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Mucosal-Associated Invariant T cells in Giant Cell Arteritis

Short running head: MAIT cells in GCA

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Maxime SAMSON: Roche-Chugaï (invitation to congress, personnel fees for symposium and boards), Abbvie (consulting), Vifor pharma (personnel fees for symposium).

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CONTRIBUTORSHIP STATEMENT

Thibault Ghesquière and Maxime Samson were the principal investigators and take primary responsibility for the paper. Thibault Ghesquière, Hervé Devilliers, François Maurier, Paul Ornetti, Valérie Quipourt, Pierre-Henri Gabrielle, Catherine Creuzot-Garcher, Sylvain Audia, Bernard Bonnotte, Maxime Samson recruited the patients. Thibault Ghesquière, Marion Ciudad, Hélène Greigert, André Ramon, Claire Gérard, Claudie Cladière, Marine Thébault, Coraline Genet, Georges Tarris and Laurent Martin performed the laboratory work for this study. Thibault Ghesquière and Agnès Soudry-Faure did the statistical analysis. Maxime Samson and Bernard Bonnotte coordinated the research. Thibault Ghesquière, Maxime Samson, Philippe Saas and Bernard Bonnotte drafted the manuscript. Thibault Ghesquière, Bernard Bonnotte and Maxime Samson contributed to data interpretation.

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ABSTRACT

This study aimed to assess the implication of mucosal-associated invariant T (MAIT) cells in GCA. Blood samples were obtained from 34 GCA patients (before and after 3 months of treatment with glucocorticoids (GC) alone) and compared with 20 controls aged >50 years. MAIT cells, defined by a $CD3^+CD4^-TCR\gamma\delta^-TCRV\alpha7.2^+CD161^+$ phenotype, were analyzed by flow cytometry. After sorting, we assessed the ability of MAIT cells to proliferate and produce cytokines after stimulation with anti CD3/CD28 microbeads or IL-12 and IL-18. MAIT were stained in temporal artery biopsies (TAB) by confocal microscopy.

MAIT cells were found in the arterial wall of positive TABs but was absent in negative TAB. MAIT frequency among total αβ-T cells was similar in the blood of patients and controls (0.52 vs. 0.57 %; P=0.43) and not modified after GC treatment (P=0.82). Expression of IFN-γ was increased in MAIT cells from GCA patients compared to controls (44.49 vs. 32.9 %; P=0.029), and not modified after 3 months of GC therapy (P=0.82). When they were stimulated with IL-12 and IL-18, MAIT from GCA patients produced very high levels of IFN-γ and displayed a stronger proliferation compared with MAIT from controls (proliferation index 3.39 vs. 1.4; P=0.032).

In GCA, the functional characteristics of MAIT cells are modified toward a pro-inflammatory phenotype and a stronger proliferation capability in response to IL-12 and IL-18, suggesting that MAIT might play a role in GCA pathogenesis. Our results support the use of treatments targeting IL-12/IL-18 to inhibit the IFN-γ pathway in GCA.

KEYWORDS: giant cell arteritis, Mucosal Associated Invariant T, invariant T-cells

1. Introduction

Giant cell arteritis (GCA) is a large vessel vasculitis that occurs in people older than 50 years and mainly affects the aorta and cranial arteries. The pathogenesis of GCA is not fully understood but major progress has been made over the last several years, leading to the emergence of new therapeutic targets in order to reduce the use of glucocorticoids (GC) [1]. Notably, previous research has demonstrated that T lymphocytes (TL) are recruited in the arterial wall by chemokines (CCL19, CCL20, CCL21) produced by activated dendritic cells [2]. Then, T cells are activated, proliferate and polarize into Th1 and Th17 cells, which produce interferon-gamma (IFN-γ) and interleukin-17 (IL-17), respectively [3-6].

MAIT cells are innate-like lymphocytes characterized by the expression of a semi-invariant T-cell receptor (TCR) composed of a constant α chain (TCRVα7.2-Jα33) and a β chain (among a limited number of variants, often Vβ2 and Vβ13), whose interaction is restricted to the major histocompatibility complex (MHC) related protein 1 (MR1). MAITs display an immediate effector function upon stimulation, and have a high clonal volume [7].

It has also been established that MAIT cells are activated during viral infections by a TCR-independent pathway [8-10]. This particular means of activating MAIT cells involves IL-12 and IL-18 [11], two cytokines that are highly expressed in GCA lesions [11,12], which made us hypothesize that they could be involved in GCA pathogenesis, as it is the case in ANCA-associated vasculitis [13]. Along this line, several studies have suggested that viral or bacterial triggering factors may be involved in triggering GCA [14-16], and recent research reported an increased frequency of GCA cases in the context of the SARS-Cov2 pandemic [14,15], an infection in which altered MAIT cell functions due to imbalances in IFN-α/IL-18 contribute to disease severity [17]. In addition, MAIT cells share phenotypic characteristics with the T cells identified in GCA lesions, including the expression of CCR6 and CD161, and the secretion of IFN-γ, IL-17 and granzyme A [6,18,19].

For all of these reasons, we hypothesized that MAIT cells could be implicated in GCA pathogenesis and play a role in the amplification of vascular inflammation through their activation by IL-12 and IL-18 in a TCR-independent pathway [11]. Therefore, we designed this study aiming to investigate the frequency of MAIT, their phenotype and especially their functional characteristics upon stimulation with IL-12 and IL-18 in GCA patients and in age-matched healthy controls.

2. Methods

2.1. Patients and controls

Thirty-four newly-diagnosed GCA patients and 20 healthy subjects (age >50 years, CRP ≤5 mg/L, no recent treatment with GC or immunosuppressants, no history of cancer, no recent acute or chronic infectious disease, and no autoimmune disease) were prospectively enrolled in this study after giving their

written informed consent in accordance with the Declaration of Helsinki. The Institutional Review Board of the Dijon University Hospital approved the study.

Blood samples were obtained in each group. For GCA patients, the first sample was obtained at diagnosis before any GC treatment. Then, GC were started at 0.7 or 1 mg/kg/day depending on the severity of the disease and progressively tapered following French guidelines [20]. A second blood sample was obtained after 3 months of treatment with GC for all GCA patients.

2.2. Cell preparation, antibodies, and flow cytometry analyses

PMBC were obtained from whole blood by Ficoll gradient centrifugation. All flow cytometry (FCM) analyses were performed on fresh peripheral blood mononuclear cells (PBMCs) using the following antibodies: anti-CD3 brilliant violet (BV), anti-CD4 phycoerythrin (PE), anti-TCRγδ fluorescein isothiocyanate (FITC), anti-TCRVα7.2 peridinin-chlorophyll-protein (PerCP), anti-CCR6 allophycocyanin (APC), anti-CXCR3 PECy7, anti-CD62L PE, anti-CCR7 APC, anti-HLA-DR APC, anti-CD69 PE, anti-granzyme B alexa fluor 647, anti-perforine PE, anti-IFN-γ APC, anti-IL17 PE, anti-Ki67 PE. Intracellular cytokine and granzyme B staining were performed starting from 3.10^6 PBMCs cultured in 24-well plates in 1 mL of RPMI 1640 (Bio Whittaker) with 10% fetal bovine serum (Gibco BRL) and stimulated with 0.1 mg/mL of phorbol 12-myristate 23-acetate (PMA) and 1 mg/mL of ionomycine (Sigma-Aldrich) in the presence of Brefeldin A (BD Golgi Plug; BD Bioscience) for 4 hours. Then, cells were fixed and permeabilized using fixation permeabilization buffer (eBioscience) before intracellular staining for granzyme B, perforin, IL-17 and IFN-γ. True Nuclear™ fixation permeabilization buffer (Biolegend) was used before Ki67 intranuclear staining. Data were acquired with a LSR-II cytometer (BD Bioscience) and analyzed with FlowJo® v7.5.6 software.

2.3. Isolation and culture of MAIT cells

For 4 patients and 5 controls, a CD8-enriched TL population was negatively sorted from PBMCs using magnetic cell sorting (Pan-T cell isolation kit and Anti-CD4 antibody from Miltenyi Biotech) using an AutoMACS pro cell sorter. Starting from this $CD8^+$ T cell enriched population, MAIT ($CD3^+CD4^-$ $TCR\gamma\delta^-TCRV\alpha7.2^+CD161^+$) and MAIT depleted $CD161^+$ T cells ($CD3^+CD4^-TCR\gamma\delta^-TCRV\alpha7.2^-CD161^+$) were sorted using fluorescent assisted cell sorting (FACS), using the same antibody as described above. Then, cells were stained with cell trace violet (invitrogen) and cultured with or without anti-CD3/CD28 microbeads or IL-12 and IL-18 (50 ng/ml each), as described by Ussher *et al* [11]. The proliferation index

was assessed by flow cytometry after 7 days of culture, and supernatants were collected to measure cytokines.

2.4. Cytokine assays

Granzyme A, granzyme B, IFN-γ, IL-17 and tumor necrosis factor-alpha (TNFα) were measured by luminex® (eBioscience) in the supernatants of the MAIT cell culture, following the provider's instructions. Data were acquired and analyzed on a Bio-Plex® 200 system for multiplex analysis (Bio-Rad).

