SUPPLEMENTARY MATERIALS FOR:

BLOCKING GM-CSF RECEPTORα WITH MAVRILIMUMAB REDUCES INFILTRATING CELLS, PRO-INFLAMMATORY MARKERS, AND NEOANGIOGENESIS IN EX-VIVO CULTURED ARTERIES FROM PATIENTS WITH GIANT-CELL ARTERITIS

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SUPPLEMENTARY METHODS

Patients

The study was performed with samples from 4 different groups of patients with suspected giant-cell arteritis (GCA). Group 1 consisted of 33 patients who donated the remnant of their diagnostic temporal artery biopsy (TAB) for research purposes as part of an approved and registered collection of frozen tissue (Vasculitis collection C.0003912). Sixteen biopsies were positive (with characteristic histopathological features of GCA) and 17 were negative and served as controls. Ten positive and 10 negative biopsies, randomly selected among those from patients who had not received glucocorticoid treatment at the time of the temporal artery biopsy, were processed for RNA extraction. The remaining 12 (6 positive and 7 negative) were used for immunofluorescence or Western-blot studies. These patients had received glucocorticoid treatment for ≤ 3 days. Group 2 consisted of 23 anonymous donors subjected to TAB for suspected GCA. Eighteen were positive and 5 negative for GCA diagnosis. Samples were formaldeyde fixed and paraffin embedded (FFPE) and were used for in situ RNA hybridization and immunohistochemical detection of the molecules of interest. These samples were purchased from Tissue for Research biobank but clinical data from their donors were limited. Group 3 consisted of 60 patients and 12 healthy donors of similar age and sex distribution who donated serum for the above-mentioned collection. Group 4 consisted of 16 patients diagnosed with biopsy-proven GCA during the study period (October 2018-August 2021) and cultured ex-vivo for the experiments described below. Clinical characteristics of patients and controls are depicted in Supplementary Table S1 and are representative of published GCA series.

Control patients who donated their biopsies for the collection, had the following final diagnosis: non-arteritic ischemic optic neuropathy (5 patients), non-specific constitutional symptoms in pluripatologic patients (7 patients), and non-specific headache (5 patients). In all of them the clinical suspicion was low and biopsies were performed to further rule-out GCA. None of them received prolonged glucocorticoid therapy.

Temporal artery culture

Serial, 1 mm thick sections of fresh temporal artery fragments from 16 patients with GCA were cultured ex-vivo on ice-cold reconstituted basement membrane Matrigel™ (BD Biosciences, San Jose, California, USA), as previously described (Corbera-Bellalta, M. et al, ARD 2014). Each section was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Waltham, Massachusetts, USA), 2mM of L-glutamine (Gibco), 50µg/mL of gentamicin (Braun, Melsungen, Germany) and 2.5 µg/mL of amphotericin B (Invitrogen). TAB sections were exposed to mavrilimumab (20µg/mL, Kiniksa, Lexington, MA, USA), placebo (mavrilimumab formulation buffer) (Kiniksa) or rhGMCSF (20ng/ml, R&D Systems). Each condition was tested in 2–3 replicate wells. TAB sections were cultured for 5 days at 37°C and 5% CO₂. The supernatant fluid was centrifuged and stored at -80°C until use. Cultured arteries were processed for RNA extraction (11 samples) or immunofluorescence (5 samples). Since the primary purpose of temporal artery biopsy is supporting diagnosis, the fragment spared for culture was small and limited number of conditions/markers per biopsy could be tested.
RNA extraction and reverse-transcription

Samples were homogenized in 1mL of TRI-Reagent (MRC Inc) prior to RNA extraction through chloroform-isopropanol separation-precipitation method. Pellets were rinsed with ethanol (70%) (Panreac Applichem, Barcelona, Spain). Total RNA was quantified by spectrophotometry (Quawell Technology, San Jose, CA, USA), and 1μg of RNA was reverse transcribed to cDNA employing High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, California, USA) in a final volume of 100μL. Samples were stored at -80°C until use.

