UNIVERSITAT DE BARCELONA

Contribution of hypoxia inducible factor, HIF1α, to vascular inflammation and remodeling in giant cell arteritis (GCA). Effects of GM-CSF receptor blockade on HIF1α stabilization

Roser Alba Rovira

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Doctoral thesis

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Contribution of hypoxia inducible factor, HIF1α, to vascular inflammation and remodeling in giant cell arteritis (GCA)

Effects of GM-CSF receptor blockade on HIF1α stabilization

Thesis presented by **Roser Alba Rovira** for the degree of Doctor by the University of Barcelona

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Abbreviations

A

ANCA: anti-neutrophil cytoplasmic antibodies

ARNT: aryl hydrocarbon receptor nuclear translocator

αSMA: alpha smooth muscle actin

B

BSA: bovine serum albumin

C

CC: co-culture

CCL: chemokine C-C motif ligand

CD: cluster differentiation

cDNA: complementary DNA

CRP: C-reactive protein

CXCL: chemokine C-X-C motif ligand

CXCR: chemokine C-X-C motif receptor

D

DAPI: 4’,6-diamino-2-phenylindole

DEPC water: diethylpyrocarbonate-treated water

DC: Dendritic cells

DMEM: Dulbecco’s modified eagle medium

DMOG: dimethyloxaloyglycine

DMSO: dimethyl sulfoxide

E

EDTA: ethylene-diamine-tetra acetic acid

EGF: epidermal growth factor

ELISA: enzyme-linked immunosorbent assay

EPAS: endothelial PAS domain

EPO: erythropoietin

ESR: erythrocyte sedimentation rate

ET-1: endothelin 1

F

FBS: fetal bovine serum

FIH-1: factor inhibiting HIF 1

G

GC: glucocorticoids

GCA: giant cell arteritis

GLUT1: glucose transporter 1

GM-CSF/CSF2: granulocyte-macrophage colony-stimulating factor

GM-CSFRα: GM-CSF receptor alpha

H

HIF: hypoxia inducible factor

HPX: hypoxia

HRE: hypoxia response elements

I

ICAM1: intracellular adhesion molecule 1

IgG: immunoglobin G

IFN: Interferon

IL: Interleukin

iNOS/NOS2: inducible nitric oxide synthase

IPAS: inhibitory PAS domain

J

JAK: Janus kinases

L

LV: large vessel

M

MMP: matrix metalloproteases

N

NFκB: nuclear factor kappa B

NRX: Normoxia

O

ODDD: oxygen dependent degradation domain

P

PBMC: peripheral blood mononuclear cells

PBS: phosphate-buffered saline

PDGF: platelet derived growth factor

PFA: paraformaldehyde

PHD: prolyl hydroxylase domain

PMR: polymyalgia rheumatic

pO_2: partial oxygen pressure

pVHL: von Hippel-Lindau protein

R

RIPA: radio-immunoprecipitation assay buffer

ROS: reactive oxygen species

RPMI-1640: Roswell park memorial institute 1640

RU: relative units

S

SCID: Severe combined immunodeficiency

SDS: sodium dodecyl sulfate

SEM: standard error of the mean

STAT: signal transducer and activator of transcription

T

TA: temporal artery

TAB: temporal artery biopsy

TCZ: tocilizumab

TF: transcription factor

TNF: tumor necrosis factor

U

UNT: untreated

V

VEGFA: vascular endothelial growth factor A

VEGFR1/FLT1: vascular endothelial growth factor receptor 1

VSMC: vascular smooth muscle cell

Doctoral thesis in classical format with 1 annexed article:

Marc Corbera-Bellalta*, **Roser Alba-Rovira***, Sujatha Muralidharan*, Georgina Espígol-Frigolé, Roberto Ríos-Garcés, Javier Marco-Hernández, Amanda Denuc, Farah Kamberovic, Patricia Pérez-Galán, Alexandra Joseph, Annalisa D'Andrea, Kent Bondensgaard, Maria C Cid**, John F Paolini**. (* Equal contribution; ** Shared senior authorship). 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. Annals of the Rheumatic Diseases Published Online First: 19 January 2022. doi: 10.1136/annrheumdis-2021-220873. IF:19.103 , D1, Rheumatology area.

Summary

A summary in Catalan of the present doctoral thesis is written below.
A continuació s'escriu un resum de la tesi doctoral en català.

CONTRIBUCIÓ DEL FACTOR INDUÏBLE PER LA HIPÒXIA (HIF1α) A LA INFLAMACIÓ I EL REMODELAT VASCULAR EN L'ARTERITIS DE CÈL·LULES GEGANTS (GCA). EFECTES DEL BLOQUEIG DEL RECEPTOR DEL GM-CSF EN L'ESTABILITZACIÓ DE HIF1α

Introducció

ARTERITIS DE CÈL·LULES GEGANTS

L'arteritis del cèl·lules gegants (GCA, per les seves sigles en anglès) és una malaltia sistèmica caracteritzada per la inflamació i el dany en les artèries. Afecta principalment l'aorta i les seves principals branques , especialment les branques cranials. Es tracta de la vasculitis més freqüent a Europa i Nord-Amèrica en individus de més de 50 anys. El símptomes més freqüents són mal de cap, claudicació mandibular, febre, anorèxia, etc. El símptoma més greu és la pèrdua de visió deguda a complicacions isquèmiques que es pot evitar amb un tractament temprà. Altres territoris vasculars també es veuen afectats, de fet, l'aneurisma d'aorta toràcica és més freqüent en pacients amb GCA que en la població general.

La prova diagnòstica de referència és la biòpsia d'arteria temporal on s'observa un infiltrat inflamatori que afecta la paret vascular. Histològicament una biòpsia d'artèria temporal presenta un infiltrat inflamatori juntament amb una disrupció de les làmines elàstiques i una hiperplàsia de la íntima que pot resultar en una oclusió de la llum.

El model fisiopatològic actual proposa que la inflamació vascular comença després d'una activació de les cèl·lules dendrítiques residents, les quals promouen el reclutament i activació de limfòcits T CD4+. A continuació, la resposta immune és extensa, exagerada i caracteritzada per una resposta Th1. Els macròfags tenen un

paper molt important en tota la cascada inflamatòria. Les cèl·lules musculars llises (VSMC) presents en la làmina mitja de les artèries tenen un paper actiu en el desenvolupament de la malaltia secretant quimiocines, i citocines que promouen i amplifiquen la inflammació. També migren a través de la paret vascular, produeixen factors de creixement, molècules profibròtiques que finalment, resultaran en un remodelat vascular aberrant.

Recentment, el factor estimulant de colònies de granulòcits i macròfags (GM-CSF) s'ha descrit com una molècula important en la inflamació crònica. L'activació del seu receptor comporta l'activació de la via JAK2/STAT5 i actua principalment sobre cèl·lules d'origen mieloide com les dendrítiques i els macròfags. El GM-CSF es troba incrementat en les lesions de GCA comparat amb artèries control.

Els models actuals per estudiar la GCA necessiten un fragment de les biòpsies d'artèries temporals dels pacients ja sigui per implantar-lo de forma subcutànea a ratolins amb una immunodeficiència combinada greu o bé per introduir-la en una matriu per a fer cultius *ex-vivo*.

La base del tractament actual de la GCA són principalment els glucocorticoids, ja que a dosis altes atenuen dràsticament els símptomes i milloren les alteracions analítiques. Però en quant es redueixen les dosis augmenta la probabilitat de recaiguda requerint més temps a altes dosis resultant en més efectes secundaris. S'han provat altres immunosupressors i el metotrexat ha resultat favorable en reduir la probabilitat de recaigudes.

Diversos fàrmacs biològics bloquejant molècules específiques estant sent o han estat recentment testats. El tocilizumab és un anticòs monoclonal que bloqueja el receptor de la IL6 (IL6R). L'assaig clínic fase 3 anomenat GiACTA va resultar en l'aprovació d'aquest fàrmac per a la GCA. Tot i això, aproximadament la meitat dels pacients tractats amb tocilizumab no mantenen la remissió en retirar els glucocorticoids pel que seria molt interessant trobar predictors de resposta. Una limitació important d'aquest tractament és, que degut al seu mecanisme d'acció que comporta inhibició de la síntesi hepàtica de proteïnes de fase aguda , que depenen de la IL-6, invalida la utilitat de la detecció d'aquestes proteïnes per avaluar l'activitat de la malaltia.

Les funcions biològiques conegudes del GM-CSF fan pensar que podria tenir un paper important en la perpetuació en la inflamació en l'ACG, d'acord amb el model

patogenètic on les cèl·lules dendrítiques i els macròfags juguen un paper rellevant. El mavrilimumab, un anticòs monoclonal que bloqueja el receptor del GM-CSF, ha aconseguit l'objectiu primari en un assaig clínic fase 2 reduint el risc de recaigudes.

FACTOR INDUÏBLE PER LA HIPÒXIA

Per una altra banda el factor induïble per la hipòxia, HIF1α, es pot activar per processos fisiològics durant el desenvolupament o processos patològics com és el cas dels tumors. L'HIF és una proteïna heterodimèrica formada per la subunitat beta expressada constitutivament i una subunitat alfa que constitutivament s'expressa però també es degrada via l'ubiquitin-proteasoma. Quan hi ha una falta d'oxigen o nutrients la subunitat alfa no es pot hidroxilar i posteriorment no es pot ubiquitinitzar pel que no es degradada i s'estabilitza; d'aquesta forma dimeritza amb la subunitat beta, entra al nucli i actua com a factor de transcripció. Hi ha 3 subunitats alfa diferents que s'expressen en diferents situacions/teixits i tenen funcions diferents. La subunitat alfa 1 (HIF1α) és la que s'expressa a totes les cèl·lules i és el focus de la present tesis.

L'HIF1α es reconegut per les PHD, encarregades d'hidroxilar-lo i poder ser així reconegut per la pVHL i tot un complex proteic encarregat d'ubiquitinitzar la subunitat alfa per a ser posteriorment degradat pel proteasoma. Les PHD són oxigen-depenents pel que en condicions d'hipòxia l'HIF1α s'estabilitza. Quan la subunitat alfa està estabilitzada s'uneix a la subunitat beta i així poden reconèixer unes regions específiques del DNA que s'anomenen elements de resposta a hipòxia (HRE) presents en el promotor dels gens diana. Varis gens diana estan relacionats amb l'adaptació a la falta d'oxigen i/o nutrients com és el cas del GLUT1 (metabolisme), el VEGFA (angiogènesi) entre d'altres.

Recentment l'HIF1α s'ha relacionat amb la inflamació. La hipòxia es un tret característic de teixits afectats per una inflamació crònica com seria el cas de la GCA. Està descrit que la inflamació (via NFκB) indueix l'expressió d'HIF1α i que el dèficit de pVHL en progenitors epicàrdics en un model de ratolí provoca una malaltia similar a una vasculitis a les arteries coronaries. L'HIF1α està relacionat amb vàries molècules implicades en la patogènesi de la GCA com és el cas del PDGF, la endotelina 1, l'angiotensina entre d'altres.

