Supplementary data

Material and methods

Peripheral blood mononuclear cell isolation

About 20-30 ml of heparinised whole blood from each individual was used to isolate peripheral blood mononuclear cells (PBMC). Using Lymphoprep (Stemcell Technologies, Cologne, Germany), the density gradient centrifugation was performed by carefully layering the blood on top of Lymphoprep. Centrifugation was done with 800g for 20 minutes followed by the lowest deceleration.

Cell culture

PBMC at a concentration of $2x10^6$ cells per milliliter (ml) were cultured in RPMI 1640 + Glutamax (Thermo Scientific, Burladingen, Germany), supplemented by Penicillin at 100 U/ml (Sigma Aldrich), Streptomycin at 100 µg/ml (Sigma Aldrich), non-essential amino acids (Thermo Scientific, Burladingen, Germany) and sodium-pyruvate (Thermo Scientific, Burladingen, Germany), as well as 10% fetal calf serum (Biowest, Nuaillé, France). To assess the expression of pro-inflammatory T-cell cytokines (IFN-γ, IL-2, GMCSF, IL-17a), cell stimulation cocktail (eBioscience, Thermo Scientific, Burladingen, Germany) was added to the PBMC followed by incubation for 4 hours at 37°C under 5% CO_2 atmosphere. To investigate the granzyme B (GrB) production of Breg, four different stimulation setups were performed. PBMC were stimulated with (I) anti-human immunoglobulin G/M (IgG/IgM, 6 µg/ml and 6,5 µg/ml, Jackson Immuno Research, Europe Ltd., Cambridge, UK), (II) anti-human IgG/IgM and interleukin-21 (IL-21, 50 ng/ml, Miltenyi Biotec), (III) anti-human IgG/IgM and IL-21 and trimeric CD40 ligand (CD40L, 100 µg/ml, Enzo Life Sciences, Lörrach, Germany), or (IV) IL-21 and anti-human CD40L, in a 24-well plate (BD Biosciences), respectively. After 16 hours of incubation at 37°C under 5% CO_2, 1 µg brefeldin A (BFA, Sigma Aldrich) was added to the culture. To determine the IL-10 production of Breg, PBMC were stimulated with CpG (ODN2006, 500µM stock solution, Invivogen) and incubated for 72 hours at 37°C under 5% CO_2, followed by restimulation with phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich), Ionomycin (Sigma-Aldrich) and BFA. After 6 hours of incubation at 37°C under 5% CO_2, the cells were harvested.

Flow cytometry

To detect T-cell cytokines, PBMC were harvested after stimulation and stained with anti-CD3 Pacific Blue (clone UCHT1, Beckman Coulter, Krefeld, Germany) and anti-CD8 APC-H7 (clone SK1, BD Biosciences). Thereafter, cells were fixed and permeabilised for intracellular staining, using the Cytofix/Cytoperm kit (BD Biosciences). The intracellular staining process contained IL-2 (clone JES6-5H4, PE, Biolegend) or GMCSF (clone BVD2-21C11, PE, Biolegend), IL-17a (clone BL168, PerCP, Biolegend) and IFN-γ (clone B27, FITC, BD Biosciences). Breg were stained with anti-CD3 Pacific Blue and anti-CD19 (clone J3-119, FITC, Beckman Coulter, Krefeld, Germany) followed by fixation and permeabilization. Thereafter, cells were intracellularly stained with either anti-GrB