2.5. Immunofluorescence staining - Confocal microscopy

Sections of 3 positive and 3 negative temporal artery biopsies were fixed in cold paraformaldehyde 4% (PFA), followed by exposure to increasing concentrations of saccharose (15-30%). Then, sections were embedded in Tissue-Tek® O.C.T.™ Compound (Sakura, Flemingweg, The Netherlands) and stored at -80°C. Ten µm cryostat sections were fixed in 4% PFA, permeabilized with 0.1% of Triton (Fisher Scientific) and blocked in a solution containing 5% of donkey serum (Merk), 1% BSA before being incubated for 12 hours with primary antibodies (anti-CD3 [1:100; Abcam], anti-IL-18R [1:200; R&D Systems], anti-TCRVα7.2 [1:100; BioLegend]). After washing, slides were incubated for 1 hour in the dark with donkey secondary antibodies conjugated with fluorochromes AF488 (green), AF555 (yellow) and/or AF647 (red) at 1:300. Slides were mounted with ProLong™ Gold Antifade Mountant with DAPI (Molecular Probes, LifeTechnologies). Acquisition was performed using a Leica TCS SP8 fluorescence microscope and data were analyzed with the Leica Application Suite X.

2.6. Ex-vivo culture of temporal arteries

Sections of 18 fresh temporal artery biopsy (TAB) specimens showing lesions of GCA (n=12) or not (n=6) were embedded in MATRIGEL® and cultured as previously described [21]. Briefly, the surrounding tissue was carefully removed to keep only the temporal artery before cutting regular sections of ~1 mm of thickness. These sections were embedded in 25 µL of MATRIGEL® in a 24-well plate (1 section/well and 2 sections/condition) and then covered by 1000 µL of RPMI 1640 medium (supplemented with 10% fetal bovine serum, L-glutamine, amphotericin B and gentamycin) with or without dexamethasone (0.5 µg/mL). After 5 days, arterial sections were collected and homogenized in TRIzol reagent using a Minilys homogenizer (Bertin instruments®) before the extraction of total mRNA.

2.7. RT-PCR analyses

Total RNA was extracted from cultured artery and cDNA obtained by reverse-transcription employing a random hexamer priming kit (Applied Bioscience) in a final volume of 100 µL. Then, specific pre-developed TaqMan probes from Applied Bioscience (TaqMan Gene Expression Assays) were used for PCR amplification: *GUSB* (Hs99999908_m1), *IL12p35* (Hs00168405_m1), *IL12p40* (Hs01011518_m1) and *IL18* (Hs01038788_m1). Fluorescence was detected with the CFX96TM Real-Time PCR Detection System and the results were analyzed with CFX ManagerTM software (Bio-Rad Laboratories). Gene expression was normalized to the expression of the endogenous control GUSB using a comparative ΔCt method. mRNA concentration was expressed in relative units with respect to GUSB expression (relative expression).

2.8. Statistical analyses

Data are expressed as medians [interquartile range] for continuous variables and numbers (%) for categorical variables. Mann Whitney U tests were used to compare data between untreated patients and controls. Wilcoxon matched-pairs signed rank tests were used to compare patients before and after 3 months of treatment. Fisher's exact tests were used to compare qualitative variables. Statistical significance was set at $P<0.05$ (two-tailed). GraphPad Prism® was used for statistical analyses.

3. Results

3.1. Population characteristics

Main clinical and biological characteristics of the 34 GCA patients and the 20 healthy controls are summarized in **Table 1.** Thirty-three GCA patients underwent a TAB, among them 21 (63.6%) were positive, 9 were negative (27.3%), and 3 (9.1%) did not yield usable material. One patient refused the procedure. Among the 13 patients without a positive TAB (not performed or negative), seven had evidence of large vessel vasculitis proven by positron emission tomography and/or CT scan, two had a temporal artery ultrasound-scan showing a bilateral halo sign, three other patients had confirmed ophthalmologic involvement of GCA together with unequivocal cephalic signs of GCA, and the last patient was treated with GC and follow-up revealed GCA relapses with evidence of large vessel vasculitis. Finally, all GCA patients met at least 3/5 ACR criteria for the diagnostic of GCA [22]. After 3 months of treatment with GC, all GCA patients achieved remission (no clinical sign of GCA and CRP level <10 mg/L). None of the GCA patients received methotrexate, tocilizumab or any other GC-sparing drug between inclusion and 3-months of follow up. Patients and controls had similar characteristics, especially regarding age and sex, which have been reported to modify MAIT frequency [18].

3.2. MAIT cells are present in the arterial wall of GCA patients

Figure 1 depicts the study of MAIT cells, as defined by a $CD3^+$ (green) IL-$18R^+$ (yellow) and TCRV$\alpha$$7.2^+$ (red) phenotype, in the arterial wall of temporal arteries. MAIT cells were identified in the 3 positive TABs we studied. They were mainly localized at the media/adventitia interface either among lymphoid infiltrates (A, D), or isolated in the arterial wall (B, C). Interestingly, we observed that the expression of IL-18R was elevated and not restricted to MAIT cells since it was also expressed by TCRV$\alpha$$7.2^-CD3^+$ cells. By contrast with GCA arteries, no T lymphocytes and no MAIT cells were observed in negative TABs (n=3; sup figure 1).

3.3. In the blood of GCA patients, MAIT cells are skewed towards a Th1 phenotype

The percentage of circulating MAIT cells ($CD3^+CD4^-TCR\gamma\delta^-$TCRV$\alpha$$7.2^+CD161^+$) among total $\alpha\beta$-T cells was similar between untreated GCA patients and controls (0.52 % [0.18-1.02] *vs*. 0.57 % [0.31-1.57]; P=0.43; **figure 2B**) and not modified after 3 months of treatment with GC (0.51 % [0.17-0.81] *vs*. 0.53 % [0.22-1.00]; P=0.82; **figure 2C**). The absolute number of circulating MAIT cells was also comparable between patients and controls (5.73/mm^3 [1.84-11.14] *vs*. 6.89/mm^3 [2.95-23.26]; P=0.29), and between patients before and after treatment (5.14/mm^3 [1.82-11.27] *vs.* 5.40/mm^3 [2.13-7.76]; P=0.098, **figure 2C**).

On the contrary, the phenotype of MAIT cells was significantly modified in GCA patients towards a decreased expression of CXCR3 (6.08 % [1.57-13.96] *vs*. 11.2 % [6.14-38.12] in total MAIT cells; P=0.036, **figure 2D**) and an increased expression of IFN-γ (44.49 % [32.18-60.59] *vs*. 32.90 % [19.75-41.02] in total MAIT cells; P=0.029, **figure 2F**). After 3 months of treatment, the percentage of $CXCR3^+$ MAIT cells increased without reaching the level of significance (from 7.05 % [2.31-15.29] to 9.56 % [2.03-27.74]; P=0.063). The percentage of IFN-γ^+ cells was not modified (43.85 % [31.06-57.27] *vs.* 51.52 % [24.85-63.72]; P=0.82) but still remained higher than healthy controls, although non significantly (51.52 % [24.85-63.72] *vs.* 32.90 % [19.75-41.02]; P=0.107). The expressions of CCR6, IL-17, CD69, granzyme A and perforin by MAIT cells were not significantly modified between groups (**Figure 2E, G, H, I, J**).

Notably, the CRP level did not correlate with the percentage of IFN-γ^+ MAIT cells at baseline (P=0.398) and after 3 months of treatment with prednisone (P=0.897) (**sup Figure 2**).

3.4. The proliferation of MAIT cells is increased in GCA patients, especially after exposure to IL-12 and IL-18

The ability of circulating MAIT to proliferate, as assessed by the expression of Ki67, was significantly increased in patients versus controls (7.93 % [4.97-10.78] *vs*. 3.19 % [2.89-5.71]; P=0.021) and tended to decrease after treatment with GC (6.95 % [4.69-9.43] *vs.* 4.21 % [2.41-6.97] P=0.062) (**Figure 3A, 3B**).

In vitro, isolated MAIT cells proliferate after TCR engagement with anti-CD3/CD28 beads and in case of stimulation with IL-12 and IL-18 without TCR engagement (**figure 3D-E**). Proliferation was significantly increased in MAIT from GCA patients when compared with MAIT from controls after activation with IL-12 and IL-18 (proliferation index = 3.39 [2.36-7.15] *vs.* 1.4 [1.2-2.42]; P=0.032) but not significantly after TCR activation (12.8 [5.64-20.6] *vs.* 2.18 [1.6-5.89]; P=0.063) (**figure 3 D-E**). Notably, MAIT-depleted $CD161^+$ T cells did not proliferate in the presence of IL-12 and IL-18 while they did proliferate after TCR stimulation with anti-CD3/CD28 beads. However, there was not a significant difference between patients and controls (4.67 [2.99-7.59] *vs.* 7.91 [3.12-15.0]; P=0.49) (**figure 3 D-E**).

3.5. MAIT cells produce high amounts of IFN-γ after exposition to IL-12 and IL-18

Figure 4 depicts the concentrations of cytokines in the supernatant of MAIT cells from patients and controls depending on the type of stimulation (anti-CD3/CD28 beads or IL-12 and IL-18). Notably, MAIT cells produced much higher amounts of IFN-γ after exposure to IL-12 and IL-18 than after engagement of the TCR by anti-CD3/CD28 beads: 155.6 ng/ml [6.2-517.3] vs. 1.1 ng/ml [0.70-5.80] (P=0.0087) in controls and 71.3 ng/ml [9.96-186.5] vs. 0.88 ng/ml [0.66-5.51] (P=0.0022) in patients, but without a significant difference between patients and controls (P=0.818 and P=0.730).