FACTOR ESTIMULANT DE COLÒNIES DE GRANULÒCITS I MACRÒFAGS

El factor estimulant de colònies de granulòcits i macròfags, GM-CSF, com el seu nom indica es va descriure com a un factor de creixement hematopoietic. No obstant, la majoria de cèl·lules mieloides no requereixen el GM-CSF per a estar en estat estacionari. De fet, durant els processos d'inflamació, el GM-CSF serveix com una via de comunicació entre els limfòcits que envaeixen el teixit i les cèl·lules mieloides. El paper fonamental del GM-CSF en la relació entre les cèl·lules T i les cèl·lules mieloides dóna suport a la seva importància en la immunopatologia. Fer diana en la comunicació entre aquests dos tipus cel·lulars, mitjançant el GM-CSF, podria ser un bon objectiu terapèutic per combatre la inflamació tissular crònica.

El receptor específic del GM-CSF és un complex heterodimeric format per una cadena alfa específica pel GM-CSF (GM-CSFRα) i una subunitat beta encarregada de transduir la senyal activant la vía JAK-STAT. El GM-CSF activa el JAK2 i l'STAT5, factors importants en la inflamació.

Diversos models animals de processos inflamatòria tenen com al GM-CSF com a factor amb un paper important. El GM-CSF pot actuar sobre cèl·lules mieloides, promovent l'activació de cèl·lules dendrítiques i macròfags, i diferenciant monòcits a cèl·lules dendrítiques, tot i que altres tipus cel·lulars també poden respondre. S'ha vist el GM-CSF localitzat en els llocs d'inflamació i la seva concentració s'ha trobat elevada en les lesions de pacients amb artritis reumatoide, esclerosi múltiple i la GCA.

Hipòtesi

En la GCA hi ha una inflamació crònica del teixit fet que pot comportar hipòxia i expressió de HIF1α en les lesions. Vies implicades en la GCA com per exemple l'angiogenesi, la inflamació o el remodelat tissular estan relacionades amb l'HIF i per aquest motiu és raonable pensar que l'HIF1α pot estar jugant un paper important en la fisiopatologia de la GCA. Tenint en compte el potencial inflamatori del GM-CSF, és assenyat hipotetitzar la seva possible implicació en la fisiopatologia de la GCA. Ambdues vies (HIF1α i GM-CSF) podrien estar relacionades.

Objectius

Determinar la presència d'hipòxia i HIF1α en les biòpsies d'artèria temporal de malalts amb GCA, estudiar possibles mecanismes que ho promouen, determinar quins són els efectes d'aquesta presència en les lesions i analitzar l'efecte d'inhibir citocines inflamatòries. Investigar l'expressió del GM-CSF i el seu receptor en les lesions dels pacients amb GCA, detectar l'activació de les vies de senyalització relacionades i la modulació dels gens implicats. Investigar l'impacte de bloquejar el GM-CSF amb mavrilimumab en la inflamació d'artèries de pacients cultivades ex-vivo.

Mètodes

De la biòpsia d'artèria temporal duta a terme per fins diagnòstics sovint hi ha material que es pot dedicar a investigació. Poder treballar amb el teixit implicat en la malaltia ens serveix per a poder esbrinar mecanismes implicats en la malaltia. A partir d'aquest teixit fem cultiu *ex-vivo* com a model de la malaltia, n'extraiem cultiu primari de VSMC per estudiar aquestes cèl·lules en concret o n'extraiem l'RNA per fer estudis d'expressió. A partir de sang de controls sans extraiem cèl·lules mononuclears de sang perifèrica (PBMC) que co-cultivem amb les VSMC com a model *in-vitro* de la malaltia.
Tant del teixit fresc o cultivat de la biòpsia com de les cèl·lules aïllades i cultivades *in-vitro* analitzem mitjançant microscòpia confocal per conèixer la localització tissular de les proteïnes d'interès; fem extracció de proteïnes per quantificar via western-blot o extracció de RNA per quantificar via PCR a temps real. Del sobrenedant dels cultius *in-vitro* o *ex-vivo* n'analitzem el nivells de certes proteïnes per establir l'expressió i secreció d'aquestes per part de les cèl·lules/teixit.

Principals resultats

Les lesions de GCA presenten zones hipòxiques i quan les VSMC estan en un ambient inflamatori (amb presència de cèl·lules CD45+, com a marcador leucocitari) aquestes tenen HIF1α a nivell proteic. Al comparar biòpsies d'artèria temporal GCA amb biòpsies no-GCA també es veu un augment d'HIF1A a nivell d'mRNA.
Un microambient inflamatori (co-cultiu de PBMC amb VSMC) promou un augment d'HIF1α en les VSMC tan a nivell d'mRNA com a nivell proteic. A més a més es

demostra que aquest augment es troba principalment al nucli on pot actuar com a factor de transcripció. Vàries molècules inflamatòries implicades en la patogènesi de la GCA (IL6, IL1β, IFNγ i TNFα) són capaces d'estabilitzar l'HIF1α a nivell d'mRNA i de proteïna en les VSMC. S'ha corroborat un augment de l'activitat com a factor de transcripció de l'HIF1α comprovant l'augment de dos gens diana diferents (SLC2A1 i EGLN3).

Estabilitzar l'HIF1α en les VSMC comporta una disminució dels nivells d'ET-1 i una disminució de la capacitat migratòria de les mateixes. També comporta un canvi en l'expressió de quimosines: una disminució de CCL2 i CCL5 i un augment de CXCL8. Finalment, també afecta a molècules relacionades amb l'angiogènesi com és el cas del VEGFA i la IL6, ambdues augmenten.

D'entre els inhibidors de citosines testats en el model *in-vitro* de la malaltia, l'infliximab, l'anakinra i el mavrilimumab (inhibidors de TNFα, IL1β i GM-CSF respectivament) són capaços de restablir els nivells basals d'HIF1α a nivell proteic; mentre que inhibir la IL6 amb el tocilizumab o inhibir l'IFNγ no van afectar els nivells d'HIF1α.

L'expressió del GM-CSF i el seu receptor estan incrementats en les lesions de GCA i les vies de senyalització implicades es troben actives. La inhibició del GM-CSFRα mitjançant un anticòs monoclonal, mavrilimumab, redueix marcadors limfocitaris i mieloides; redueix l'expressió de molècules involucrades amb l'activació de cèl·lules T i relacionades amb la diferenciació Th1; disminueix els nivells de citocines pro-inflamatòries; disminueix mediadors del dany vascular; i finalment també redueix l'angiogènsi

Inhibir el GM-CSF disminueix els nivells de citocines proinflamatòries com és el cas de la IL6, la IL1β, l'IFNγ i el TNFα i també disminueixen els nivells de VEGFA, una molècula important en l'angiogènesi, en el model *ex-vivo* de la GCA; es tracta de proteïnes inductores de l'estabilització de HIF1α i/o gens diana del mateix.

Conclusions

Les lesions de pacients amb GCA presenten zones hipòxiques i amb presència d'HIF1α a la làmina mitja de la paret vascular. Sota un microambient inflamatori les VSMC

augmenten els seus nivells d'HIF1α. L'augment d'HIF1α afecta al seu transcriptoma afectant vies importants en la GCA com són la migració cel·lular, l'angiogènesi, la quimiotaxis i la inflamació. Inhibir l'efecte del TNFα, la IL1 o el GM-CSF disminueix els nivells d'HIF1α en les VSMC. Les artèries de GCA tenen més GM-CSF i receptor, així com més activació de molècules implicades en la via de senyalització comparat amb controls. El bloqueig d'aquesta via amb el mavrilimumab (anti-GM-CSFRα) comporta una reducció de: marcadors limfocitaris i mieloides; molècules involucrades en l'activació de cèl·lules T; citocines pro-inflamatòries; i també redueix l'angiogenèsi en un model *ex-vivo* de la malaltia. El mavrilimumab també disminueix l'expressió de molècules implicades tant en l'estabilització de HIF1α com gens diana d'aquest factor de transcripció.

1

Introduction

Giant cell arteritis

Definition, epidemiology and clinical manifestations

Systemic vasculitides are a heterogenous group of immune-mediated diseases characterized by inflammation and damage of blood vessels. Classification of systemic vasculitis is primarily based upon the predominant size of the involved vessels (Figure I) (1).

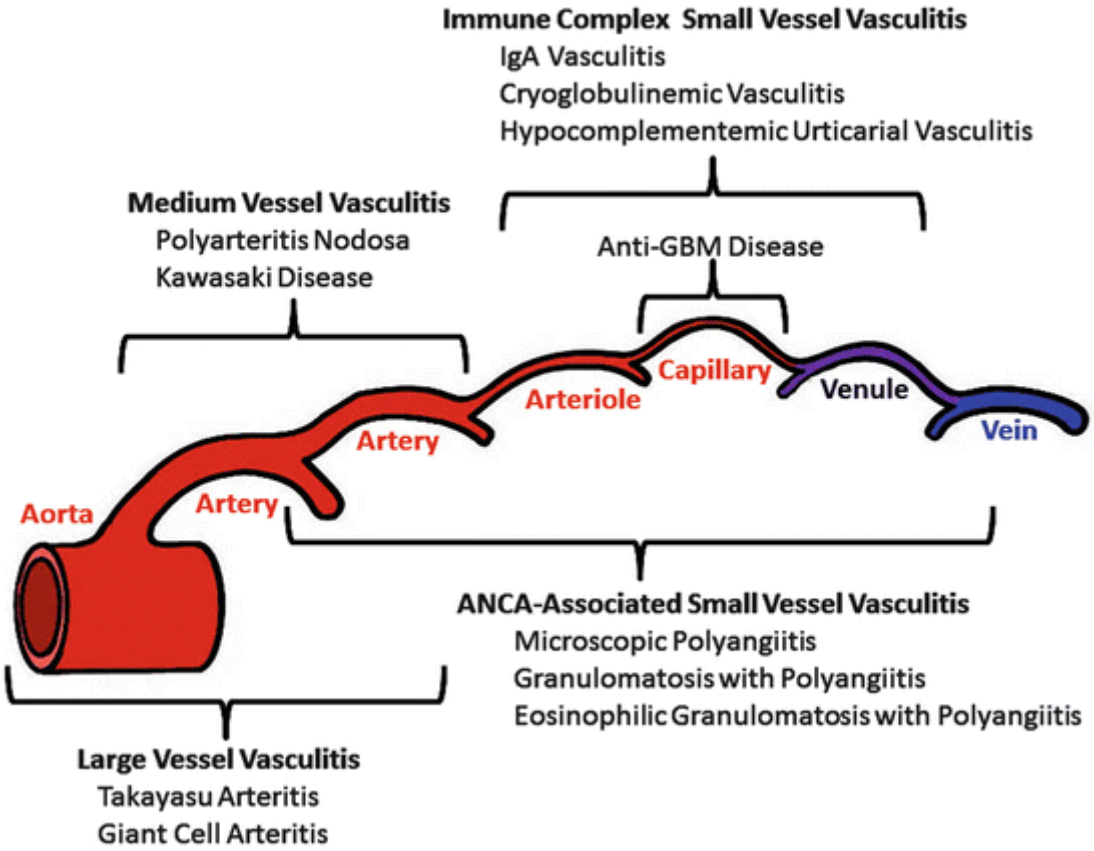


Figure I (2). Distribution of vessel involvement by large vessel, medium vessel, and small vessel vasculitis. Large vessel vasculitis affects large arteries more often than other vasculitides. Medium vessel vasculitis predominantly affects medium arteries. Small vessel vasculitis predominantly affects small vessels, but medium arteries and veins may be affected, although immune complex small vessel vasculitis rarely affects arteries. Not shown is variable vessel vasculitis, which can affect any type of vessel, from aorta to veins.