Candidate gene expression analysis

Total RNA was extracted from cryostat sections of OCT-embedded frozen biopsies or cultured biopsies, homogenized with Bullet Blender (Next Advance, Troy, NY, USA) using TRI-Reagent (MRC Inc, Cincinnati, OH, USA).

cDNAs were obtained by reverse-transcription and measured by quantitative real-time PCR with specific pre-developed TaqMan probes (Applied Biosystems) (Supplementary Table S3).

Fluorescence was detected using ABI Prism 7900 Hardware Real-Time PCR System and results were quantified and analyzed with Sequence Detector software v.2.4 (Applied Biosystems). Gene expression was normalized to the expression of the endogenous control gene GUSB using comparative ΔCt method and expressed in relative units to GUSB expression.

Immunofluorescence of TABs

Fresh-frozen or cultured biopsies were fixed with 4% paraformaldehyde (PFA), pre-rinsed with increasing concentrations of sucrose, 15% and 30%, before being embedded in Tissue-Tek OCT Compound (Sakura, Flemingweg, The Netherlands) and preserved at -80°C until use. Sections of 7µm were obtained with a cryotome cryostat (Leica Microsystems), re-fixed with 4% PFA, permeabilized with 0.1% Triton solution and blocked with 5% donkey serum (Sigma) in 0.1% Triton PBS1X. Primary and secondary antibodies used and their concentrations or specific dilutions and corresponding sources are detailed in Supplementary table S4. Mounting medium with 4′,6-diamidino-2-phenylindole (DAPI Fluoromount-G, Southern Biotech, Birmingham, AL, USA) was used to stain the nuclei and to preserve fluorescence. To control for non-specific background, for each condition, some sections were processed omitting the primary antibody. Immunofluorescence samples were observed with confocal microscopy SP5 Leica (Leica Microsystems) and LSM880 (ZEISS) and the images were analyzed using the ImageJ software (National Institutes of Health, Wayne Rasband, Bethesda, Maryland, USA).

Modified RIPA buffer composition

Commercial RIPA lysis buffer (Sigma-Aldrich, Ayrshire, UK) was supplemented with phenylmethylsulfonyl fluoride (PMSF) (1mM) (Sigma), benzenesulfonyl fluoride hydrochloride (1mM) (Roche), orthovanadate (2nM) (Sigma), leupeptin (Sigma), aprotinin (Thermo Scientific), and pepstatin (Sigma) (0.5ug/mL), ethylene-diamine-tetraacetic acid (EDTA) (1mM) (Sigma), ethylene-glicol-tetraacetic acid (EGTA) (1mM) (Sigma), sodium fluoride (NaF) (50mM) (Sigma), and NP-40 detergent (1%) (Abcam).
Western Blot

TABs were homogenized with Bullet Blender (Next Advance, Try, NY) in complete RIPA buffer supplemented with protease and phosphatase inhibitors. Protein lysates were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis gels (BioRad) under reducing conditions and transferred to nitrocellulose membranes. Primary and secondary antibodies used with their specific dilutions and corresponding sources are detailed in Supplementary table S4. When necessary, membranes were treated with stripping buffer (100mM glycine (Sigma) and 100mM NaCl (Sigma); pH 2.5). Chemiluminescence signal was measured with the ImageQuant LAS-4000 imaging system (GE HealthCare Life Science, Pittsburgh, PA, USA). Images were analyzed and quantified using the ImageJ software (National Institutes of Health, Wayne Rasband, Bethesda, Maryland, USA).