On the contrary, production of granzyme B and TNF-α were higher after activation of the TCR pathway than after treatment with IL-12 and IL-18, again without a difference between patients and controls. Granzyme B was measured at 325.1 ng/ml [249.7-1003] *vs* 12.38 ng/ml [3.70-28.38] (P=0.0022) in GCA patients and 642.1 ng/ml [217.3-814.2] *vs* 9.80 ng/ml [4.27-32.16] (P=0.0022) in controls. TNF-α was measured at 14.27 ng/ml [2.92-33.9] *vs* 0.48 ng/ml [0.26-1.13] (P=0.0043) in GCA patients and 36.88 ng/ml [23.6-43.1] *vs* 0.62 ng/ml [0.32-1.50] (P=0.0043) in controls.

Production of granzyme A was constrictively high without difference between studied groups, and production of IL-17 remained very low whatever the culture conditions.

3.6. Assessment of IL12 and IL18 mRNA in GCA lesions

Figure 5 depicts the expression of the mRNA of *IL18*, and *p35* and *p40* (the two subunits of *IL12)* in the arterial wall of TAB cultured in MATRIGEL for 5 days. *IL-18* expression was higher in positive TABs than in negative ones (23.25 [11.92-38.11] *vs* 5.95 [1.88-11.22]; P=0.015) and significantly decreased after in vitro treatment with dexamethasone (10.23 [5.38-15.16] *vs* 23.25 [11.92-38.11]; P=0.031). The expression of the p40 subunit was similar in positive and negative TAB (P=0.407) and decreased in the presence of dexamethasone (P=0.062). By contrast, the expression of the p35 subunit was higher in negative TAB than in positive TAB (22.77 [16.3-28.34] *vs* 3.98 [1.76-5.11]; P=0.022) and was not modified in the presence of dexamethasone.

It is worth noting that the expression levels of the mRNA of *IL18* and the *p35* subunit were strongly influenced by the duration of treatment with GC before TAB was performed. Of the 6 cultured TAB, only one was performed on the day GC treatment was started whereas the five others were performed ≥2 days after the initiation of treatment (range: 2 - 186 days). *IL18* and the *p40* subunit mRNA levels were strongly decreased in the 5 TABs performed after at least 2 days of GC therapy in comparison with the remaining one, whereas expression of the *p35* subunit was not impacted (sup Figure 3).

4. Discussion

Although MAIT cells have been implicated in several autoimmune diseases [13,23-25], no data were available regarding their role in the pathogenesis of GCA before this study. In polymyalgia rheumatica (PMR), which is often associated with GCA [26], Nakajima *et al.* studied innate-like lymphocytes and showed that the frequency of circulating MAIT cells was decreased in PMR when compared to controls and that MAIT lymphocytes were enriched in HLA-DR$^+$CD38$^+$ cells in patients with an inactive PMR compared to patients with active disease and controls [27]. Even if MAIT cells were found to be decreased in other autoimmune diseases such as ANCA-associated vasculitis, systemic lupus and

inflammatory bowel diseases [13,23,24], we did not find that MAIT were decreased in GCA patients. This difference may be explained by the fact that aging induces a significant decrease in MAIT frequency, and that healthy controls and GCA patients were matched for age in the present work, unlike in previous studies [13,23,24]. Along this line, the percentage of MAIT cells in total CD3 T cells was actually similar between PMR patients and elderly healthy controls in the study by Nakajima *et al.*[27], which is more consistent with our results in GCA.

Although MAIT are not the main cell type infiltrating TAB, our results demonstrate that these cells are present in a higher proportion in the arterial wall than in the blood, in which they are very rare. This suggests that they could be recruited in GCA lesions. Considering that the expression of CXCR3 was decreased in circulating MAIT from GCA patients, we hypothesize that this receptor could be implicated in their recruitment in the arterial wall since the ligands of CXCR3 (CXCL9, CXCL10 and CXCL11) are expressed in GCA lesions and have been shown to be produced by macrophages and vascular smooth muscle cells when they are activated by IFN-γ [19,28]. In addition, IFN-γ is produced by Th1 [5,6], Tc1 [19] and MAIT cells from GCA patients activated with IL-12 and IL-18, as we demonstrated in this study. Furthermore, we observed in GCA-TABs that MAIT cells were both present in lymphocyte infiltrates and also in an isolated manner in the media and the adventitia, which is consistent with their ability to infiltrate tissues at the early stages of the inflammation process [18].

Interestingly, we observed dramatic differences in how MAIT cells function in GCA patients. Notably, they had a greater proliferation and more frequently produced IFN-γ than the MAIT cells obtained from controls. The absence of correlation between the level of CRP and the percentage of IFN-γ^+ MAIT cells suggests that the increase in the percentage of IFN-γ^+ MAIT is more related to GCA itself than simply a consequence of systemic inflammation. In addition, *in vitro* experiments revealed that circulating MAIT from GCA patients were more sensitive to TCR-independent activation through IL-12 and IL-18, which increased proliferation in GCA patients and led to stronger production of IFN-γ than TCR engagement, thus matching with the phenotype of circulating MAIT cells that we observed in the blood of GCA patients. Therefore, we hypothesize that MAIT cells could participate in an antigen-independent amplification of vascular inflammation and remodeling through their ability to proliferate and produce IFN-γ in the presence of IL-12 and IL-18 without TCR engagement. Along this line, and as reported earlier [12], we found that IL-18 was expressed in GCA arteries but that it decreased to the levels found in healthy arteries after treatment with GC. Concerning IL-12, the results were more difficult to analyze. IL-12 is a heterodimeric cytokine composed of two subunits (p35/p40) which is involved in Th1 polarization [29]. The p35 subunit is also expressed by IL-35 (p35/EBI3), an anti-inflammatory cytokine whose function has not been described in GCA. Espígol-Frigolé *et al*. previously demonstrated that the mRNA expression of p40 was moderately expressed in temporal arteries from GCA patients and rapidly decreased after 2 days of treatment with GC [30]; this was also observed in the present work, thus explaining why we did not find an increased expression of p40 in the six TABs we studied. Contrary to

p40 and p19, Espígol-Frigolé *et al*. also reported that p35 was constitutively expressed in the temporal arteries, with no difference between treated GCA patients, untreated GCA patients, or healthy controls [30]. Similarly, we found that the mRNA of the p35 subunit was expressed in healthy arteries. However, we also found that its expression was lower in GCA arteries, which may be explained by the fact that our patients had been receiving GC treatment for a longer period before TAB than in the study by Espígol-Frigolé *et al*. [30].

Interestingly, we observed that the percentage of circulating IFN-γ^+ MAIT cells did not decrease after 3 months of treatment with GC. This was surprising considering the decrease in *IL18* and the *p40* subunit observed in TABs treated with dexamethasone. This result may be explained by a difference in the concentration of steroids to which MAIT cells are exposed (higher in *ex vivo* cultures of TAB than in patients treated *in vivo*) and/or to a potential resistance to steroids since MAIT cells are known to be xenobiotic-resistant because they express the ABCB1 efflux pump [18].

Our study has major strengths: the number of patients, the fact that all patients were included before receiving GC and the fact that they were able to compared these same patients after 3 months of treatment (pairing). In addition, controls were carefully selected to limit the factors that are known to influence the proportion and phenotype of MAIT cells, such as age, presence of chronic diseases and systemic inflammation [31,32]. However, we also recognize that our study has limits. First, we did not performed adjustment for multiple comparisons but this kind of analysis is less critical in exploratory studies involving a limited number of patients, as it was the case in the present study. Anyway, the possible impact MAIT cells on the phenotype and outcome of GCA should be confirmed in subsequent studies with preplanned hypotheses [33-35]. The second limit is related to the phenotypic definition of MAIT cells that we used instead of identifying them using MR1 tetramers [36]. Accordingly, we excluded the $CD4^+$MAIT-cell subset to limit the risk of misidentifying non-MAIT $CD4^+$ cells as MAIT cells. Furthermore, recent data have clearly demonstrated that CD161 and Vα7.2 allowed MAIT cell identification similar to that found with MR1 tetramers loaded with the active ligand 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil [17]. In addition, the functional characteristics of isolated $CD3^+CD4^-$TCRγδ$^-$TCRVα7.2$^+$$CD161^+$ cells fit perfectly with usual functional characteristics of MAIT [11], ensuring our phenotypic definition of MAIT cells.

5. Conclusion

This study demonstrates that MAIT cells are present in GCA lesions and that their functional characteristics are dramatically modified toward a pro-inflammatory phenotype: increased IFN-γ expression and greater proliferation in the presence of IL-12 and IL-18. Seeing as IL-12 and IL-18 are produced in GCA lesions, MAIT cells could be activated by a TCR-independent pathway and thus participate in sustaining vascular inflammation through their ability to produce of IFN-γ. In addition, our

results suggest that MAIT cells are not sensitive to GC. These findings highlight the importance of developing therapies targeting the IL-12 and/or IL-18 axis in order to better control the IFN-γ pathway, which is clearly involved in GCA pathogenesis [28].

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Table 1
Clinical and biological characteristics of GCA patients and healthy controls.