Giant cell arteritis (GCA) in particular, is a chronic granulomatous vasculitis usually affecting the aorta and its major branches, with a predilection for the branches of the carotid and vertebral arteries (1,3,4). Epidemiological studies report an estimated annual incidence ranging from 1.1 to 32.8 cases per 100.000 individuals aged more than 50 years; incidence varies according to geographic location (5,6). GCA is the most

frequent systemic vasculitis in Europe and North America in individuals older than 50 years-old (7). Average age at diagnosis is approximately 70 years old with a slight predominance of women over men (8,9).

Even though GCA is a systemic disease, it has a predominance for supra-aortic branches from cranio-cervical areas; this leads to common clinical manifestations, due to persistent inflammation, such as headache, and ischemic complications such as mandibular claudication, or vision loss (3,4). Visual manifestations represent the most severe complications, and they usually range from transient diplopia to complete permanent visual loss. Permanent visual loss still affects approximately 15% of patients and once it is established, it is usually permanent; although, it can be prevented by early treatment intervention (10,11).

Nevertheless, other vascular territories like the aorta and its proximal branches are also affected (11). In fact, aortic aneurysm is frequent among patients with GCA (12). Moreover, the GCA is usually accompanied by a remarkable systemic inflammatory response, which includes symptoms like fever, anorexia, malaise, progressive weight loss, and/or increased acute-phase proteins (3). GCA patients may also develop polymyalgia rheumatica (PMR), a related inflammatory disease, consisting of synovitis of the proximal joints and periarticular structures, clinically characterized by severe aching pain and morning stiffness in the neck, shoulders and pelvic girdle (4,11).

Diagnosis and histopathology

Giant cell arteritis is suspected on the basis of clinical evaluation in combination with laboratory and imaging tests, and it may be confirmed by histologic examination. Combination of several of the aforementioned clinical symptoms raises suspicion of GCA if patient age is over 50 years old. The histopathological substrate of GCA consists of mononuclear cell infiltration of the involved arteries. Temporal artery biopsy (TAB) was the first method used to confirm GCA, because of the common involvement of the superficial temporal artery and its ease of access (13). In the histopathological study of the affected temporal artery tissue characteristic findings include a disrupted artery wall with an intense inflammatory infiltrate, multinucleated giant cells, disruption of the elastic laminas, and intimal hyperplasia (14) (Figure II).

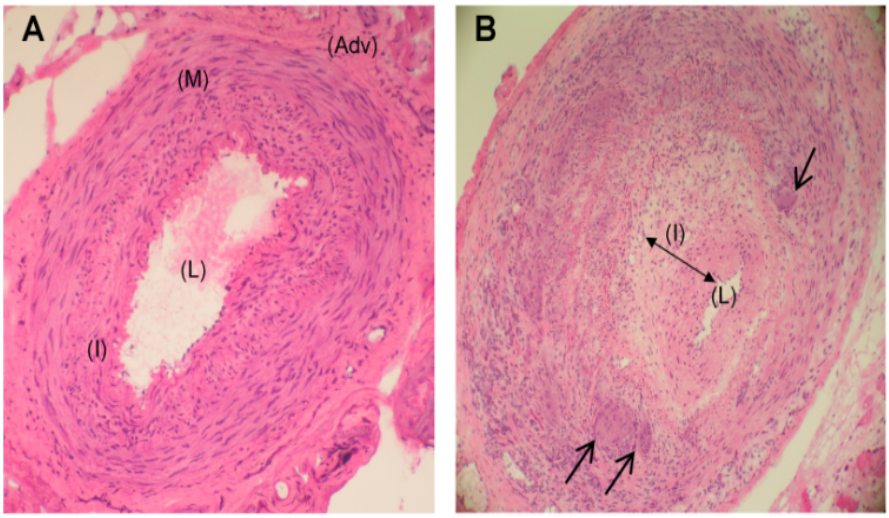


Figure II (14). Normal temporal artery biopsy (left) as opposed to a temporal artery biopsy from a patient with giant-cell arteritis (right) disclosing typical transmural mononuclear cell infiltration, internal elastic lamina breakdown and intimal hyperplasia. Double head arrow remarks the thickened intima and single head arrows indicate the presence of giant cells. Hematoxylin-eosin staining. (L) lumen; (I) intima; (M) media; (Adv) adventitia.

Imaging is an emerging tool in the management and diagnosis of patients with GCA (15). Despite imaging tools have proved to be useful(16), abnormal TAB findings still provide the best diagnostic specificity (17–19). Beyond their diagnostic value, TAB samples are an important source of tissue with great utility for investigating the pathogenesis of GCA.

In terms of histology, in normal conditions three different layers can be differentiated in the artery wall (Figure III, left). The inner layer is called tunica intima and it is formed by endothelial cells, extracellular matrix and some myofibroblast-like-cells. The intima and the media are separated by the internal elastic lamina. The tunica media is mainly formed by smooth muscle cells responsible for vasoconstriction and vasodilatation. The external elastic lamina limits de tunica media and the adventitia. In this external layer there's connective tissue, collagen fibers, nervous fibers, resident dendritic cells, fibroblasts and small vessels called *vasa vasorum* involved in the feeding of the artery wall from medium and large vessels (14,20).

Unlike what is found in a normal artery wall, the classic histologic alterations of GCA consist of an inflammatory infiltrate throughout all three layers of the arterial wall with disruption of the internal elastic lamina and intimal thickening (21) (Figure IIB and Figure III right). Intimal hyperplasia may result in partial or complete lumen occlusion

leading to the ischemic complications typically observed in GCA. The infiltrating cells mainly include $CD4^+$ lymphocytes and macrophages. Giant cells are present in half of the patients. These multinucleated cells resulting from the fusion of macrophages are usually located at the intima-media junction (4,11). Considerable histological variations can be found in GCA samples, not only among different patients, but also within the same sample, partially due to the segmental distribution of the inflammatory infiltrate (21).

According to the distribution of inflammatory infiltrates through the artery wall, 4 different histological patterns, representing successive stages of artery inflammation, can be differentiated (21,22): 1) adventitial pattern: when inflammatory cells are restricted to the adventitia, with preservation of the media and intima layers; 2) adventitial invasive pattern: when the adventitial infiltration is followed by local invasion of the muscular layer, with integrity of the intima; 3) concentric bilayer pattern: when inflammatory cells are infiltrating the adventitia and the intima (or the intima/media junction), with a preserved media; and 4) panarteritic pattern: when the inflammatory infiltrates were distributed through the 3 arterial layers.

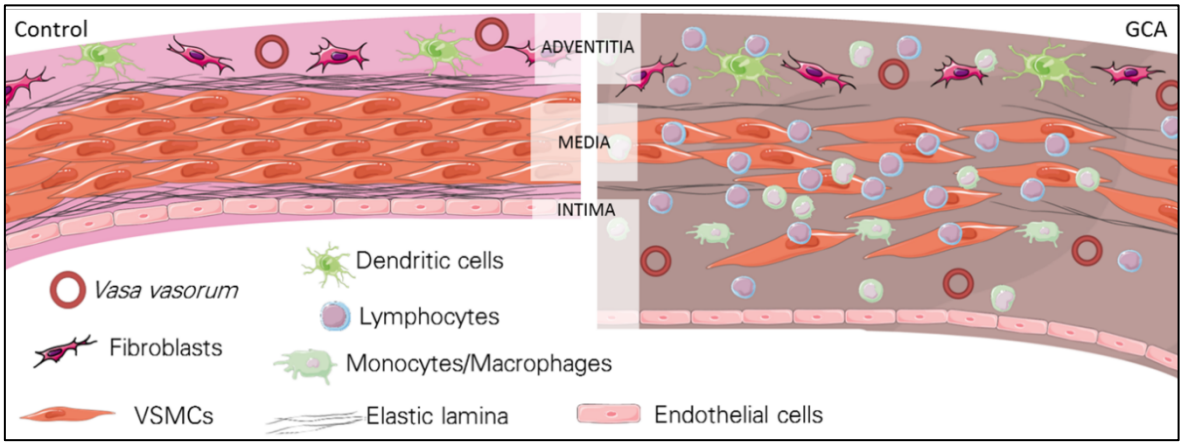


Figure III. Schematic representation of a medium or large vessel wall from a healthy control (left) and from a GCA patient (right). In a normal (control) artery, three different layers can be differentiated and separated by elastic laminas (intima, media and adventitia). In a GCA lesion, elastic laminas are disrupted, and inflammatory infiltrate is diffuse and evident. Adapted from Planas-Rigol (2016) (14).

Etiology, triggers and genetic background

Although the etiology of GCA and the antigen(s) involved in the development of the autoimmune response are still unknown, several advances have been made regarding the importance of the genetic background in the pathogenesis of the disease with a variety of molecules now being associated with the development and persistence of the inflammatory response in GCA (23).

The relevance of a genetic contribution in this vasculitis is supported by the marked differences in geographic variation with the highest incidence demonstrated in northern European countries and individual from northern European descent along with reports of sporadic family clustering of affected members (24).

In candidate gene studies, an increase in GCA risk has been associated with polymorphisms in genes encoding molecules participating in immune, inflammatory, and vascular responses such as IL-10 (25), VEGF (26–28), NOS2 (28), IL-6 (29), TNF (30) or ICAM-1 (31). However, some contradictions emerged in the case of ICAM-1, and some results were not confirmed in independent cohorts (32).

A robust association has been found between GCA and genetic variants in the major histocompatibility complex (MHC) region, particularly with class II HLA-DRB1*04 alleles (23,33). Importantly, a large-scale fine mapping of genes related to immune responses has confirmed a strong association between GCA susceptibility and variants in the class II MHC region in which the resulting risk amino acids are located in the antigen-binding cavity of human leucocyte antigen (HLA) molecule (34). This finding reinforces the role of the adaptive immune system in the development of the disease and suggests that GCA may be an antigen-driven disease. Furthermore, variants in plasminogen and prolyl 4-hydroxylase subunit alpha 2 (P4HA2) genes were related to increased GCA risk at genome-wide significance (35). The role of the adaptive immune system is supported by the HLA variant associated with GCA risk (34) and observed oligoclonal T cell expansion in GCA lesions (36), reinforcing the participation of antigen-specific adaptive immune responses in GCA. The presence of different auto-antibodies have been observed in GCA patients sera including different antigens (e.g., vinculin, annexin V) (37,38); although its relevance in the disease is controversial as many of these

antibodies may be generated as a consequence of the intense inflammatory response and tissue injury rather than having a pathogenic role in the disease.

In terms of environment exposures, and because of some reported cyclic patterns, geographical variations and seasonal fluctuations in the incidence of GCA, infectious agents have been long suspected in the etiology of the disease but, has not been confirmed (39). In this line, several viruses (e.g. *varicella zoster*) have been associated with increased incidence of GCA, but a clear causal relationship has not been demonstrated (40–42).

The rationale why GCA affects people over 50 years and its increased incidence with age is not completely understood, but aging may play an important role in the pathogenesis of the disease (43). In this sense, T cells, dendritic cells (DC), endothelial cells and vascular smooth muscle cells (VSMC) involved in GCA immune response and vascular remodeling seem to be modified by the normal aging process (43). Moreover, blood vessels suffer structure and tissue modifications with age (44,45) that, together with cellular alterations, might partially explain the impact of age in GCA development.