T-cell analysis in GCA patients

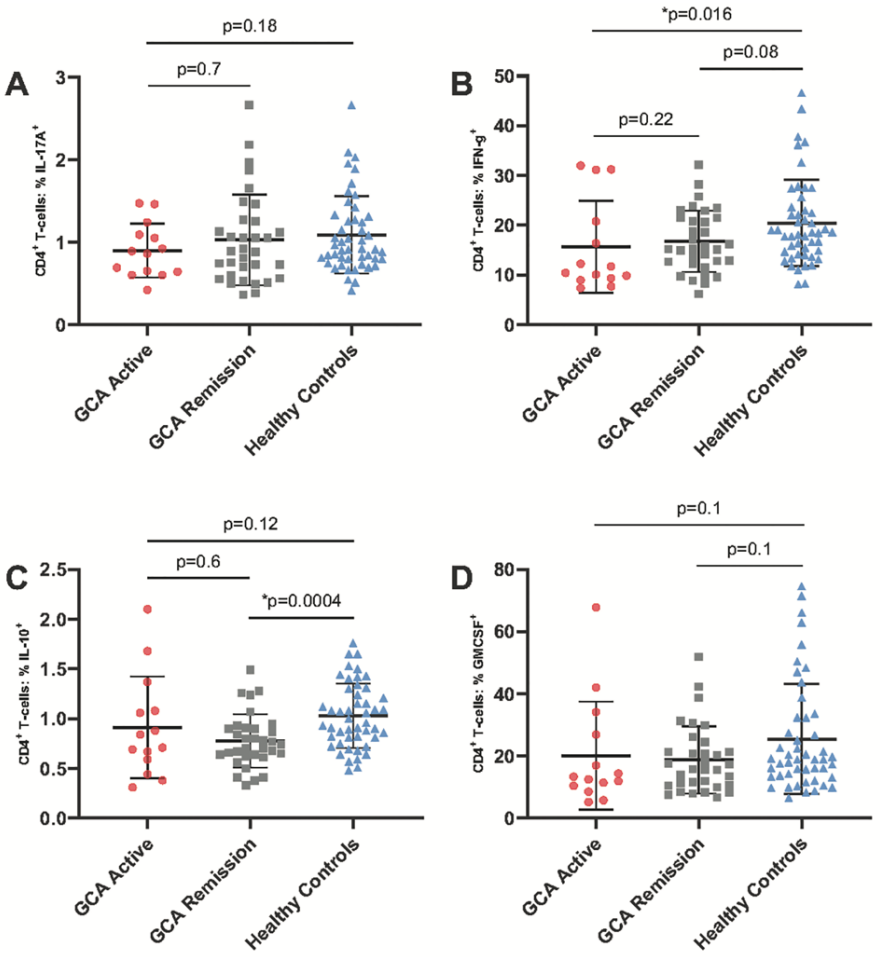

Fig. 1. CD4+ T-cell analysis in GCA. PBMC were stimulated with for four hours and cytokine expression of T-helper-cells was determined by flow cytometry. Cytokine expression was analysed within the T-helper-cell population and was given as relative fraction to the whole T-helper-cell population. Th17 cells were similar in in HC and GCA patients (A). The fraction of Th1 cells was reduced in patients with active disease as compared to HC (B). In quiescent GCA patients, the fraction of Th10 cells was reduced as compared to HC (C). GMCSF producing T-helper-cells were comparable among the groups (D).

(clone GB11, PE, Thermo Scientific, Burladingen, Germany) or anti-IL-10 (clone JES3-9D7, APC, Biolegend). 7AAD was used as a dead cell marker prior to intracellular staining. To ensure the specificity of stainings, appropriate isotype controls were used. Flow cytometric measurements were performed by using the NAVIOS™ fluorescence activated cell scanner (FACS) from Beckman Coulter. The data analysis was based on the Kaluza™ Software Version 2.1 by Beckman Coulter.

Statistical analyses

All values are expressed as mean ± standard deviation (SD). Data are displayed as scatter-plots. Statistical significance between the groups was determined by using the Mann-Whitney-U-Test. Correlation coefficients were calculated with Spearman rank correlation coefficient. A p-value ≤ 0.05 was considered significant.

Correlation analysis between Breg and Th1 / Th17 cells

Table I. Correlation analysis of Granzyme B positive B-Cells and Th1/Th17 cells in patients with active disease. Correlation analysis was performed using spearman's rank correlation test.

	IFN-γ$^+$ CD4$^+$	IL-17a$^+$ CD4$^+$
IgG + IgM (B_{reg})	R = - 0.22 P = 0.45	R = - 0.35 P = 0.22
IgG + IgM + IL-21 (B_{reg})	R = - 0.34 P = 0.23	R = - 0.46 P = 0.1
IgG + IgM + IL-21 + CD40L (B_{reg})	R = - 0.19 P = 0.56	R = - 0.35 P = 0.27
IL21 + CD40L (B_{reg})	R = - 0.47 P = 0.09	R = - 0.42 P = 0.14

Table II. Correlation analysis of Granzyme B positive B-Cells after different *in vitro* stimulations to Th1 and Th17 cells in patients with GCA in remission. Correlation analysis was performed using spearman's rank correlation test.