	GCA patients		**Healthy controls**	***P value***	
	Untreated (n = 34)	Treated (n = 34)	(n = 20)	Untreated GCA *vs* Controls	Treated GCA vs Untreated GCA
Age (years), median [IQR]	69.8 [64.6-77.3]	—	68.2 [63.1-77.4]	0.6635	
Sex ratio (F/M)	21/13	21/13	10/10	0.569	
Clinical findings, n (%)					
Weight loss (> 2kg)	23 (67.6)	0	—		
Headaches	21 (61.8)	0	—		
Jaw claudication	15 (44.1)	0	—		
Scalp tenderness	16 (47.1)	0	—		
Abnormal TA palpation	15 (44.1)	0	—		
Transient vision loss	2 (5.9)	0	—		
Transient diplopia	2 (5.9)	0	—		
AAION	1 (2.9)	0	—		
Polymyalgia rheumatica	17 (50)	0	—		
GCA related stroke	3 (8.2)	0	—		
Evidence of vasculitis, n (%)					
Positive TAB[a]	21 (61.8)	0	—		
Aortitis[b]	17 (50)	0	—		
Biology, median [IQR]					
Hemoglobin (g/dL)	11.6 [10.8-12.5]	13.4 [12.4-14.5]	14.6 [13.4-15.3]	**<0.0001**	**<0.0001**
Leukocytes (.10^9/L)	7.5 [6.5-9.5]	10.0 [8.1-11.7]	6.2 [5.7-7.9]	**0.0233**	**0.0325**
Neutrophils (.10^9/L)	5.1 [4.3-6.8]	7.3 [6.0-9.4]	4.1 [3.1-4.7]	**0.002**	**0.0305**
Lymphocytes (.10^9/L)	1.4 [1.3-2.3]	1.3 [0.8-2.0]	1.8 [1.2-2.2]	0.7125	**0.0451**
CD3 (% of total lymphocytes)	76.5 [72.7-83.7]	66.0 [62.5-75.5]	74.5 [69.2-81.2]	0.2154	**0.0006**
CD4 (% of total lymphocytes)	54.5 [51.0-64.0]	46.0 [39.5-56.5]	48.0 [37.2-53.7]	**0.0015**	**0.0004**
CD8 (% of total lymphocytes)	17.0 [11.7-21.2]	19.0[14.0-22.5]	21.1 [17.0-34.0]	**0.0325**	0.4656
NK (% of total lymphocytes)	10.5 [6.75-14.0]	13.0 [10.0-24.5]	13.5 [10.2-18.0]	**0.0378**	0.0593
CD19 (% of total lymphocytes)	9.0 [5.0-11.25]	13.0 [9.0-20.0]	10.0 [5.0-14.0]	0.6758	**0.0004**
Monocytes (.10^9/L)	0.66 [0.54-1.02]	0.42 [0.28-0.65]	0.55 [0.44-0.62]	**0.0025**	**<0.0001**
ESR (mm/h)	17.0 [11.7-21.2]	21.0 [8.0-32.5]	14.5 [10.7-21.2]	0.5294	0.3025
CRP (mg/L)	61.0 [21.3-102.9]	4.0 [2.9-7.4]	2.9 [2.9-2.9]	**<0.0001**	**<0.0001**
Fibrinogen (g/L)	6.7 [5.5-7.8]	4.0 [3.3-4.8]	3.4 [2.8-3.7]	**<0.0001**	**<0.0001**
Prednisone (mg/day), median [IQR]	0	16.7 [15.0-20.0]	0	—	—

AAION: acute anterior ischemic neuropathy; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; TA: temporal artery; TAB: temporal artery biopsy.
P values are in bold when P < 0.05.
a TAB was considered positive if it showed transmural granulomatous vasculitis (with or without giant cells).
b Aortitis was proven by PET-CT or angio-CT scan

FIGURE LEGENDS

Figure 1: Confocal microscopy analysis of MAIT cells in a temporal artery biopsy specimen from a GCA patient

Representative TAB from a GCA patient. CD3 appears in green, IL-18R in yellow, TCR Vα7.2 in red and DAPI depicts nuclei and the collagen structure of the arterial wall. Magnification x 40. Higher magnification views of the boxed area show co-localization of the 4 MAIT-specific markers used. Image on the right panel (control) shows the result of a staining with DAPI and secondary antibodies without specific primary antibodies (control).

Figure 2: Frequency and phenotype of MAIT cells in controls, untreated GCA patients, and treated GCA patients.

A: Gating strategy for identification of circulating MAIT cells ($CD3^+CD4^-TCR\gamma\delta^-TCRV\alpha7.2^+CD161^+$). **B:** MAIT frequency among αβ T Lymphocytes, **C:** absolute number of circulating MAIT cells. The following figures show MAIT's expression of CXCR3 **(D)**, CCR6 **(E)**, IFN-γ **(F)**, IL-17 **(G)**, CD69 **(H),** Perforin **(I),** Granzyme B **(J)**. P is the result of Mann Whitney tests (healthy vs. untreated patients) or Wilcoxon tests (untreated vs. treated patients). (Horizontal bars represent the median and error bars the interquartile range)

Figure 3: Study of the proliferation ability of circulating MAIT cells

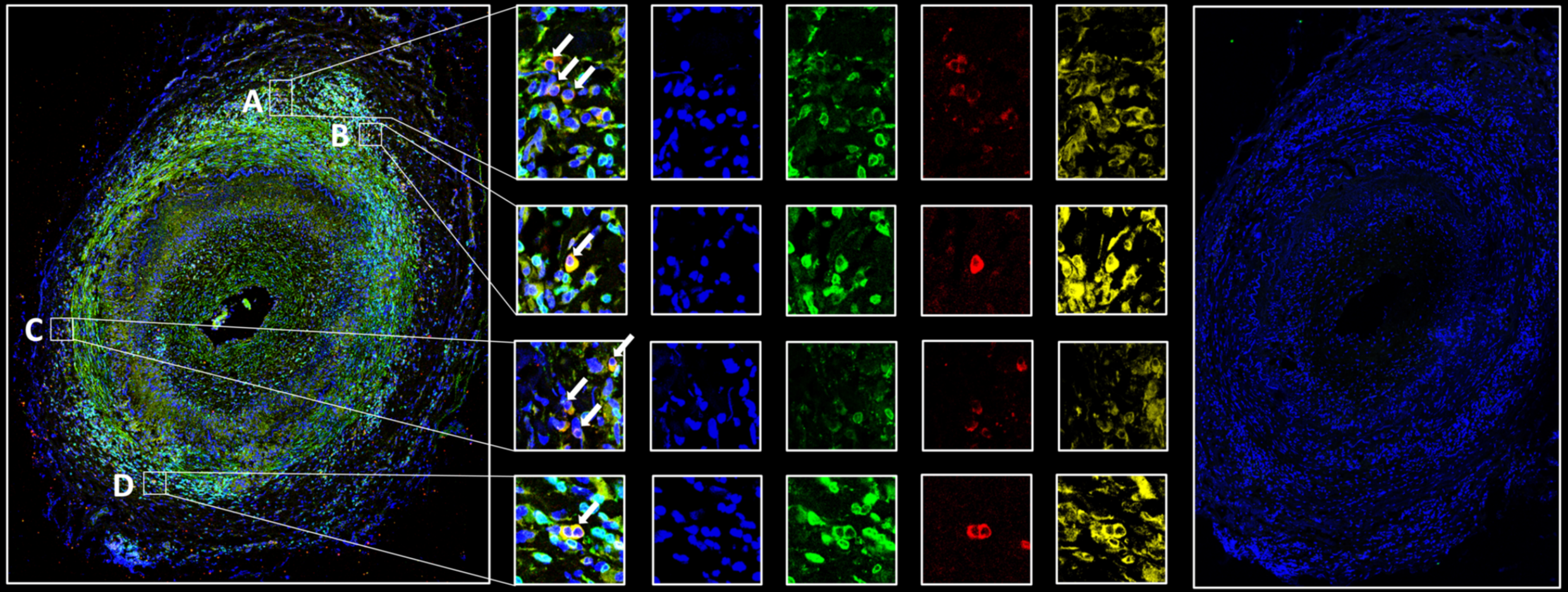
A & B: Assessment of the expression of Ki67 in circulating MAIT cells by flow cytometry. C-E: $CD161^+$ MAIT-depleted αβ T lymphocytes (D) and MAIT cells (E) were separated using a two-step sorting. Proliferation was assessed by flow cytometry analysis (cell trace violet incorporation) after 7 days of culture (healthy controls n=5, patients n=4). Results were analyzed with ModFit. Horizontal bars represent the median and error bars the interquartile range. P is the result of Mann Whitney tests (patients vs controls).