Finally, altered DNA methylation levels have been reported in several pro-inflammatory genes in temporal arteries from GCA patients. Thus, environmental factors may modify gene expression through epigenetic modifications which, then, may increase the predisposition to GCA (20).

Pathophysiology of GCA

The current accepted model about the generation of inflammatory lesions in GCA is based on evidence obtained from comparisons between inflamed and normal arteries and extrapolation of known biologic activities of certain cytokines, proteases, or growth factors differentially expressed in GCA temporal biopsies versus control arteries. The current consensus regarding the sequence of events that occurs in GCA starts with an initial immune activation, followed by arterial infiltration, damage and repair response (46,47).

IMMUNE ACTIVATION

Even though the initial triggering event is still unknown, some pathogenic sequences may play a role in the firsts steps of GCA given that innate immune activation mechanisms also contribute to GCA (48,49). IL6 is produced by monocytes, macrophages and dendritic cells within the immune system (as well as by endothelial cells, VSMC among other non-immune cells) as a signal of damage. IL6 has a key role in T cell differentiation through the activation of STAT3 transcription factor (50). This interleukin also stimulates production of acute phase response protein such as CRP and fibrinogen resulting in an increased ESR levels. IL6 serum levels are associated with relapses in GCA but negatively associated with ischemic complications (46).

ARTERIAL INFILTRATION

Arterial invasion requires activation of both circulating and vascular cells. In this sense, it has been proposed that inflammation in GCA temporal arteries initiates after resident dendritic cells present in the adventitia are activated by an unknown trigger (22,51–53). Once activated, dendritic cells act as antigen presenting cells (APC) inducing the activation and recruitment of $CD4^+$ T cells through the expression of CD83 and CD86 as stimulatory molecules (53) and the activation of the *vasa vasorum* endothelial cells which express ICAM-1 and VCAM-1 (54).

From there on, the immune response is extensive and exaggerated, and is mainly characterized by a Th1 response involving IFNγ production by activated lymphocytes (55–57). Th17 immune response is also engaged with concomitant pro-inflammatory cytokine production (58–63). Increased secretion of the pro-inflammatory cytokine IFNγ has been shown in GCA-involved arteries (64,65). Likewise, several IFNγ related products have been found in lesions, including class II MHC (55), endothelial adhesion molecules (54), inducible nitric oxide synthase (66) and chemokines (65,67,68) responsible for monocyte and T cell recruitment. IFNγ is a key cytokine in macrophage activation and its granulomatous differentiation forming typical giant cells present in GCA lesions (47,55).

AMPLIFICATION OF THE INFLAMMATORY RESPONSE

T cells and macrophages have a leading role in the feed-forward inflammatory infiltrate. Activated CD4+ T cells invade GCA arteries homing mostly to the adventitial-medial border but present in all three layers. While a comprehensive assessment of infiltrating T cells is lacking, there are varying levels of IFN-γ, IL-17, IL-21, and IL-9 production (20,46).

Myeloid cells also infiltrate all three layers of the artery on TAB and populate granulomas formed by monocytes, macrophages and giant cells that produce IL-6 and IL-1β, MMP9, MMP2, VEGF, and PDGF (46). Other myeloid generated cytokines elevated in TAB that contribute to the pro-inflammatory environment include TNF, IL-12, and IL-23 (58,61,63). Various cytokines (such as IFNγ, IL-1β, and IL-6) can stimulate macrophage fusion into giant cells but correlation between IFN-γ levels and number of giant cells on TAB suggests this is the primary mechanism in GCA (69,70). Collectively, these data suggest that monocytes, which are transcriptionally primed to produce pro-inflammatory cytokines and gelatinases in circulation, are recruited from the peripheral blood, differentiate in the inflamed vessel into macrophages, and further combine to form giant cells in response to IFN-γ.

Multiple cell types generate positive feedback chemokine loops that enhance T cell and myeloid recruitment. Remarkably, different cell types including DC, macrophages, Th17 cells, and VSMC can produce CCL20 which is overexpressed on TAB. Important players in this inflammatory network are VSMC which are able to express CX3CL1, CCL2 among other T cell and monocyte recruiting chemokines upon stimulation with inflammatory molecules such as TNF. Vascular endothelial cells also play an important role recruiting leucocytes. Therefore, once T cells and monocytes have entered into the vascular tissue, interactions with resident vascular cells maintain inflammation (46).

VASCULAR REMODELING

In healthy vessels, pro-MMP2 and its natural inhibitor TIMP2 are constitutively produced by VSMC resulting in a quiescent tissue with no proteolysis. In contrast, in inflammatory conditions, macrophages and giant cells express MMP2 and MMP9 overriding its inhibitors. Moreover, these MMPs become activated by cleavage in an

inflammatory environment. This results on extracellular matrix degradation, generating a tissue more easily infiltrated by T cells. Tissue destruction takes place near macrophages. Along with giant cells, macrophages produce gelatinases, developing in the cleavage and destruction of the elastic laminas (66,71,72). Intimal hypertrophy and neovascularization occurs as a result of vascular remodeling initiated by macrophages and Th1 inflammation. In response to PDGF, and other mitogenic signals, VSMC become proliferative and invasive myointimal cells (73). These invasive muscular cells are able to migrate from the media and invade the intima where they produce extracellular matrix proteins, such as collagen I and II, strengthening the hypertrophic neointima which in some patients ends in lumen occlusion and derived ischemic complications (73,74).

Patients with ischemic symptoms have higher levels of ET-1, a potent vasoconstrictor mainly generated by endothelial cells. ET-1 is also expressed by infiltrating immune cells and is able to produce intimal-invasive switch from quiescent VSMC (75,76). The intensity of intimal hyperplasia correlates with the amount of neovascularization in the intima and media layers, and is also associated with VEGF levels, suggesting this phenomenon may be hypoxia-driven (77). Moreover, macrophages and giant cells, highly metabolically active cells, co-localize in areas of new vessels generation, suggesting also hypoxia as a mediator (77).

Altogether, vascular remodeling results in thickened vessel walls that could cause symptomatic ischemia and generates leaky vasculature facilitating the recruitment of additional inflammatory cells (46,78).

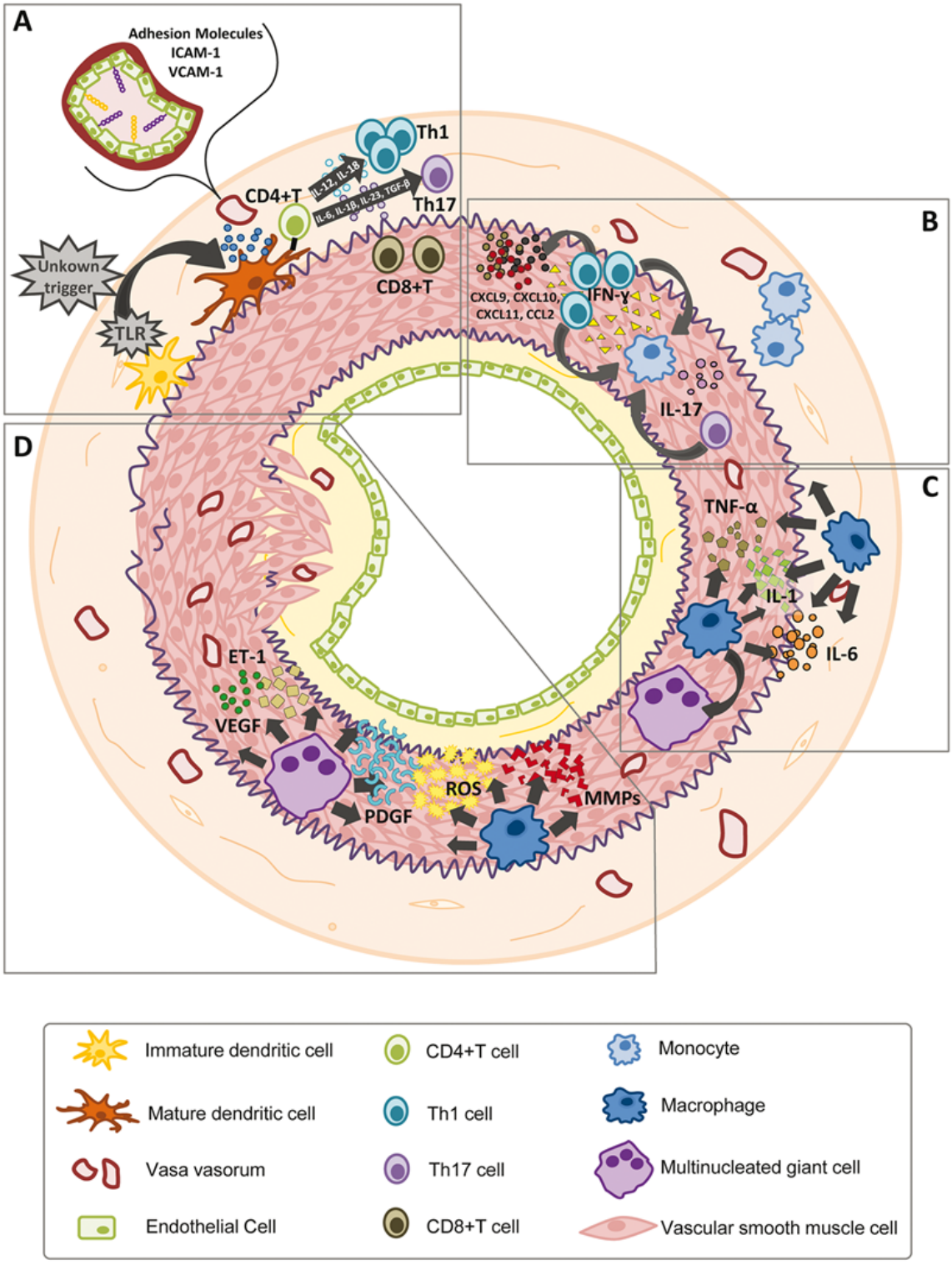


Figure IV (47). Schematic representation of immunopathogenic mechanisms involved in inflammation and vascular remodeling in GCA. (A) Activation of dendritic cells and recruitment, activation and differentiation of CD4+ T cells and CD8+ T cells. (B) Recruitment and activation of monocytes and differentiation into macrophages. (C) Amplification of the inflammatory response. (D) Vascular remodeling and vascular occlusion.

ADDITIONAL MECHANISMS

Another mechanism contributing to GCA pathogenesis is related to B-cell lymphocytes. B cell lymphocytes are not abundant in GCA, however, their presence has been observed in GCA lesions (51,79) even forming tertiary lymphoid structures(80). Although GCA has been essentially considered a T-cell mediated disease, B-lymphocytes are crucial to T-cell activation.

Another cytokine is emerging as a key player in chronic inflammation, granulocyte-macrophage colony-stimulating factor (GM-CSF; also known as CSF-2). The role of GM-CSF and its importance in GCA pathogenesis is explained in depth in pages 50-52.

Functional models

Most of the conclusions related to GCA pathogenesis were formerly based on the previously known functions of molecules identified in observational studies and their correlation with clinical or histological abnormalities. In addition, two functional models, based on the use of human temporal arteries obtained from biopsies, have contributed to gain a better understanding of the GCA pathogenic process.

