.1–2.5)
CRP (mg/l) – median (IQR)	-	56.9 (13.7–68)	1.3 (0.5–1.8)
ESR 1 hour (mm) – median (IQR)	-	29 (23.8–50.5)	6.5 (4.3–18.5)
Relapsed cases – no (%)	-	1 (16.7)	-
TCZ – no (%)	-	-	2 (33.3)
Corticosteroids as monotherapy – no (%)	-	1 (16.7)	3 (50)
Prednisolone or prednisolone equivalent dose (mg) – median (IQR)	-	0 (0–11.3)	5 (1.9–7.5)
csDMARD – no (%)	-	1 (16.7)	1 (16.7)

CRP, c-reactive protein; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; IQR, interquartile range; TCZ, tocilizumab

Reference

i. Schirmer JH, Aries PM, Balzer K, *et al.* S2k-Leitlinie (Kurzfassung): Management der Großgefäßvaskulitiden [S2k guidelines (executive summary): management of large-vessel vasculitis]. *Z Rheumatol* 2020;79:937-942.