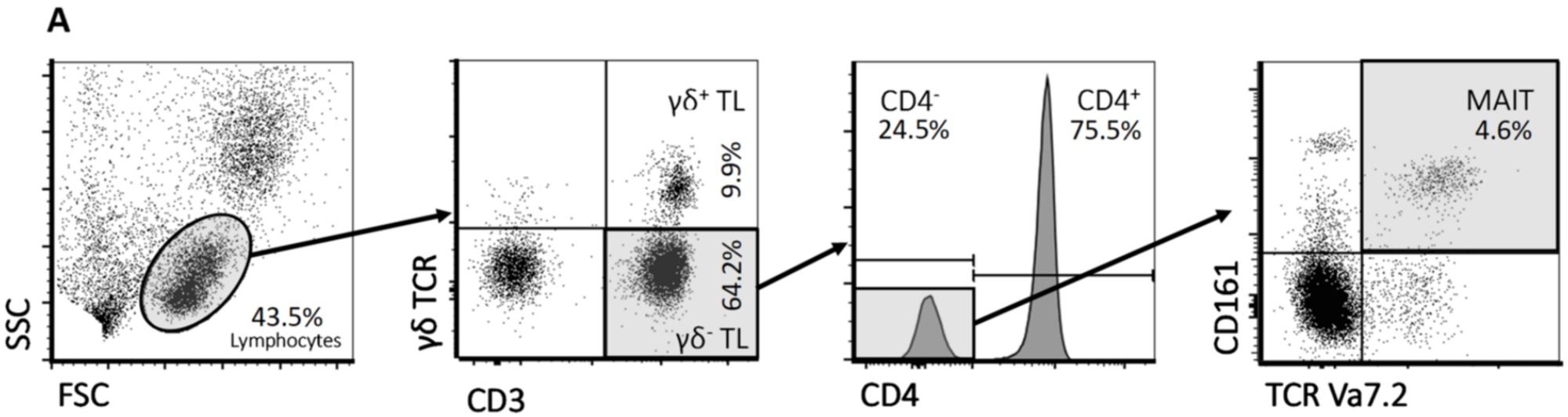
Figure 4: MAIT produce cytokines and cytotoxic molecules under stimulation

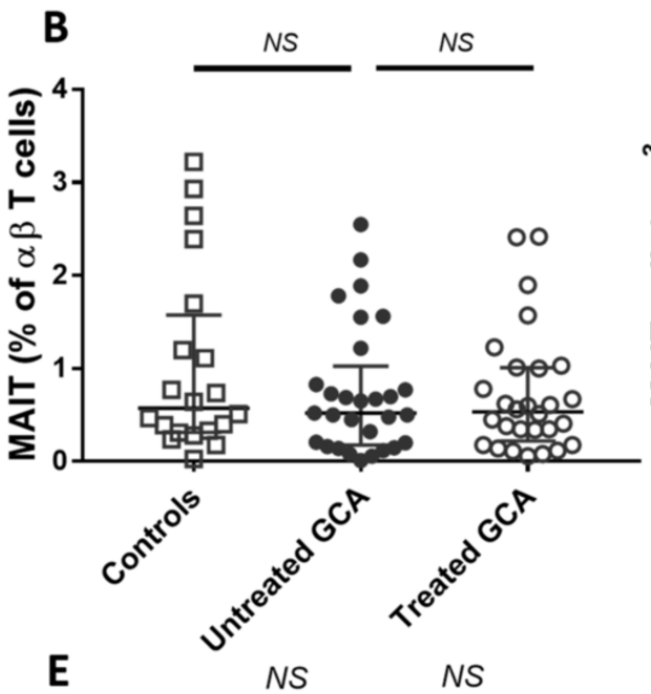
Measurements of the concentration of cytokines (IFN-γ [A], IL-17 [B], granzyme A [C], granzyme B [D] and TNF-α [E]) in the supernatant of in vitro cultivated MAIT cells, alone (negative control) or in the presence of anti CD3/CD28 beads or IL-12 and IL-18 (n= 5 healthy controls and 4 GCA patients). P is the result of Mann Whitney tests.

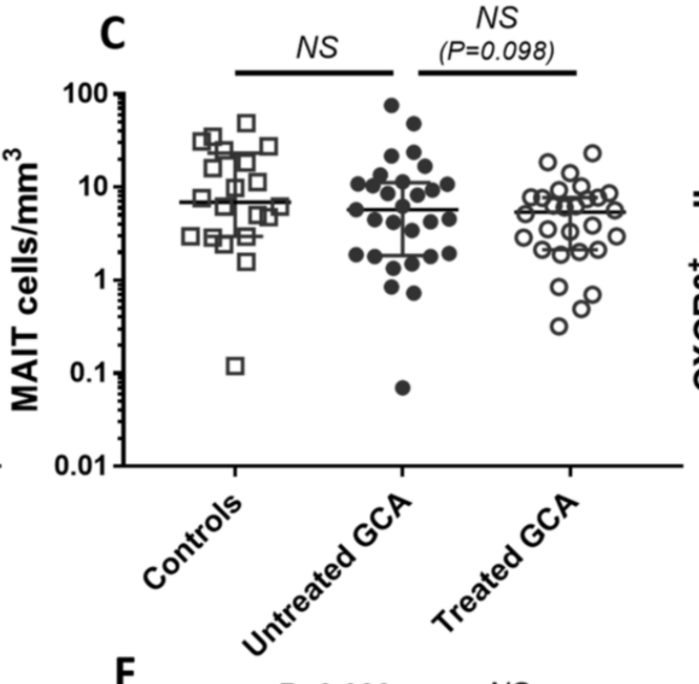
Figure 5: Level of expression of mRNA of IL12 subunits and IL18 in GCA-affected and healthy TABs

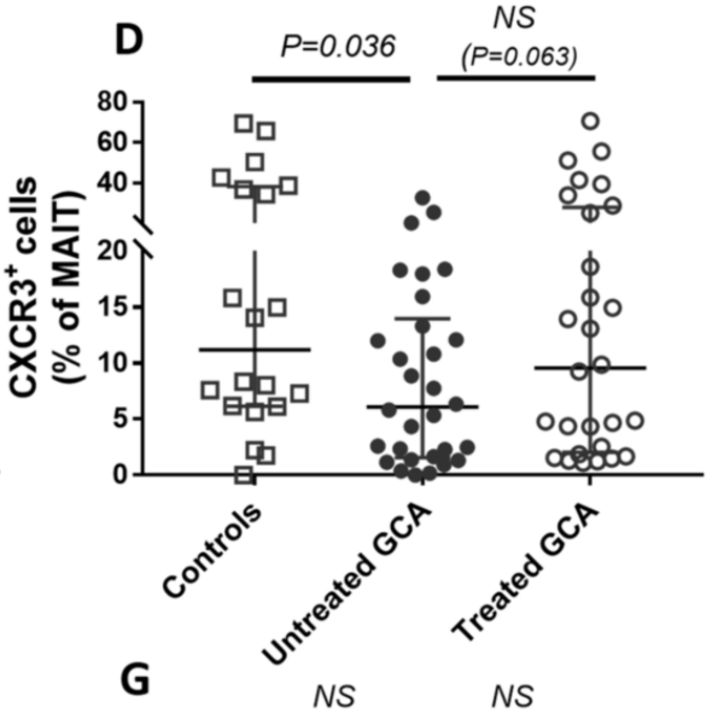
mRNA levels of IL12p35 (A), IL12p40 (B) and IL18 (C) in fresh temporal artery biopsy (TAB) specimens showing GCA lesions (TAB+ n=12) or not (TAB- n=6) that were cultured in MATRIGEL® for 5 days with or without dexamethasone (0.5 µg/mL). Horizontal bars represent the median and error bars the IQR. P is the result of Mann Whitney (TAB- vs TAB+) and Wilcoxon tests (TAB+ vs TAB+ with dexamethasone).
TAB: temporal artery biopsy, DEXA: Dexamethasone

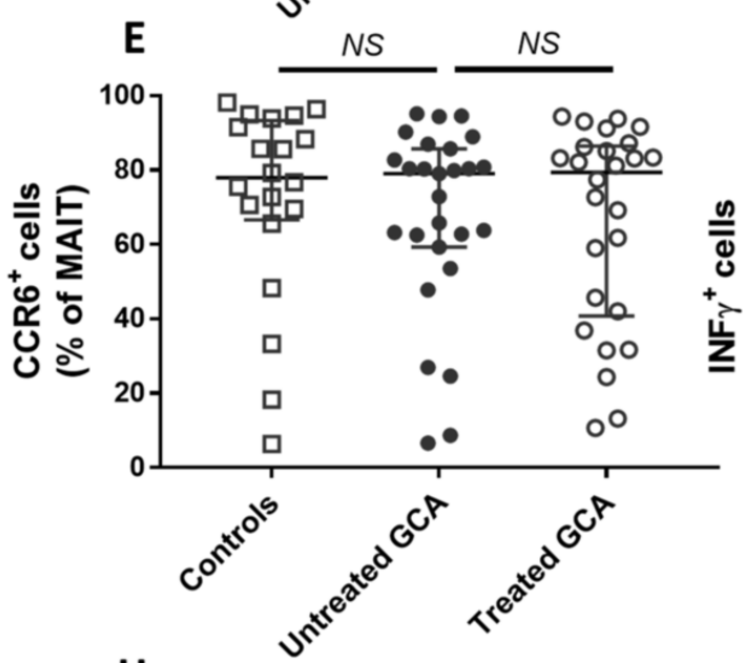

A
B
C
D
MERGE
MERGE
DAPI
CD3
TCR Vα7.2
IL-18R
MERGE (CONTROL)