In the first model, GCA-involved temporal artery fragments were implanted subcutaneously into SCID mice. Using this model, it was possible to demonstrate an important role of dendritic cells in GCA pathogenesis since their depletion reduces inflammation in the explants (53). In another study, T-cell depletion with T-cell specific antibodies reduced T-cell dependent cytokines showing the importance of this population in disease pathology (81). This model has also been useful to detect changes in cytokine expression in TA after pharmacological treatment of engrafted mice with corticosteroids (82,83).

More recently, we have developed in our group a TA culture in 3D to investigate pathogenic pathways. As an advantage, this model substitutes the use of SCID mice by Matrigel^{TM} as the biological support, reducing the complexity and expense (68). This model has allowed us to demonstrate that glucocorticoids (GC) decrease the production of inflammatory cytokines but are not able to influence factors involved in vascular remodeling (68) and that IFNγ may play an important role in the recruitment of macrophages in GCA by inducing the production of specific chemokines such as

CXCL9, CXCL10 and CXCL11 (65). In addition, blocking PDGF receptor signaling with imatinib or ET-1 signaling with receptor antagonists reduced myointimal cell outgrowth (76,84).

One of the main drawbacks of this model is the lack of a functional immune system. Nevertheless, these functional models have proven to be useful to study the impact of therapeutic drugs on vascular inflammation and remodeling. These data, together with those derived from clinical trials, will help to better understand the mechanisms of some specific therapies. In addition, functional models will provide unique insights into pathogenic mechanisms of vascular inflammation and repair.

Treatment

Glucocorticoids (GC) are still the cornerstone of remission induction in GCA, but their side effects remain a problem for patients with GCA. Therefore, there is an unmet need for more effective and safer therapies in GCA. Reducing relapses as well as GC exposure and side effects are the main goals of new therapies. Several immunosuppressive agents and targeted therapies have been or are being tested in order to improve this issue.

There are still many challenges in the treatment of GCA. About 10-17% of patients with visual impairment at diagnostic continued to lose vision during the first weeks of the treatment, which is a very traumatic situation. Between 3-6% of patients develop stroke and more than half develop stroke after the initiation of low corticosteroid therapy. Between 18-33% of patients develop aortic dilatation over time with potentially severe complications. Between 10-50% of patients suffer from LV-stenosis and between 40-80% of patients relapse during GC tapering. The higher frequency of relapses is found in clinical trials with rapid GC reduction. Relapses require treatment and are associated with higher accumulated GC doses and major side effects (85).

GLUCOCORTICOIDS

GCA has been considered the paradigm of a GC responsive disease, since high-dose GC dramatically relieves symptoms and markedly improves blood test abnormalities in most patients. For this reason, GC are still the cornerstone of remission induction (11).

Unfortunately, as soon as GC are tapered the probability of relapses increases. This requires prolonged retreatment, thus resulting in well-known GC related side effects (hypertension, hypercholesterolemia, fractures, Cushing and weight gain, cataracts, serious infections…). Although we have learned to manage and prevent it, GC side-effects are still a problem for patients with GCA and are recently addressed in several important publications (86–90).

A recent meta-analysis demonstrates that the probability of relapse is lower in older cohorts compared with more recent cohorts where investigators have tried to minimize GC exposure (91). Another study focused on clinical trials shows that when cumulative doses of GC are lower the percentage of relapsing patients is higher. Usually, relapses are more frequent in clinical trials that includes a rapid GC-tapering than in real life (92).

IMMUNOSUPPRESSIVE DRUGS

Several broad-spectrum immunosuppressive agents have been used in patients with GCA in an attempt to spare GC including leflunomide, mycophenolate, cyclophosphamide and methotrexate, among others, but only methotrexate has been tested in randomized clinical trials. However, a meta-analysis of 3 different clinical trials demonstrated that methotrexate results in a reduced probability of first and secondary relapse, and an increased probability of complete GC discontinuation (93). Methotrexate is now widely used in GCA clinical practice and it is supported by several observational studies (94–96).

TARGETED THERAPIES

More recently, targeted therapies are addressed to block specific pathways though to be of relevance in the pathogenesis of GCA. Several molecules have been targeted and their pharmacological approaches have been tested or are currently being tested. Some of the targeted molecules participated mainly in Th1/Th17 differentiation and others in macrophage activation at different levels. Tocilizumab, mavrilimumab, abatacept and TNF blockers stand out among biological treatments.

Abatacept is a recombinant molecule (CTLA-4-IgG1) that prevents CD28 mediated T cell activation during initial steps in adaptive immune response and probably will act at

initial steps in the inflammatory cascade leading to GCA. Abatacept has been tested in a phase II clinical trial including 41 patients and the primary endpoint of this trial was sustained remission at 12 months. Abatacept was slightly more effective than placebo in achieving this primary endpoint (97). A phase III randomized clinical trial is currently ongoing (NCT04474847).

TNF blockers were the first biologic agents ever tested in GCA. Unfortunately, 3 clinical trials did not show any sign of efficacy in newly diagnosed patients at the expenses of more side effects, mainly infections and two of them were too small to yield a meaningful result (98–100).

TOCILIZUMAB

IL-6 pathway can be blocked pharmacologically by interfering with IL-6R such as tocilizumab (TCZ) or sarilumab or interfering with IL-6 cytokine itself such as sirukumab. These 3 monoclonal antibodies have been tested in GCA and entered phase III clinical trials but only the clinical trial with tocilizumab was completed as the others were terminated by the sponsors (not due to safety issues). A phase II trial with intravenous tocilizumab was published in 2016 with 30 patients and a primary endpoint of relapse-free survival at week 52 was accomplished (101). TCZ yield a dramatic benefit in this clinical trial. The phase III GiACTA trial with tocilizumab is the largest performed thus far in GCA, enrolling 250 patients. The trial fulfilled spectacularly the primary endpoint and more than 50% of patients in the tocilizumab arm were able to endure sustained remission at week 52, time to relapse also favored tocilizumab over placebo and cumulated GC doses were significantly lower in the TCZ arms (102).

This trial led to the approval of tocilizumab for the treatment of newly diagnosed or relapsing patients with GCA. Since then, it has been a substantial experience accumulated in clinical practice around the world and several papers have been published. These studies include a higher proportion of relapsing patients than in clinical trials and higher proportions of pre-treated patients that were given tocilizumab due to a high risk or of GC side effects. In real world clinical practice, the percentage of responders is higher because the response criteria is not so stringent and frequently tocilizumab is an add-on treatment on patients with concomitant GC or

other drugs which are not completely discontinued. In clinical practice there are also more side effects, perhaps due to the load of previous or concomitant medications (103–105).

GiACTA trial (102) lead to an extension of the study showing interesting results regarding the duration of the treatment. As soon as tocilizumab is discontinued, the probability of relapses increases but still more than 40% of patients that initially responded were able to maintain treatment-free remission. In this extension phase, weekly tocilizumab seems to have better efficacy and accumulated prednisone was also significantly lower in the tocilizumab arms (106).

An important limitation in tocilizumab treatment is the monitoring of disease activity due to the mechanism of action of tocilizumab that interferes with the hepatic synthesis of C-Reactive Protein (CRP) and other acute phase proteins responsible for erythrocyte sedimentation rate (ESR) acceleration. These useful markers, CRP and ESR, are widely used for patient evaluation and are not reliable under tocilizumab treatment. An unanswered question is whether tocilizumab masks or controls the disease. Tocilizumab has a deep impact on the systemic inflammatory response, which is a strong source of discomfort for patients with GCA, with fatigue, fever and/or weight loss. Since all these symptoms are relieved by tocilizumab it is still not very well known if it masks or controls GCA. Moreover, there is still a need to further investigate the impact of tocilizumab on vascular inflammation, remodeling, occlusion and subsequent aortic complications.

In conclusion, tocilizumab has been a major advance in the treatment of GCA and has been approved by several regulatory agencies for the treatment of patients with new or relapsing GCA. Nearly half of patients relapse when GC are withdrawn, and sustained treatment-free remission can be expected in 42% of responders (107).

MAVRILIMUMAB

Mavrilimumab is a fully human IgG4 monoclonal antibody able to neutralize GM-CSF effects by binding to the GM-CSF receptor alpha chain (GM-CSFRα) (108). In, a phase 2b trial in patients with rheumatoid arthritis, mavrilimumab showed comparable efficacy to anti-TNFα blocker golimumab and superior efficacy compared to placebo, as well as a good safety profile (109–111).

Along with the pre-clinical data generated in the present study, a phase II clinical trial with mavrilimumab for the treatment of GCA has been recently completed. This trial enrolled 70 patients, half newly diagnosed and half relapsing patients, who were in GC-induced remission and randomized to mavrilimumab or placebo (3:2) along with pre-specified 26-week GC tapering. The primary efficacy endpoint evaluated at 26 weeks was time to first relapse resulting in a 62% of risk reduction in mavrilimumab treatment compared to placebo. Sustained remission at week 26 was achieved by 83.2% of patients in the mavrilimumab arm compared to about 50% of patients in the placebo arm (112). These results are highly encouraging for further development.

OTHER TARGETED THERAPIES

Clinical trials with other targeted therapies: secukinumab (anti IL-17 antibody), anakinra (IL1 receptor antagonist recombinant molecule), guselkumab (anti IL-23p19 antibody) and upadacitinib (JAK1 inhibitor) are currently ongoing and may expand the therapeutic armamentarium for GCA (113).

The different targeted therapies mentioned intend to control the aberrant over activation of the immune system happening in GCA, however pathogenic mechanisms involved are not clearly understood. Based on vascular wall thickening, increased metabolic activity and angiogenesis, which is known to be present in GCA, it is reasonable to hypothesize implication of hypoxia mechanisms, and, more specifically, HIF1α as a relevant factor, known to be implicated in inflammation and tissue remodeling in other diseases.

HIF1α

Oxygen concentration in tissues is a key factor for cell and organ survival. In normal conditions, partial oxygen pressure (pO2) results in the balance between oxygen delivery and its consumption. Oxygen is transported, in mammals, by circulating red blood cells. PO_2 in tissues varies widely, depending on their respective metabolic requirements and their functional status. In normal physiological conditions, pO_2 is called physioxia. Any alteration of tissue environment leading to a decrease in pO_2 is called hypoxia. Hypoxic conditions have been observed in many different pathological situations like tumor development, obesity or transient ischemia (114).

Hypoxia inducible factor (HIF) is the main cell factor responsible for the cell response to low oxygen concentrations (115,116). This hypoxia response can be activated in physiological or in pathophysiological processes (117) such as development (118) or tumors (119), respectively, changing gene expression profile.

Description, regulation and mechanism of action

HIF, a transcription factor, induces the expression of genes related to cellular adaptation in conditions of low oxygen (115). HIF is a heterodimeric protein formed by an alpha subunit and a beta subunit (115). Whereas there is only one beta subunit, which is constitutively expressed, there are three different alpha subunits (120). Alpha subunits are constitutively expressed at mRNA level but are quickly degraded at protein level in the presence of oxygen (121), and, therefore, the heterodimer cannot be constructed. In the context of oxygen deprivation, alpha subunits are stabilized and HIF can act as a transcription factor (115).