Detection of proteins in the supernatants of cultured arteries and patient sera

Cytokines, chemokines, or membrane-bound molecules released into artery culture supernatants or present in serum were detected by immunoassay. Sources and characteristics of the commercially available immunoassays used are depicted in Supplementary Table S5. All procedures were performed according to the instructions of the respective manufacturers.
## Supplementary Table S1: Clinical characteristics of GCA and control sample donors

|                          | Fresh-frozen GCA arteries (n=16)* | Fresh-frozen control arteries (n=17)** | Formaldehyde fixed, paraffin embedded GCA arteries (n=12)† | GCA Serum (n=60) | Cultured GCA arteries (n=16)*** |
|--------------------------|-----------------------------------|----------------------------------------|-------------------------------------------------------------|------------------|---------------------------------|
| Age, median (range) years| 80 (69-90)                        | 78.5 (62-89)                           | 75 (64-88)                                                  | 77 (57-90)       | 80 (66-93)                      |
| Sex                      |                                   |                                        |                                                             |                  |                                 |
| Male, n (%)              | 6 (37.5%)                         | 7 (41%)                                | 4 (33.3%)                                                   | 21 (35%)         | 5 (31.25%)                     |
| Female, n (%)            | 10 (62.5%)                        | 10 (59%)                               | 8 (66.7%)                                                   | 39 (65%)         | 11 (68.75%)                    |
| Clinical data at diagnosis|                                  |                                        |                                                             |                  |                                 |
| Cranial symptoms (%)     |                                   |                                        |                                                             |                  |                                 |
| Headache, n (%)          | 12 (80%)                          | 7 (46.7%)                              | 8 (66.7%)                                                   | 49 (81.7%)       | 14 (87.5%)                     |
| Scalp tenderness, n (%)  | 6 (40%)                           | 3 (20%)                                | N/A                                                         | 24 (40%)         | 8 (50%)                        |
| Jaw claudication, n (%)  | 7 (46.7%)                         | 2 (13.3%)                              | N/A                                                         | 25 (41.7%)       | 10 (62.5%)                     |
| Stroke/visual events, n (%)| 4 (25%)                           | 5 (33.3%)                              | 4 (33.3%)                                                   | 17 (28.3%)       | 5 (31.25%)                     |
| Systemic symptoms, n (%) |                                   |                                        |                                                             |                  |                                 |
| Fever, n (%)             | 4 (26.7%)                         | 1 (6.7%)                               | N/A                                                         | 21 (35%)         | 3 (18.75%)                     |
| Weight loss, n (%)       | 7 (46.7%)                         | 9 (60%)                                | N/A                                                         | 23 (38.3%)       | 5 (31.25%)                     |
| Polymyalgia rheumatica, n (%)| 4 (26.7%)                         | 3 (20%)                                | N/A                                                         | 22 (36.7%)       | 3 (18.75%)                     |
| Laboratory findings at diagnosis|                                  |                                        |                                                             |                  |                                 |
| ESR, mm/h                | 81.5 ± 25                         | 89 ± 39                                | N/A                                                         | 90 ± 32          | 77 ± 47                        |
| CRP, mg/dL               | 5.20 ± 4.23                       | 2.97 ± 4.05                            | N/A                                                         | 9.41 ± 7.61      | 11.97 ± 11.3                   |
| Haemoglobin, g/L         | 108.5 ± 12                        | 112 ± 19                               | N/A                                                         | 112 ± 15         | 118.9 ± 19                     |
| Prednisone treatment pre-biopsy or serum extraction, n (%) | 4 (25%)                           | 5 (33.3%)                              | N/A                                                         | 5 (8.5%)         | 12 (75%)                       |

* 10 for RNA extraction, 3 for protein extraction (western-blot), and 4 for immunofluorescence.

** 10 for RNA extraction, 3 for protein extraction (western-blot) and 4 for immunofluorescence.

*** 11 for RNA extraction, 5 for confocal microscopy and 13 for ELISA.

† Some relevant clinical information was not available from purchased samples.
Supplementary Materials: Mavrilimumab in GCA Arteries

Supplementary Table S2: Quantitation of RNAscope signal

Expression score was calculated as RS score (dots/cell) multiplied by Positivity score (% cells positive with >1 dot/cell).