	IFN-γ$^+$ CD4$^+$	IL-17a$^+$ CD4$^+$
IgG + IgM (B_{reg})	R = - 0.2 P = 0.26	R = - 0.02 P = 0.9
IgG + IgM + IL-21 (B_{reg})	R = - 0.3 P = 0.09	R = - 0.18 P = 0.32
IgG + IgM + IL-21 + CD40L (B_{reg})	R = - 0.18 P = 0.42	R = - 0.004 P = 0.97
IL21 + CD40L (B_{reg})	R = - 0.07 P = 0.68	R = - 0.016 P = 0.93

Table III. Correlation analysis of Granzyme B positive B-Cells after different *in vitro* stimulations to Th1 and Th17 cells in healthy controls. Correlation analysis was performed using spearman's rank correlation test.

	IFN-γ$^+$ CD4$^+$	IL-17a$^+$ CD4$^+$
IgG + IgM (B_{reg})	R = - 0.25 P = 0.085	R = - 0.05 P = 0.7
IgG + IgM + IL-21 (B_{reg})	R = - 0.45 P = 0.001	R = - 0.18 P = 0.2
IgG + IgM + IL-21 + CD40L (B_{reg})	R = - 0.49 P = < 0.001	R = - 0.16 P = 0.3
IL21 + CD40L (B_{reg})	R = - 0.23 P = 0.11	R = 0.02 P = 0.9

Impact of treatment on regulatory B-cell populations

The impact of treatment on Breg was analysed (Fig. 2-5).

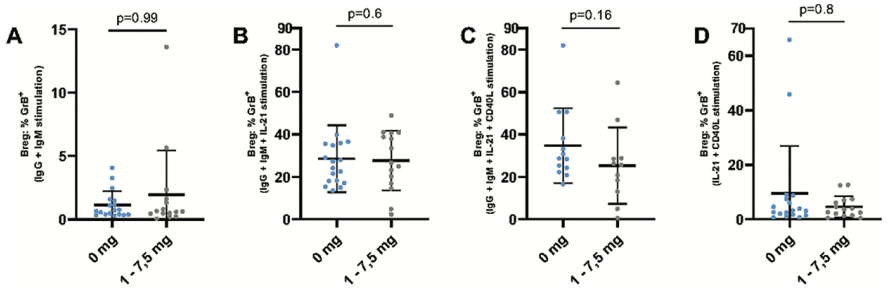

Fig. 2. Low dose steroids do no impact GrB producing Breg. PBMC from patients were stimulated with anti-human IgG and IgM (A), anti-human IgG and IgM plus IL-21 (B), IgG and IgM plus IL-21/CD40L (C) or IL-21 plus anti-CD40L (D). Patients were stratified according to steroid dosage at the time of sampling. There were no significant differences between the treatment groups. Statistical significance between the groups was determined by using the Mann-Whitney-U-Test.

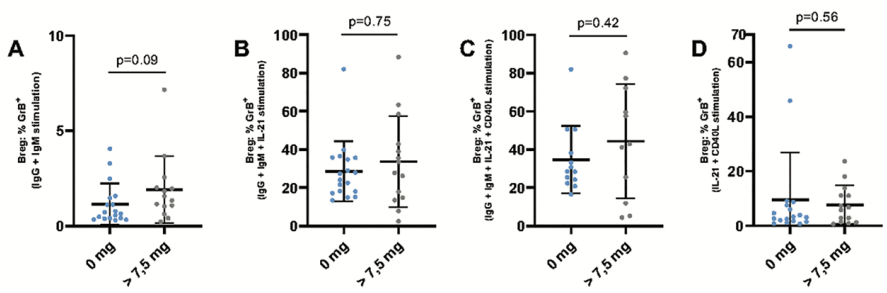

Fig. 3. Steroids do no impact GrB producing Breg. PBMC from patients were stimulated with anti-human IgG and IgM (A), anti-human IgG and IgM plus IL-21 (B), IgG and IgM plus IL-21/CD40L (C) or IL-21 plus anti-CD40L (D). Patients were stratified according to steroid dosage at the time of sampling. There were no significant differences between the treatment groups. Statistical significance between the groups was determined by using the Mann-Whitney-U-Test.