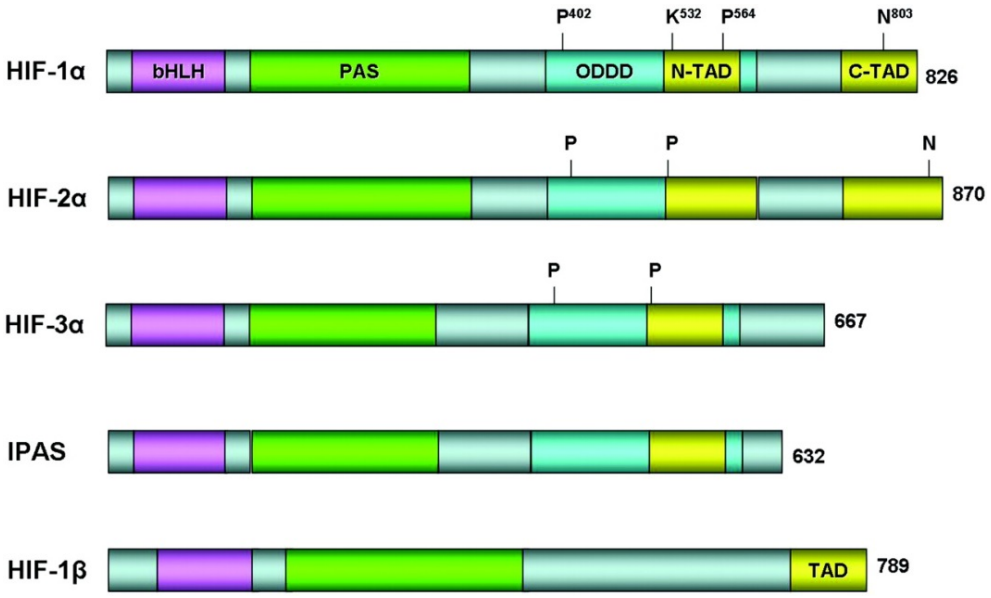


Figure V (122). Domain structure of human HIF-α and HIF-1β. HIF-α (HIF-1α, HIF-2α, HIF-3α, IPAS (Inhibitory PAS domain)) and HIF-1β belong to the bHLH and PAS protein family. HIF-α contains an ODDD that mediates oxygen-regulated stability through the hydroxylation of two proline (P) residues and the acetylation of a lysine (K). The proline residues are conserved in HIF-2α and HIF-3α. HIF-1α and HIF-2α also contain two transaction domains (C-TAD and N-TAD), whereas HIF-1β has only one TAD. The total number of amino acids of each subunit is marked at the end of the domain structure.

PAS domains, present in all subunits, have been reported to contribute to HIF heterodimer formation (121,123,124). Other common domains are HLH (involved in DNA interaction), TAD (associated to transactivation) and ODDD (Oxygen dependent degradation domain). Beta subunit, also called ARNT (aryl hydrocarbon receptor nuclear translocator), share PAS, HLH and TAD domains but lacks ODDD, therefore it cannot be degraded the context of oxygen deprivation (122).

The most studied subunit is HIF-1α, the focus of this thesis. HIF2α was discovered later and was also named EPAS (Endothelial PAS domain protein) as a result of being discovered in endothelial cells, where is mainly expressed; unlike HIF1α which is expressed in all mammalian cells. HIF3α is the least studied subunit but a splicing variant with inhibitory properties called IPAS (Inhibitory PAS domain) has been described. IPAS does not contain the transactivation domain; therefore, it acts as a competitive inhibitor of HIF1α and HIF2α.

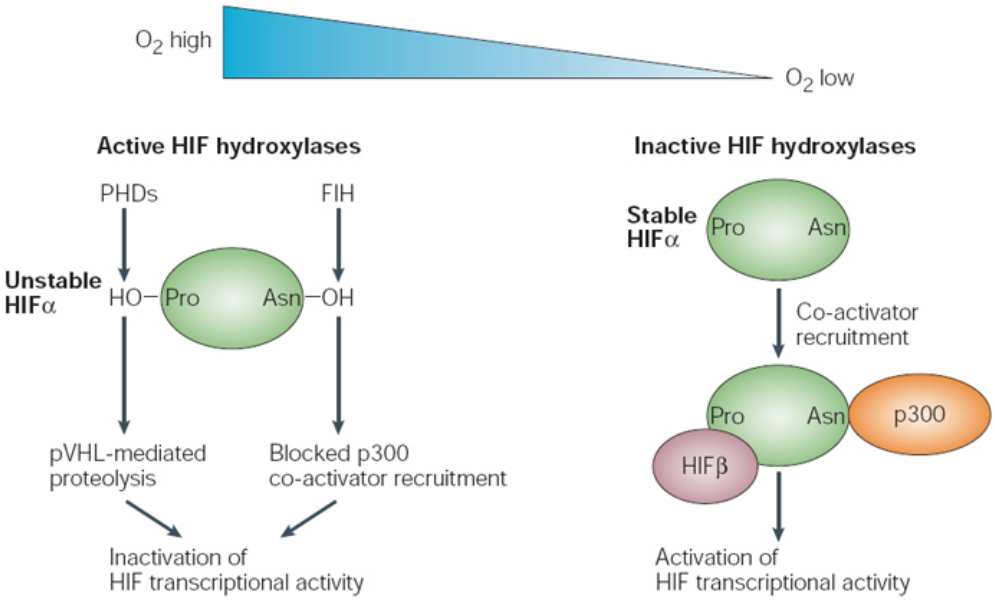


Figure VI (120). Dual regulation of HIFα subunits by prolyl and asparaginyl hydroxylation. In the presence of oxygen, active hypoxia-inducible factor (HIF) hydroxylases (prolyl hydroxylase domains (PHDs) and factor inhibiting HIF (FIH-1)) downregulate and inactivate HIFα subunits. PHDs hydroxylate a prolyl residue in the amino- and the carboxy-terminal oxygen- dependent degradation domains (NODDD and CODDD, respectively), which promotes von-Hippel–Lindau-tumour-suppressor (pVHL)-dependent proteolysis and results in the destruction of HIFα subunits. FIH, on the other hand, hydroxylates an asparaginyl residue in the carboxy-terminal activation domain (CAD), which blocks p300 co-activator recruitment and results in the inactivation of HIFα-subunit transcriptional activity. In hypoxia, HIF hydroxylases are inactive, and these processes are suppressed, which allows the formation of a transcriptionally active complex.

HIF1A gene is constitutively expressed but its highly complex protein regulation make levels variable depending on the microenvironment (125). The heterodimers formed by HIF1/2α together with HIFβ, act as gene transcription activator in response to cellular hypoxia.

Alpha subunit stability is the main way of HIF transcription factor activity regulation. This subunit can be affected by several post-transcriptional modifications such as phosphorylation, acetylation or hydroxylation, among others, which alters its activity and its degradation susceptibility. Hydroxylation is the most studied modification; two different hydroxylases protein are known to modify HIFα. PHD (prolyl-hydroxylase) can add an hydroxyl radical to P402 and P564 amino acids of HIFα (126) that labels HIFα for its degradation by proteasome, and constitutes the fundamental HIF regulation system.

HIFα can also be hydroxylated by an aspariginyl hydroxylase named FIH-1 (factor inhibiting HIF) at N803 amino acid located at the transactivation domain inhibiting coactivators union and consequent activity. Both hydroxylases, PHD and FIH, are inhibited during hypoxia promoting HIF transcriptional activity in the context of low oxygen levels (120).

This inhibition has double control on HIF transcriptional activity. Both, PHD and FIH are oxygen- and iron-dependent enzymes. Thus, they are inactivated when there is a lack of substrate, co-substrate, or product excess. Each enzyme has its own kinetics. In the context of hypoxia, being PHD the first to be inhibited, followed by FIH, proving the sensitivity and complexity of the system (127,128).

Oxygen-depending degradation of HIFα

PHD hydroxylation followed by HIFα degradation through the ubiquitin-proteasome system, also determine the regulation of HIFα subunit in normoxia. HIF1α is regularly produced but in the presence of oxygen, PHD marks the subunit with a hydroxyl residue. This hydroxylation allows the recognition of HIF1α by the pVHL (Von Hippel-Lindau protein) (126,129). pVHL is part of E3 ubiquitin-ligase complex and its function is to add several ubiquitin proteins to HIF1α subunit (129). These ubiquitins mark the protein for posterior proteasomal degradation.

Under hypoxia, hydroxylation mechanisms are paused, HIFα is not hydroxylated, and therefore, it cannot be recognized and ubiquitinated by pVHL. HIFα can form an active heterodimer in hypoxia/anoxia condition in minutes (130). This huge speed in the response is due to the already synthetized protein, just its degradation needs to be inhibited. In case of re-oxygenation the degradation reactivation occurs rapidly being HIFα degraded within few minutes (131).

PHDs are much more than oxygen sensors, having into account its reaction, they link hypoxia with cell metabolism alteration. PHDs are not equally distributed. PHD1 is mainly expressed in testicles, PHD3 is present in smooth and cardiac muscle and PHD2 is constitutive (132). Hypoxia induces PHD2 and PHD3 expression generating a negative feedback, yet PHD1 is not affected (126,133,134). PHD2 is considered to be predominantly controlling HIF1α levels in normoxia (133).

Hydroxylation reaction requires 2-oxoglutarate as a substrate and, Fe(II) and oxygen as cofactors. The lack of a co-substrate or a cofactor, or product excess, inhibits the reaction under normoxia and cause a pseudohypoxia phenomenon. Since PHD are dependent on 2-oxoglutarate and iron, nowadays, not only oxygen is considered the regulator of HIFα and PHD are described as general cellular metabolic sensors (135).

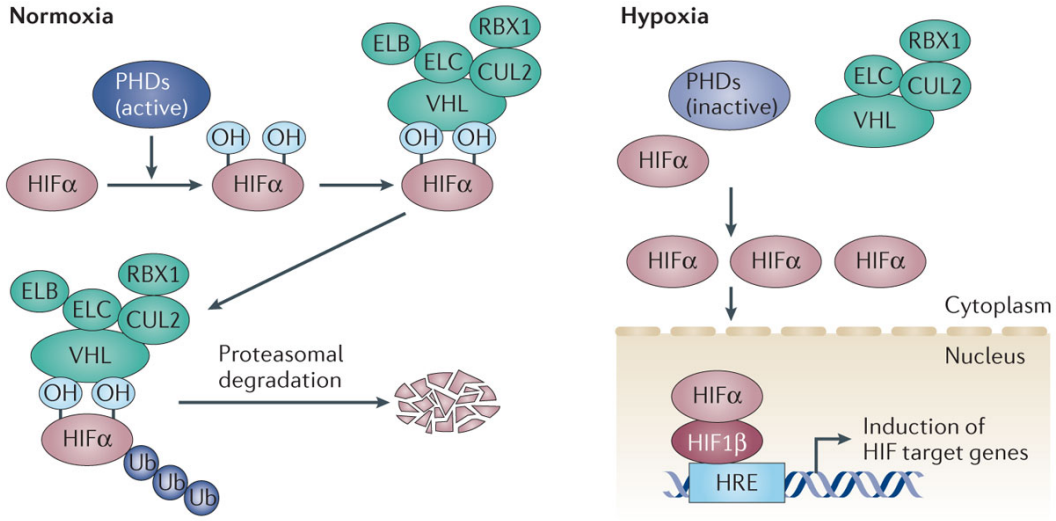


Fig VII (129). Oxygen-dependent hypoxia-inducible factor regulation. In normoxic conditions, HIF1α and HIF2α are hydroxylated on one or both of two conserved proline residues by PHD1, PHD2 and PHD3. Prolyl-hydroxylated HIFα is recognized by the pVHL–elongin C (ELC)–elongin B–cullin 2 (CUL2)–RBX1 (VCB–CR) E3 ubiquitin ligase complex and targeted for ubiquitylation (Ub) and proteasomal degradation. In hypoxic conditions, PHD1, PHD2 and PHD3 are inactive (oxygen is an essential cofactor). HIFα therefore accumulates and forms heterodimers with HIF1β. These heterodimers translocate to the nucleus, bind to hypoxia-response elements (HRE) and induce the transcription of genes involved in adaptations to hypoxia.