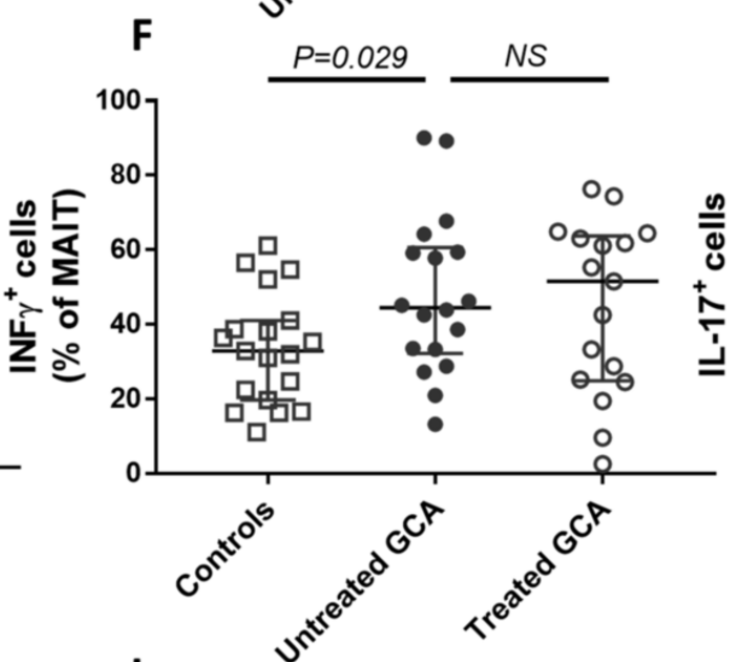

A
SSC
FSC
43.5%
Lymphocytes
γδ TCR
CD3
γδ+ TL
9.9%
γδ- TL
64.2%
CD4-
24.5%
CD4+
75.5%
CD4
CD161
TCR Va7.2
MAIT
4.6%

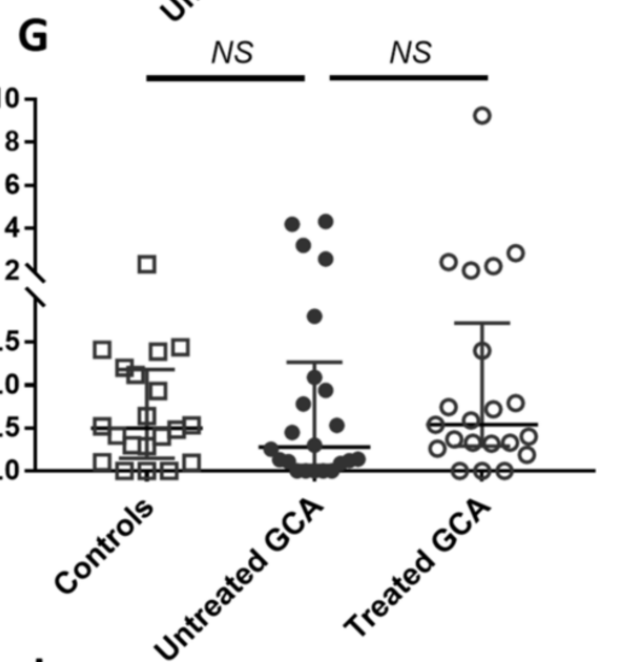

B
NS
NS
MAIT (% of αβ T cells)
Controls
Untreated GCA
Treated GCA

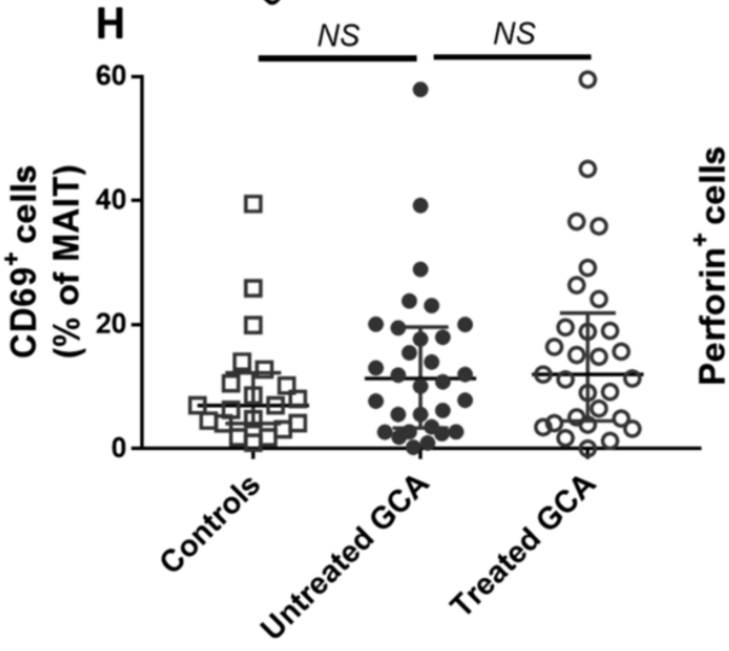

C
NS
NS
(P=0.098)
MAIT cells/mm3
Controls
Untreated GCA
Treated GCA

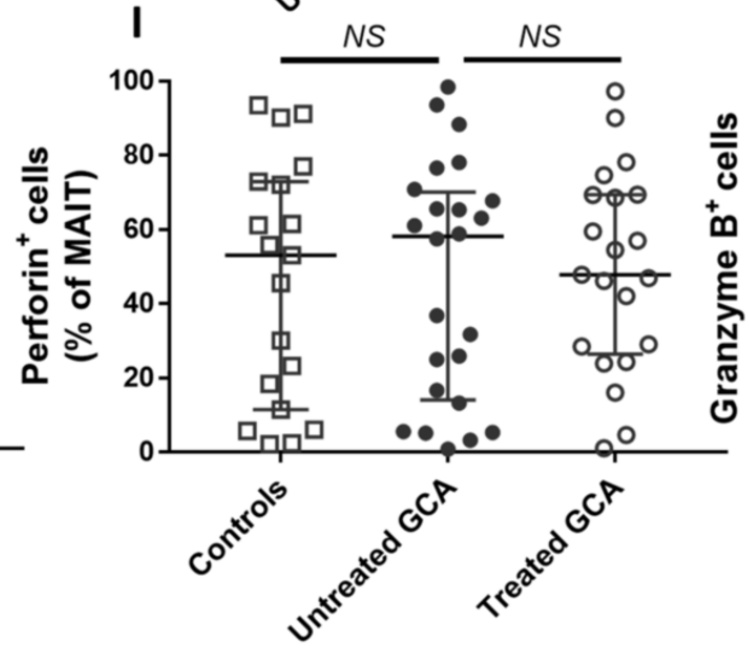

D
P=0.036
NS
(P=0.063)
CXCR3+ cells
(% of MAIT)
Controls
Untreated GCA
Treated GCA