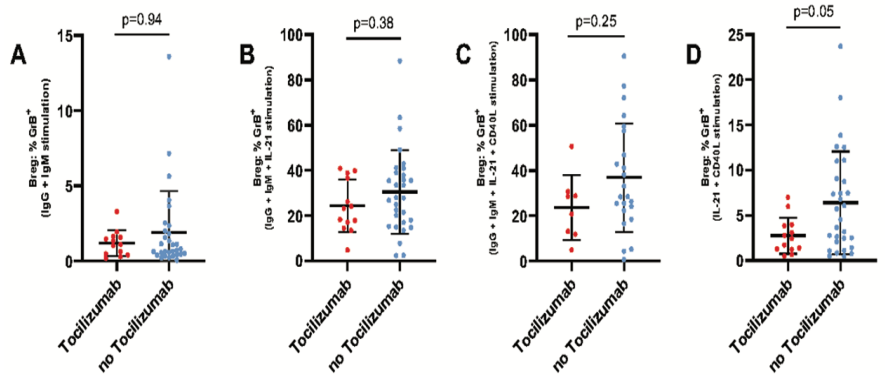

Fig. 4. Influence of tocilizumab treatment on GrB producing Breg. PBMC from patients were stimulated with anti-human IgG and IgM (A), anti-human IgG and IgM plus IL-21 (B), IgG and IgM plus IL-21/CD40L (C) or IL-21 plus anti-CD40L (D).Patients were stratified according to treatment at the time of sampling. There were no significant differences between the treatment groups. However, Patients with tocilizumab tended to harbor less GrB producing B-cells upon stimulation with IL-21 plus anti-CD40L ($CD19^+$ B-cells: $\%GrB^+$, 2.78±1.99% vs. 6.4±5.68%, p=0.05). Statistical significance between the groups was determined by using the Mann-Whitney-U-Test.

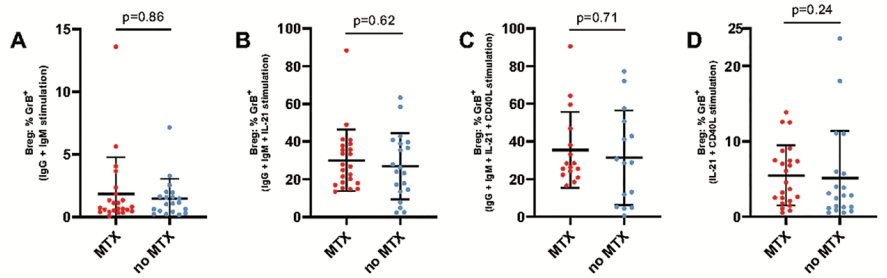

Fig. 5. Association of methotrexate treatment and GrB producing Breg. PBMC from patients were stimulated with anti-human IgG and IgM (A), anti-human IgG and IgM plus IL-21 (B), IgG and IgM plus IL-21/CD40L (C) or IL-21 plus anti-CD40L (D). Patients were stratified according to treatment at the time of sampling. There were no significant differences between the treatment groups. Statistical significance between the groups was determined by using the Mann-Whitney-U-Test.

Impact of gender on regulatory B-cell populations

We compared females (n=23) to males (n=10) among patients in remission. There was no difference in expression of Granzyme B within CD19⁺ B-cells independent of the stimulus used. (CD19⁺ B-cells: %GrB+, 1.91%±2.88% *vs.* 1.64 %±2.26% p=0.5 (Fig. 1A); CD19+ B-cells: %GrB+, 33.7%±19.5% *vs.* 26.3%±15.3% p=0.14 (Fig. 1B); CD19+ B-cells: %GrB+, 38.82%±22.92% *vs.* 23.34%±5.5% p=0.1 (Fig. 1C; CD19+ B-cells: %GrB+, 6.62%±9.2% *vs.* 9.73%±19.9% p=0.6 (Fig. 1D).

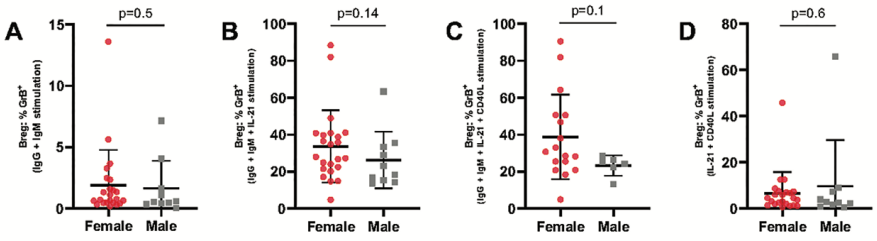

Fig. 6. Granzyme B expression of B-Cells does not dependent on gender in GCA patients. PBMC from patients with quiescent disease were stimulated with anti-human IgG and IgM (A), anti-human IgG and IgM plus IL-21 (B), IgG and IgM plus IL-21/CD40L (C) or IL-21 plus anti-CD40L (D). The Granzyme B expression showed no significant difference between males and females. Statistical significance between the groups was determined by using the Mann-Whitney-U-Test.