| RS score | Criteria               |
|----------|------------------------|
| 0        | No staining or <1 dot/10 cells |
| 1        | 1-3 dots/cell          |
| 2        | 4-9 dots/cell          |
| 3        | 10-15 dots/cell        |
| 4        | >15 dots/cell          |

| Positivity score | Criteria             |
|-----------------|----------------------|
| 1               | <25% cells positive  |
| 2               | 25-50% cells positive|
| 3               | 50-75% positive      |
| 4               | >75% cells positive  |
## Supplementary Table S3: Probes used for real-time quantitative RT-PCR

| Gene name       | Probe reference | Gene name       | Probe reference |
|-----------------|-----------------|-----------------|-----------------|
| GUSB            | Hs99999908_m1   | CD14            | Hs00169122_g1   |
| TBX21 (T-bet)   | Hs00894392_m1   | CD16            | Hs04334165_m1   |
| RORC (ROR-γ1)   | Hs01076112_m1   | CD3E            | Hs01062241_m1   |
| IL6             | Hs00985639_m1   | CD20            | Hs00544818_m1   |
| IL1B (IL-1β)    | Hs01555413_m1   | CD68            | Hs00154355_m1   |
| TNFα (TNFα)     | Hs00174128_m1   | CD83            | Hs00188486_m1   |
| IFN-γ (IFN-γ)   | Hs00174143_m1   | SPI1 (PU.1)     | Hs02786711_m1   |
| IL17A           | Hs00174383_m1   | HLA-DRA         | Hs00219575_m1   |
| IL23a           | Hs00372324_m1   | NOS2 (iNOS)     | Hs01075529_m1   |
| CXCL10          | Hs00171042_m1   | GM-CSF          | Hs00929873_m1   |
| IL10            | Hs00961622_m1   | GM-CSFRA (GM-CSF) | Hs00538896_m1   |
| CD163           | Hs00174705_m1   | MMP-9           | Hs00234579_m1   |
| CD206           | Hs00267207_m1   | TIMP1           | Hs00171558_m1   |
| PECAM-1 (CD31)  | Hs00169777_m1   | VWF             | Hs01109446_m1   |
| CD34            | Hs00990732_m1   | VEGF-A          | Hs00900055_m1   |
Supplementary Materials: Mavrilimumab in GCA Arteries

Supplementary table S4: List and characteristics of antibodies

A. Primary antibodies used for immunofluorescence

| Antibody     | Company            | Working concentration or dilution | Host animal | Clonality (clone/ref) |
|--------------|--------------------|----------------------------------|-------------|-----------------------|
| Anti-CD68    | DAKO               | undiluted                        | Mouse       | Monoclonal (KP1)      |
| Anti-CD3     | Thermo Fisher      | 1:200                            | Mouse       | Monoclonal (F7.2.38)  |
| Anti CD3ε    | Raybiotech         | 1μg/ml                           | Rabbit      | Polyclonal (119-11933) |
| Anti-CD16    | BioRad             | 40                               | Mouse       | Monoclonal (2H7)      |
| Anti-CD20    | DAKO               | undiluted                        | Mouse       | Monoclonal (L26)      |
| Anti-CD31    | DAKO               | undiluted                        | Mouse       | Monoclonal (JC70A)    |
| Anti-α-SMA   | Abcam              | 10μg/ml                          | Mouse       | Monoclonal (ab54723)  |
| Anti-GMCSF   | Abcam              | 1μg/mL                           | Rabbit      | Polyclonal (ab220888) |
| Anti-GMCSFRα | Biorbyt            | 10μg/ml                          | Rabbit      | Polyclonal (orb157207) |
| Anti-HLA-DRA | Beckton Dickinson  | 100                              | Mouse       | Monoclonal (G46-6)    |
| Anti-HNE     | Bios Antibodies    | 20μg/ml                          | Rabbit      | Polyclonal (bs6313R)  |
| Anti-pSTAT5  | Cell Signaling     | 1:200                            | Rabbit      | Polyclonal (C71E5)    |
|              | (Tyr694)           |                                  |             |                       |
| Anti-CD34    | Ventana            | 0.8μg/ml                         | Mouse       | Monoclonal            |
| Anti-VEGFA   | Santa Cruz Biotechnology | 4μg/ml                | Rabbit      | Polyclonal (sc-152)   |