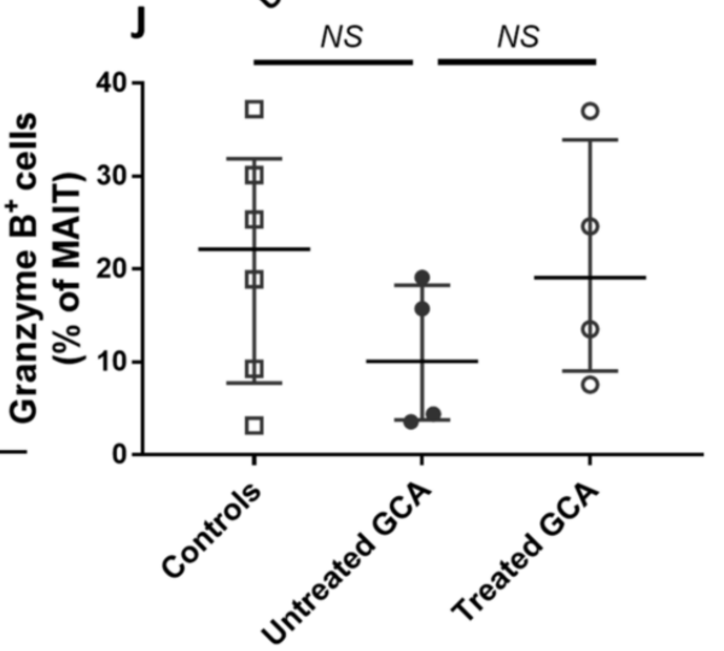

E
NS
NS
CCR6+ cells
(% of MAIT)
Controls
Untreated GCA
Treated GCA


F
P=0.029
NS
INFγ+ cells
(% of MAIT)
Controls
Untreated GCA
Treated GCA

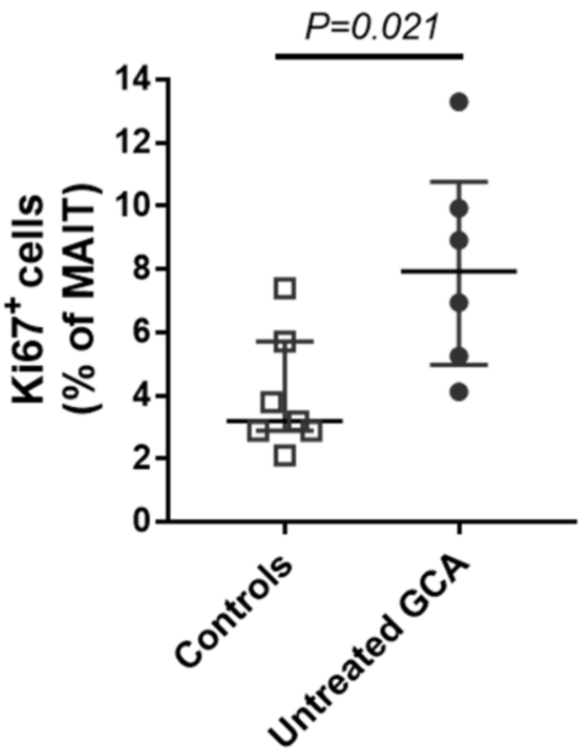

G
NS
NS
IL-17+ cells
(% of MAIT)
Controls
Untreated GCA
Treated GCA


H
NS
NS
CD69+ cells
(% of MAIT)
Controls
Untreated GCA
Treated GCA

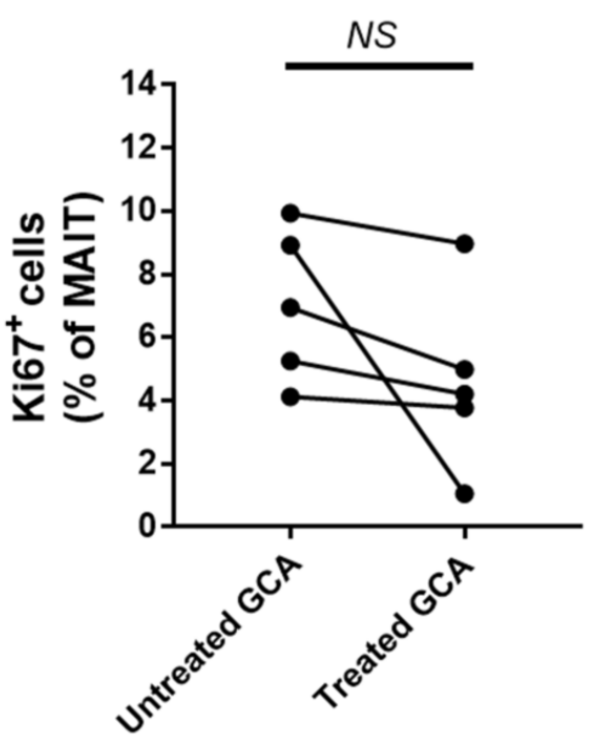

I
NS
NS
Perforin+ cells
(% of MAIT)
Controls
Untreated GCA
Treated GCA


J
NS
NS
Granzyme B+ cells
(% of MAIT)
Controls
Untreated GCA
Treated GCA

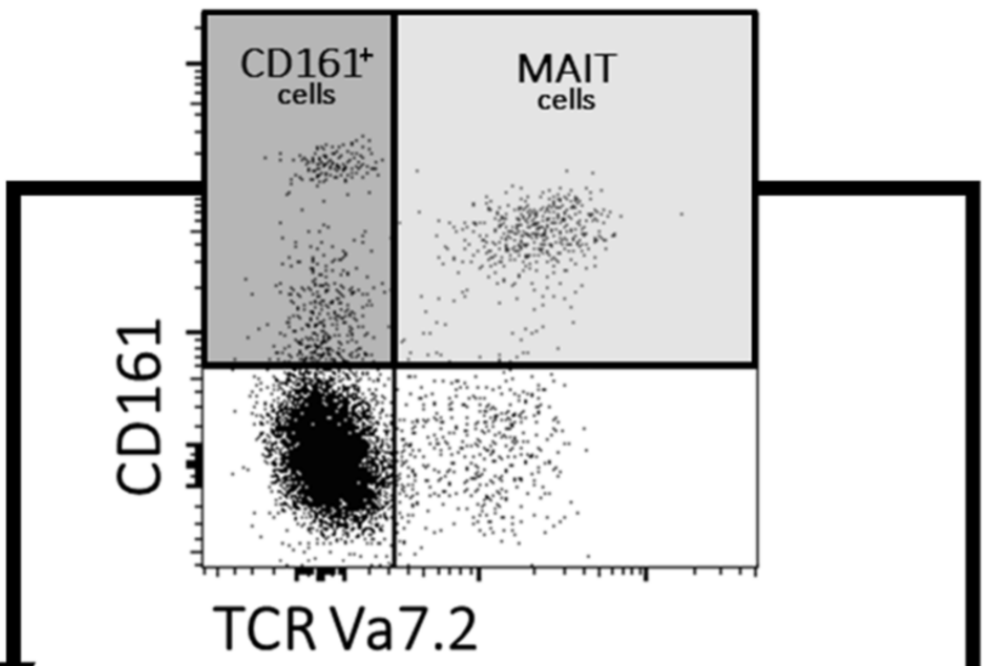
A



B



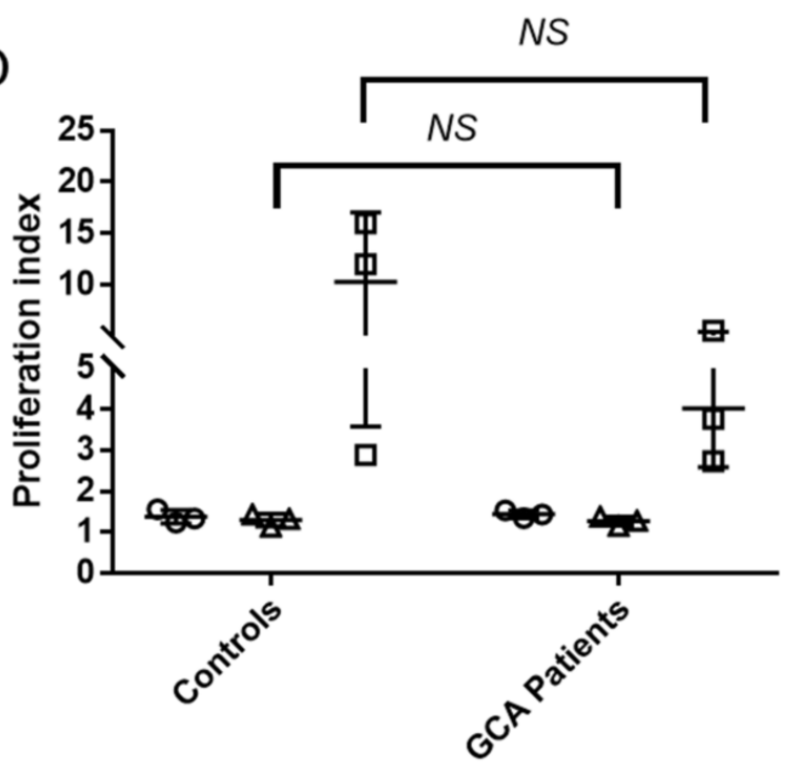
C



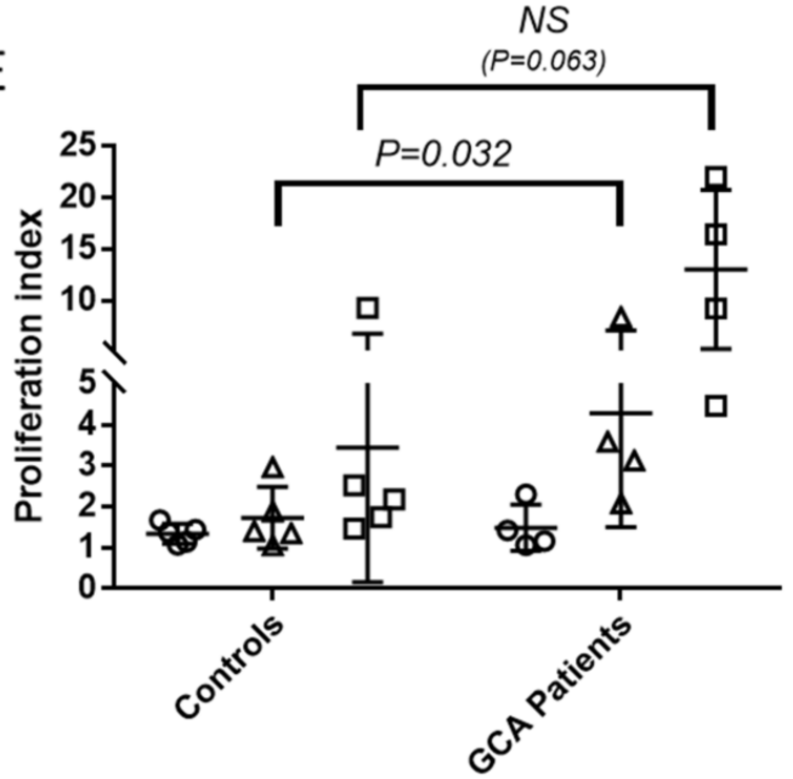
MAIT-depleted CD161+ αβT cells

MAIT cells

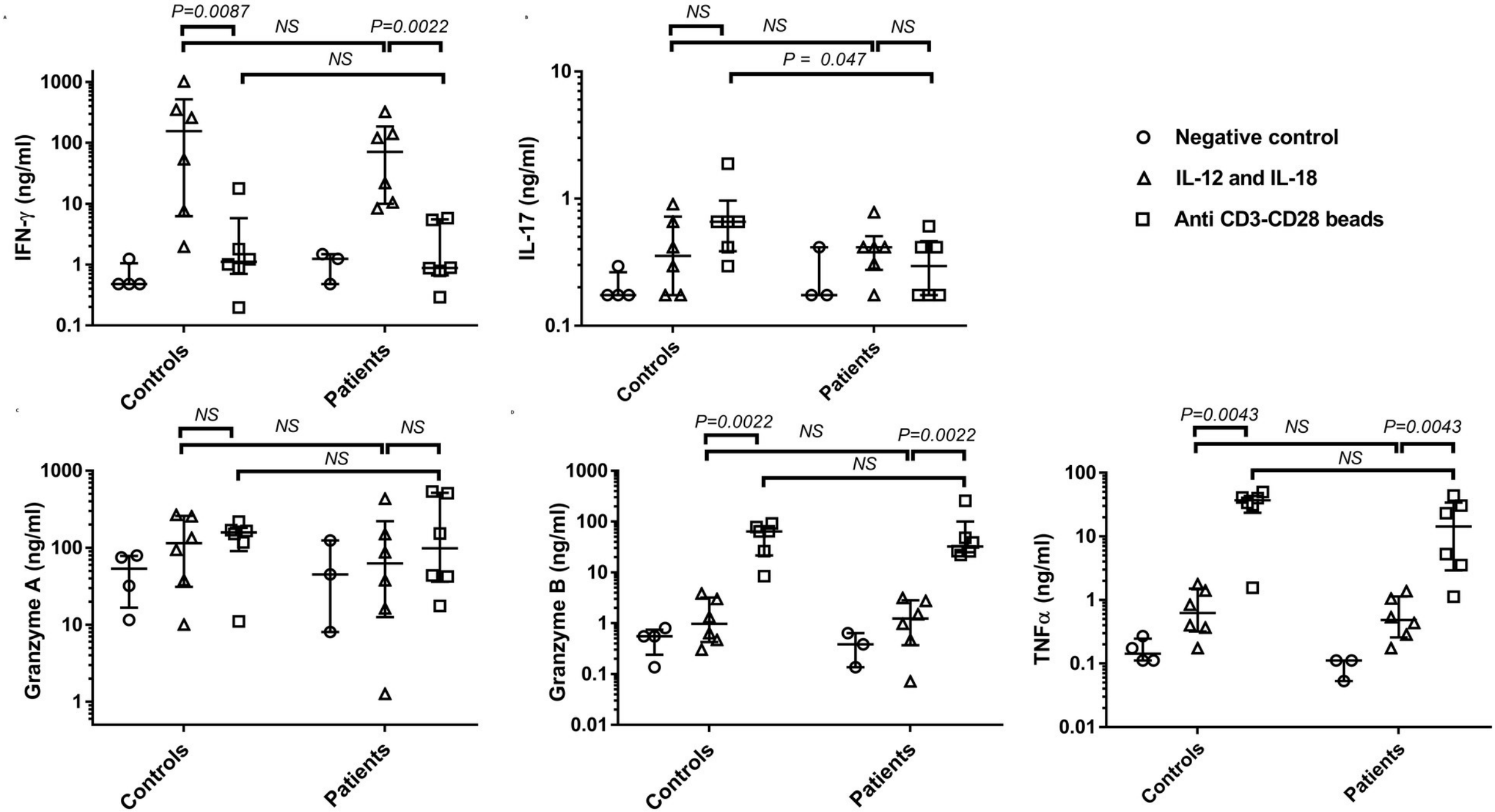
D

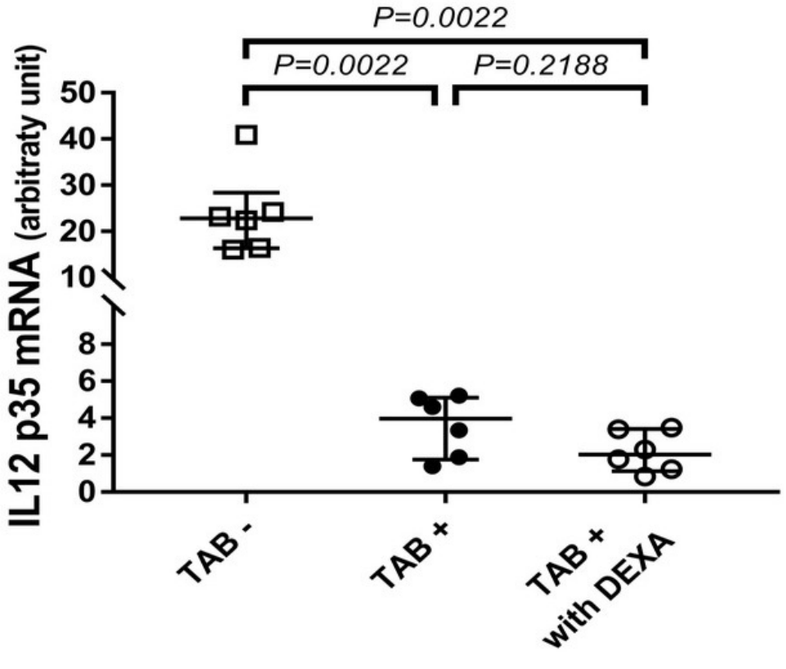


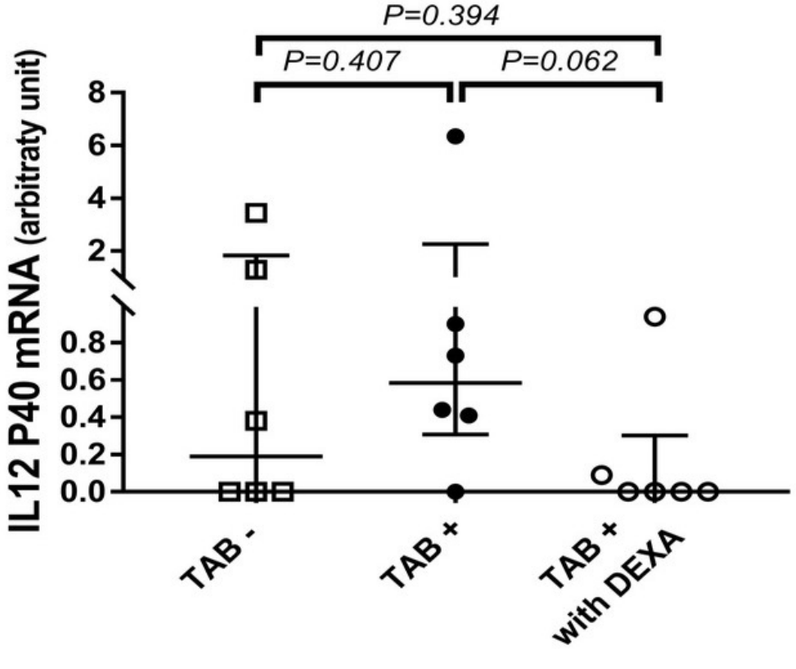