Impact of age on regulatory B-cell populations

We compared groups of GCA Patients and HC of respectively over and under 70 years of age. (GCA <70 n=20; GCA ≥70 n=27; HC <70 n=36; HC ≥70 n=13). There were no statistical significant differences considering the GrB expression of B-cells.

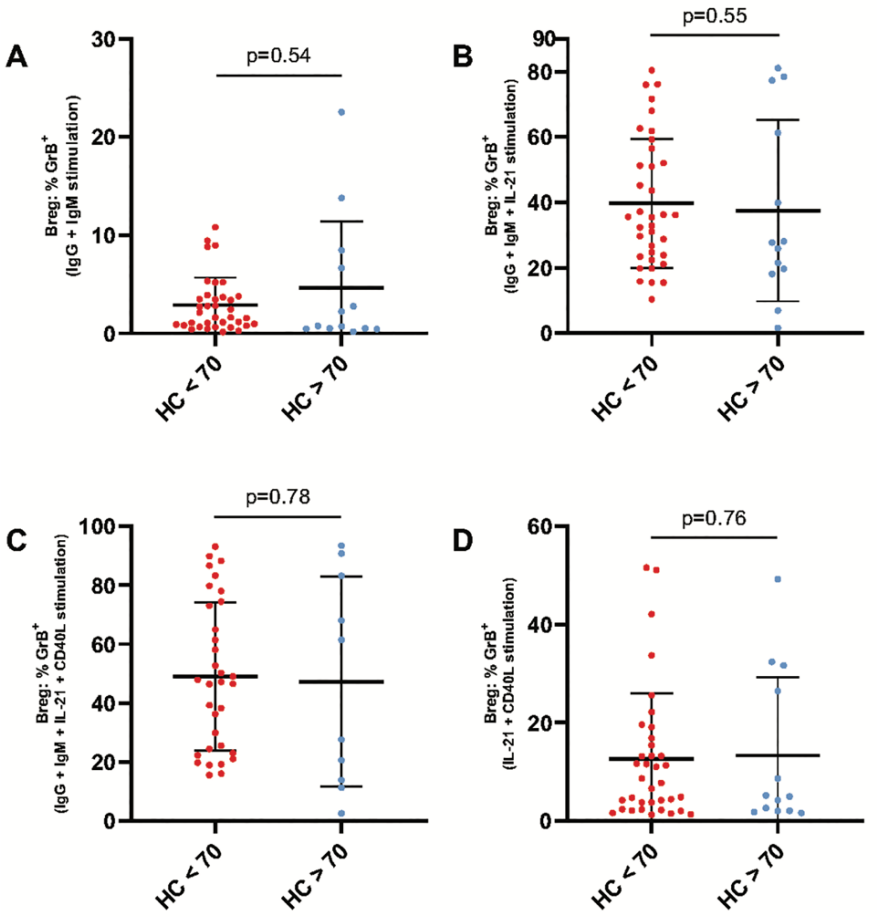

Fig. 7. Granzyme B expression of B-Cells is independent from age in HC. PBMC from HC were stimulated with anti-human IgG and IgM (A), anti-human IgG and IgM plus IL-21 (B), IgG and IgM plus IL-21/CD40L (C) or IL-21 plus anti-CD40L (D). There were no significant differences observed between the age groups of healthy controls. Statistical significance between the groups was determined by using the Mann-Whitney-U-Test.