B. Secondary antibodies used for immunofluorescence

| Antibody         | Company          | Working concentration or dilution | Host animal | Clonality (clone/ref) |
|------------------|------------------|----------------------------------|-------------|-----------------------|
| Anti-Mouse-Alexa | Molecular Probes | 6,6μg/ml                         | Donkey      | Polyclonal (A31570)   |
| Fluor 555        |                  |                                  |             |                       |
| Anti-Rabbit-Alexa| Molecular Probes | 6,6μg/ml                         | Donkey      | Polyclonal (A21206)   |
| Fluor 488        |                  |                                  |             |                       |
B. Primary antibodies used for Western-Blot

| Antibody                  | Company      | Working concentration or dilution | Host animal | Clonality (clone/ref) |
|---------------------------|--------------|----------------------------------|-------------|-----------------------|
| Anti-pSTAT5 (Tyr694)      | Cell Signaling | 1:1000                           | Rabbit      | Polyclonal (C71E5)    |
| Anti-STAT5                | Cell Signaling | 1:1000                           | Rabbit      | Polyclonal (D3N2B)    |
| Anti-β-ACTIN              | Sigma        | 1:5000                           | Mouse       | Monoclonal (AC-15)    |

Secondary antibodies used for immunoblot

| Antibody                  | Company      | Working concentration or dilution | Host animal | Clonality (clone/ref) |
|---------------------------|--------------|----------------------------------|-------------|-----------------------|
| Anti-Rabbit-HRP           | Cell         | 1:2000                           | Goat        | Polyclonal (7074S)    |
| Anti-Mouse-HRP            | BioRad       | 1:2000                           | Goat        | Polyclonal (170-6516) |

C. Primary antibodies used for immunohistochemistry

| Antibody                  | Company      | Working concentration or dilution | Host animal | Incubation time | Clonality (clone/ref) |
|---------------------------|--------------|----------------------------------|-------------|-----------------|-----------------------|
| Anti-CD83                 | BioRad       | 100μg/ml                         | Mouse       | 1 hour          | Monoclonal (HB15-E)   |
| Anti-GMCSFRα              | Biorbyt      | 10μg/ml                          | Rabbit      | 1 hour          | Polyclonal (orb157207) |
| Anti-GMCSF                | My Biosource | 10μg/ml                          | Mouse       | 2 hours         | Monoclonal (7U1)      |
| Anti-pJAK2                | Abcam        | 2.5μg/ml                         | Rabbit      | 1 hour          | Polyclonal (ab32101)  |
| Anti-PU.1                 | My Biosource | 2.5μg/ml                         | Goat        | 1 hour          | Polyclonal            |
| Anti-pSTATS               | Cell Signaling | 1:150                           | Rabbit      | 1 hour          | Polyclonal (C71E5)    |

Note: Concentration of some antibodies was not detailed in the technical information, and the working dilution of the purchased material is provided.
Supplementary Table S5: ELISA kits used for immunoassay

| Target protein | Source            | Sensitivity threshold | Samples used         |
|----------------|-------------------|-----------------------|----------------------|
| GM-CSF         | R&D Systems       | > 0.26 pg/mL          | Serum                |
| IL-6           | R&D Systems       | > 0.7 pg/mL           | Culture supernatants |
| TNFα           | R&D Systems       | 0.011-0.049 pg/mL     | Culture supernatants |
| IL-1β          | R&D Systems       | > 1 pg/mL             | Culture supernatants |
| IFNγ           | R&D Systems       | 0.025-0.173 pg/mL     | Culture supernatants |
| CXCL10         | R&D Systems       | 0.41-4.46 pg/mL       | Culture supernatants |
| MMP-9          | R&D Systems       | 0.002-0.01 ng/mL      | Culture supernatants |
| IL-10          | R&D Systems       | >3.9 pg/mL            | Culture supernatants |
| sCD83          | Antibodies Online | > 1.42 pg/mL          | Culture supernatants |
| TIMP-1         | R&D Systems       | > 0.08 ng/mL          | Culture supernatants |
| VEGF-A         | Invitrogen        | > 7.9 pg/mL           | Culture supernatants |