E

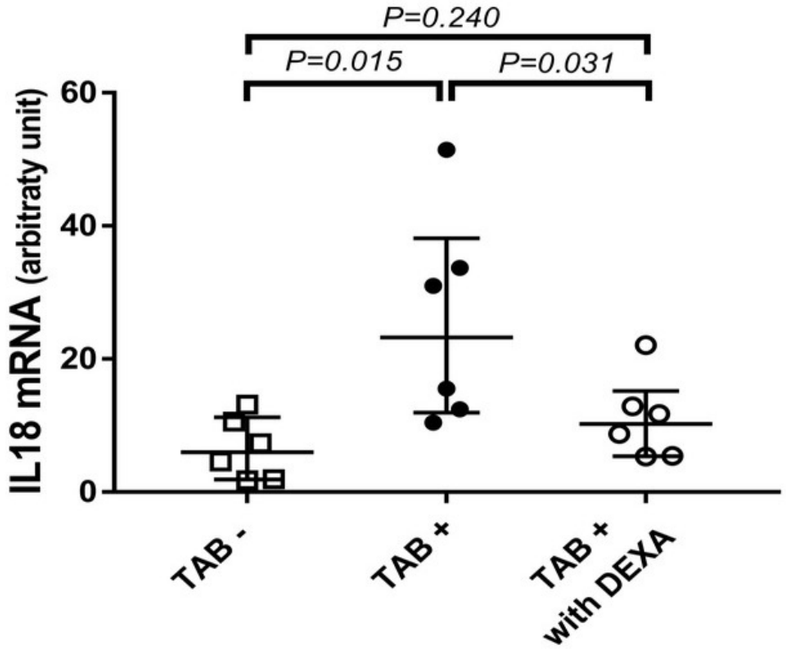


○ Negative control △ IL-12 and IL-18 □ Anti CD3-CD28 beads


P=0.0087
NS
P=0.0022
NS
IFN-γ (ng/ml)
1000
100
10
1
0.1
Controls
Patients
NS
NS
NS
P = 0.047
IL-17 (ng/ml)
10
1
0.1
Controls
Patients
Negative control
IL-12 and IL-18
Anti CD3-CD28 beads
NS
NS
NS
NS
Granzyme A (ng/ml)
1000
100
10
1
Controls
Patients
P=0.0022
NS
P=0.0022
NS
Granzyme B (ng/ml)
1000
100
10
1
0.1
0.01
Controls
Patients
P=0.0043
NS
P=0.0043
NS
TNFα (ng/ml)
100
10
1
0.1
0.01
Controls
Patients


P=0.0022
P=0.0022
P=0.2188
IL12 p35 mRNA (arbitraty unit)
50
40
30
20
10
8
6
4
2
0
TAB -
TAB +
TAB + with DEXA


P=0.394
P=0.407
P=0.062
IL12 P40 mRNA (arbitraty unit)
8
6
4
2
0.8
0.6
0.4
0.2
0.0
TAB -
TAB +
TAB + with DEXA


P=0.240
P=0.015
P=0.031
IL18 mRNA (arbitraty unit)
60
40
20
0
TAB -
TAB +
TAB + with DEXA