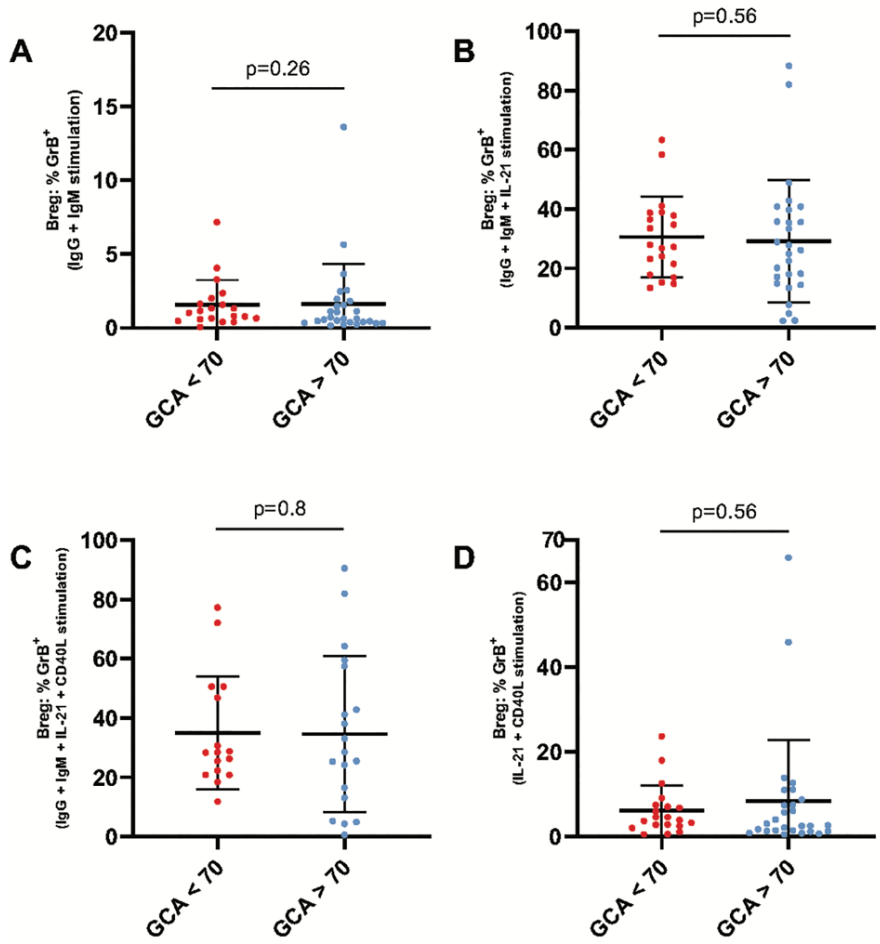

Fig. 8. Granzyme B expression of B-Cells is independent from age in GCA patients. PBMC from patients were stimulated with anti-human IgG and IgM (A), anti-human IgG and IgM plus IL-21 (B), IgG and IgM plus IL-21/CD40L (C) or IL-21 plus anti-CD40L (D). There were no significant differences observed between the age groups of GCA patients. Statistical significance between the groups was determined by using the Mann-Whitney-U-Test.

GrB producing Breg induced by IL-21 plus CD40L stimulation

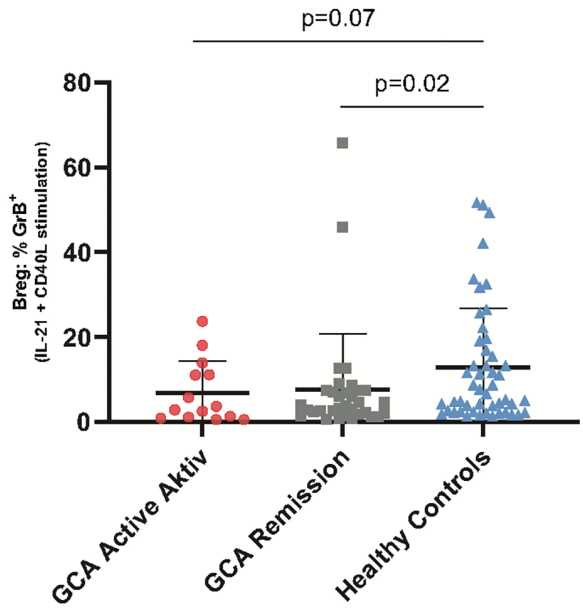

Fig. 9. Granzyme B expression of regulatory B-cells upon stimulation with IL-21 and CD40L. GrB expression was determined by flow cytometry after stimulation. Patients with quiescent disease showed a reduced fraction of GrB+ Breg. Statistical significance between the groups was determined by using the Mann-Whitney-U-Test.