Several PHD inhibitor mechanisms able to stabilize HIFα have been described *in vitro*. For example adding cobalt displaces Fe(II) stopping PHD activity (136). Another system consists of adding a 2-oxoglutarate competitive inhibitor named DMOG (dimethyloxaloylglycine) which also impairs PHD, in this case FIH is also inhibited (137). Under hypoxia, or when PHD/FIH are inhibited by other mechanisms, HIF heterodimer binds to DNA specific regions, HRE, present in the promoter or regulatory regions of target genes such as SLC2A1 (138), EGLN3(139) among others. SLC2A1 is the gene name for GLUT1, a glucose transporter, and EGLN3 is the gene name for PHD3 a HIF regulator.

Hypoxia adaptation response

HIF transcription factor was discovered in the 90s and was described as an erythropoietin (EPO) interacting factor in hypoxia (140). Described HIF transcriptional targets are related to energy metabolism (i.e., GLUT1), angiogenic signaling (i.e., VEGFA, EGF), growth and apoptosis (i.e., Endoglin), cell migration (i.e., CXCR4), matrix and barrier functions (i.e., procollagen prolyl hydroxylase α_1), vasomotor regulation (i.e., ET-1, iNOS), and many other cell functions (120). During fetal development, HIF plays an important role establishing an efficient vascular system through the regulation of EPO and VEGF.

VEGF is one of the most studied HIF target gene. HIF directly induces VEGF (141), stabilizes its mRNA (142) and increases its activity upregulating its receptor (FLT1/VEGF-R1) (143). HIF relation with VEGF connects directly hypoxia with angiogenesis (144).

HIF and inflammation

HIF relation with inflammation has been largely discussed, however, little is known (145–153). A non-resolved or uncontrolled inflammation can be the cause of several diseases like GCA.

Hypoxia is a prominent characteristic of chronic inflamed tissues due to high oxygen consumption by metabolically active resident cells and an active inflammatory infiltrate added to an insufficient oxygen supply. This deficient supply and/or increased consumption have a significant impact in signaling pathways affecting disease progression (151).

NFκB (nuclear factor kappa B) is a TF involved in innate immunity, inflammation and apoptosis, activated by a phosphorylation cascade usually initiated by an inflammatory stimulus like IL1β and TNFα among others. NFκB plays an important role in HIF1α mRNA expression (151,154). While NFκB role in immunity and inflammation has been studied, HIF role in the regulation of inflammation is not so well defined (151).

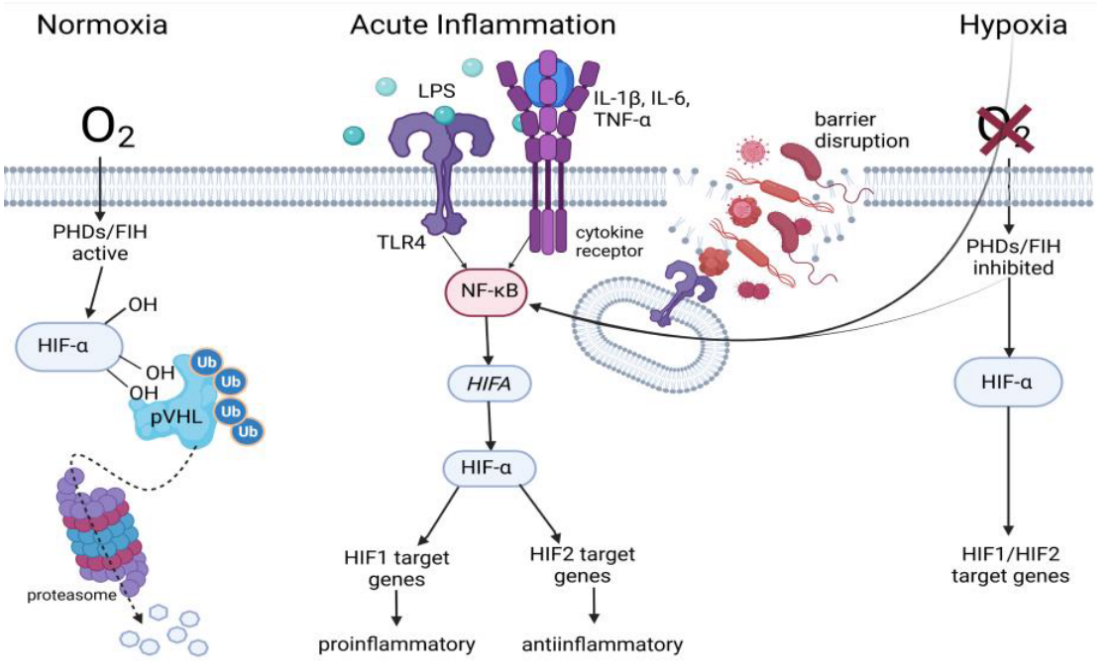


Figure VIII (154). Hypoxia-inducible factor activation under acute inflammation. When oxygen is present, hydroxylation and subsequent degradation of the HIFα protein by the proteasome occurs (left). Under hypoxia, the inhibition of HIF hydroxylases allows a stabilization of HIF-α. Under conditions of acute inflammation, bacterial stimuli bind to membrane-bound and intracellular toll-like receptors and inflammatory cytokines recognize their receptors on the cell surface. Barrier disruption allows the contact of microbial peptides with the respective TLR. This merges in the activation of NF-κB, the transcription factor controlling inflammatory gene expression. Both HIFA isoforms are target genes of NF-κB, thus enabling increased transcription of HIFA mRNA, higher stabilization of HIF-α protein, and target gene transcription without the necessity of oxygen deprivation.

Recently, it has been described in a mouse model with specific VHL-deficiency in cardiac progenitors, which in turn stabilize HIF1/2α, suffer inflammation, dilatation and aneurisms in coronary arteries, cardiac inflammatory infiltrate, fibrosis and ventricular hypertrophy (155). All these aberrant features clearly resemble a vasculitis highlighting a possible relation between HIF1α and vascular inflammation. Mechanisms underlying this observation need to be investigated.

Moreover, HIF1α is known to be expressed by VSMC (156). HIF1α has been described to regulate molecules or processes implicated in GCA pathogenesis such as PDGF (157), endothelin (158), vascular calcification (159), angiotensin (156) and its converting enzyme, ACE and ACE2 (160). Since VSMC play an important role in GCA pathogenesis it is reasonable to hypothesis HIF1α may play a significant role in this disease.

GM-CSF

During the development of the present thesis GM-CSF gain interest related to GCA pathogenesis and its relation to HIF is hypothesized.

GM-CSF, as its name suggests, was originally discovered as a protein capable of generating both granulocyte and macrophage colonies from myeloid precursor cells in vitro. However, in spite of its designation as a hematopoietic growth factor, it does not appear to play a major role in steady-state myelopoiesis (161).

CSF family and their receptors

Macrophage colony stimulating factors (M-CSF or CSF-1), granulocyte colony stimulating factor (G-CSF or CSF-3) and granulocyte-macrophage colony stimulating factor (GM-CSF or CSF-2) and their receptors form the CSF system (Figure VIII).

The high-affinity receptor for GM-CSF (GM-CSFR) is a heterodimeric complex consisting of a GM-CSF-specific α chain and a signal-transducing β subunit, which is shared with IL-3 and IL-5 receptor. Both GM-CSFR and G-CSFR lack intrinsic catalytic activity but trigger the activation of JAK-STAT pathway. GM-CSF signaling activates JAK2 and results in phosphorylation of STAT5, which transcriptional activity appears to govern the GM-CSF inflammatory signature (161).

CSF proteins have structural differences but also divergent spatiotemporal expression patterns. While M-CSF and G-CSF can be detected in the circulation, GM-CSF is virtually absent. The CSF receptors are expressed in myeloid cells but each one of them is restricted to different lineages. GM-CSFR is mostly expressed by DCs and their precursors as well as monocytes, macrophages, granulocytes, and eosinophils. GM-CSF, despite of being part of the CSF family, plays only a minor role in the physiological support of myeloid cells. Instead, it is becoming increasingly clear that GM-CSF is a major mediator of tissue inflammation.

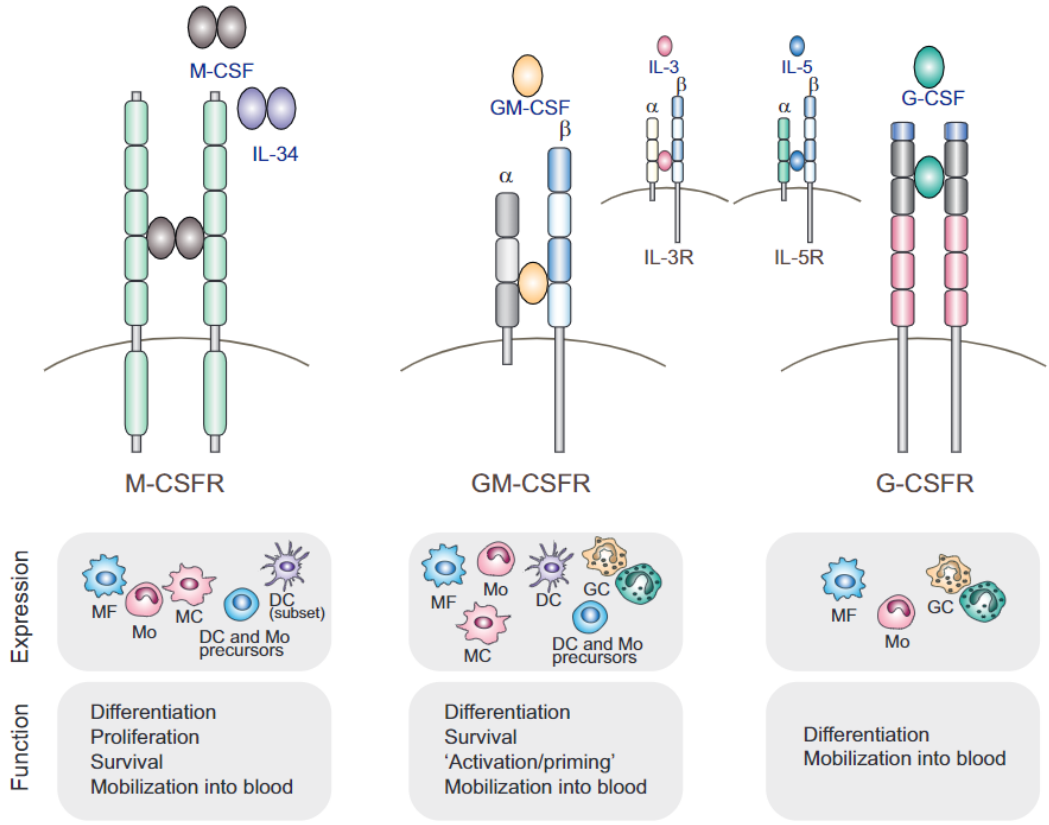


Figure VIII (161). The CSF Super Family. The M-CSFR is a homodimer that can be expressed across most members of the mononuclear phagocyte system and can be triggered by its canonical ligand M-CSF as well as IL-34. The latter has been shown to be involved in the homeostasis of both Langerhans cells (LCs) and microglia. Most members of the myeloid compartment likewise express the GM-CSFR. The beta chain can pair with the GM-CSF-specific alpha-chain or the alpha-chains forming the receptors for IL-3 and IL-5. GM-CSFR engagement leads to activation and cell differentiation and, in some cells (e.g., MCs), to the initiation of a proinflammatory expression signature. The G-CSFR homodimer is expressed primarily on GCs and is important for the mobilization of granulocytic cells from the BM. Abbreviations are as follows: MF, macrophage; Mo, Monocyte; MC, Monocyte-derived cell; DC, dendritic cell; GC, granulocyte (only in this figure, along the thesis GC stands for glucocorticoids).

GM-CSF and inflammation

Epithelial cells, endothelial cells, fibroblasts, myeloid and T cells produce GM-CSF upon stimulation by other cytokines or pathogen-associated molecular pattern molecules (PAMPs). GM-CSF has a seminal role in the progression of disease in animal models of inflammatory conditions (161–163).

GM-CSF acts primarily on myeloid cells, promoting activation of dendritic cells and macrophages, and differentiation of monocytes into dendritic cells, although other cell types may also respond to this cytokine (161–163).

There is evidence that GM-CSF, virtually absent in circulation, is produced and active locally at sites of inflammation. Thus, increased GM-CSF concentrations are found in lesions from patients with rheumatoid arthritis, multiple sclerosis, and GCA (64,161). Moreover, GM-CSF has been found to drive disease progression in preclinical models of arthritis and multiple sclerosis (161). In addition, GM-CSF mRNA has been detected in arterial lesions of GCA and GM-CSF protein production is increased in circulating peripheral blood mononuclear cells from GCA patients compared to healthy controls (59,64). According to its known biologic functions, GM-CSF may have a role in promoting and amplifying vascular inflammation and injury in GCA.

Based on animal models several disorders may potentially benefit of GM-CSF blockade such as cardiac inflammation in Kawasaki disease (164) or aortic aneurism formation (165), which is an important GCA outcome.

2

Hypothesis

GCA pathophysiology is not fully understood. Moreover, current treatment of GCA is based on GC meaning that patients suffer from serious GC side-effects. Thus, there is an urgent unmet-need in this condition and further studies need to be undertaken to establish curative therapies.

First, GCA involved arteries undergo thickening and remodeling of the arterial wall due to an aberrant inflammatory infiltrate. Angiogenesis, a cellular process promoted by hypoxia is a remarkable finding in GCA lesions. HIF1α stabilization is one of the principal consequences of hypoxia, and HIF1α transcriptional activity is a seminal mediator of cell response to hypoxia. Inflammation and HIF1α are closely related via several signaling pathways and recently various inflammatory molecules are described as capable of stabilizing HIF1α.

As mouse models have demonstrated that HIF1α stabilization leads to a vasculitis-like disease, it is conceivable that HIF1α may have an important role in the development and/or maintenance of vascular inflammation and might have a role in GCA. Moreover, VSMCs express HIF1α which regulates molecules or processes implicated in GCA pathogenesis. It is important to highlight that VSMCs play an important role in GCA where they acquire pro-inflammatory functions (chemokine, cytokine and adhesion molecule expression) and differentiate into myofibroblasts generating intimal hyperplasia. Therefore it is reasonable to hypothesize an important role of HIF1α in this disease.

Second, GM-CSF is emerging as a key cytokine in inflammation for several reasons; 1) its transcripts have been detected in GCA temporal arteries; 2) GM-CSF has been shown to be produced and secreted by PBMC from patients with active GCA and detected in GCA-involved temporal arteries at protein level. However, expression of GM-CSF receptor and its functional role has not been previously explored. The putative role of GM-CSF in critical steps of GCA pathogenesis suggests therapeutic potential for mavrilimumab in this disease, supported by a recent phase 2 trial.

In summary, HIF1α and GM-CSF may play an important role in vascular inflammation and remodeling in GCA, through independent or inter-related mechanisms.

3

Objectives

To assess the validity of the hypothesis of this thesis the following objectives were established:

- To determine the presence/absence of hypoxia and HIF1α expression and their tissue distribution through the study of temporal artery biopsies from patients with GCA compared to controls.
- To study if the potential deregulated signaling pathways in the disease affect the presence of HIF1α in GCA lesions.
- To analyze the effects of HIF1α stabilization in VSMC on molecules related to biologic processes implicated in GCA pathophysiology such as migration, angiogenesis, inflammation, adhesion, and vascular remodeling.
- To analyze different anti-cytokine targeted therapies and its effect modulating HIF1α stability in a GCA context and further analyze their effect on HIF1α pathway.
- To investigate the expression of GM-CSF and GM-CSFRα in inflamed arteries from patients with GCA and to determine GM-CSFR-related actived signalling pathways and modulation of downstream gene expression.
- To investigate the impact of GM-CSFRα blockade with mavrilimumab on inflammation in ex vivo cultured arteries from patients with GCA.
- To elucidate if HIF1α and GM-CSF have any effect on each other relevant in GCA pathogenesis.

4

Methods

Funding and patient samples

The aim of this thesis is part of a project funded by Ministerio de Ciencia e Innovación (SAF2017-57708-R and AEI//10.13039/501100011033 [PID2020-114909RB-I00]), La Marató de TV3 (MTV3 2014/201507), the International Vasculitis Foundation, and Kiniksa Pharmaceuticals. This project was approved by the ethics committee of the Hospital Clinic de Barcelona and patients signed informed consent.

Temporal artery (TA) biopsy processing

TA biopsies were performed to patients with suspected GCA for diagnostic purposes. After the histopathological examination and confirmation or exclusion of GCA, the remaining tissue was processed in the laboratory. GCA was confirmed if typical lesions (inflammation of the vessel wall by mononuclear cells with or without giant-cells) were observed. If inflammatory infiltrates could not be observed, biopsies were considered as control biopsies. Control arteries, then, were not obtained from healthy donors; they were obtained from patients with initially suspected, but eventually excluded GCA. Other conditions able to explain patient symptoms were subsequently diagnosed. These TA biopsies were homogenized for further extraction of mRNA, using Tri-Reagent (MRC, Inc), or cryopreserved in Tissue-Tek OCT compound (Sakura) to investigate protein by immunofluorescence.

Moreover, it is also feasible to culture TA biopsies under different conditions. The possibility of culturing TA biopsies led to the development of an *ex vivo* model, as well as to the extraction of primary cultures of VSMC.

Temporal artery biopsy culture

1mm sections from TA biopsies were embedded into *Matrigel*™ (BD Biosciences), a widely used basement membrane secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, that contains a mixture of ECM proteins which preserves the viability of the artery *ex vivo*, as previously described (68). Each section was cultured in complete RPMI-1640 (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; from Gibco, Life Technologies, Waltham, Massachusetts, USA), 2mM of L-

glutamine (Gibco), 50ug/ml of gentamicin (Braun, Melsungen, Germany) and 2,5ug/ml of amphotericin B (Invitrogen). TABs were cultured at 37ºC with 5%CO_2

At the end of the experiment (2 hours for pimonidazole (hypoxiprobe) or 5 days for mavrilimumab experiments) sections were recovered and processed for RNA extraction or cryopreserved to detect protein by immunofluorescence. Supernatants were also collected and preserved for detection of secreted/shed molecules. All samples were stored at -80ºC.

Cell culture

Primary culture of VSMC

As decribed above, 1mm sections from TA biopsies were embedded into *Matrigel*™ (BD Biosciences). To generate primary cultures of VSMC these sections were cultured with DMEM medium (Lonza, Veviers, Belgium) supplemented with 10% FBS (Gibco, Life Technologies, Waltham, Massachusetts, USA), 2mM of L-glutamine (Gibco), 50ug/ml of gentamicin (Braun, Melsungen, Germany) and 2,5ug/ml of amphotericin B (Invitrogen); at 37ºC and 5% CO_2 (84). Under these conditions, VSMC, from the media layer of the artery, can expand and colonize the culture well. VSMC usually start growing out of the artery after 3 to 6 days of culture, and they reach confluence after approximately 1 month. Subcultures were performed using 0.05%-Trypsin EDTA (Gibco) when cells reached confluence, at 1:2 ratio. VSMC were used between passage 3 and 8 for *in vitro* experiments.

Peripheral blood mononuclear cells (PBMC)

PBMC of healthy donors were isolated by density gradient centrifugation using Ficoll (Histopaque® -1077, Sigma Diagnostics, St. Louis, MO) in sterile conditions using buffy coats (leucocyte concentrated from total peripheral blood) obtained through a Blood and Tissue Bank (Banc de Sang i Teixits; BST). Cells were first diluted 1:1 in PBS, and 20ml of the diluted buffy coats were carefully deposited on top of 10-15ml of Ficoll to avoid mixing the two phases. Then, tubes were centrifuged for 30 minutes at 600G at

room temperature (RT), without brake. Using this procedure, PBMC, because of their density, stay in the interphase with the Ficoll and blood plasma. PBMC were recovered and washed three times with PBS. Viability of PBMC was assessed by trypan blue exclusion (solution 0.4% (Sigma)) and counted with Neubauer chamber. PBMC were cultured with complete RPMI-1640 medium at 37ºC and 5% CO_2.

Co-Culture of VSMC and PBMC

In order to study cell-cell interactions between VSMC and inflammatory cells we cultured VSMCs together with PBMC mimicking the inflammatory microenvironment present in GCA. VSMC were seeded on 6-well plates at 85-95% confluence and cultured overnight with DMEM medium supplemented as previously described. The day after, media from the VSMC was replaced by PBMC in RPMI medium supplemented as described. 1 million PBMC were seeded per well. The co-culture conditions were compared with situations containing PBMC or VSMC only.

After 24- or 48-hours supernatants were collected, and cells were processed to obtain RNA or protein lysates as explained below. Different cell population of the co-culture were obtained and processed separately. Supernatants from wells containing PBMC were centrifuged at 360G for 10 minutes at 4ºC to recover non-attached cells and supernatants. In co-culture conditions, before collecting VSMC, cells were rinsed with cold Versene® (Invitrogen) to remove attached PBMC.

Cell culture reagents

Mostly VSMC but also co-cultures and TA biopsy culture experiments were performed with the presence of several reagents to test the stabilization or inhibition of HIF1α. $CoCl_2$ and DMOG are PHD inhibitors; therefore HIF1α is stabilized after exposure to these reagents. IL6, sIL6R, IL1β, IFNγ and TNFα were used as activators of different inflammatory intracellular pathways to investigate whether these conditions may stabilize HIF1α. To check the effect of IL6 downstream pathway, IL6 is used in combination with sIL6R because previous experiments have demonstrated the lack of IL6 response in VSMC without adding the soluble receptor (166).

Several neutralizing monoclonal antibodies (tocilizumab, infliximab, mavrilimumab, mab285) and other molecules (anakinra) were used to inhibit IL6, TNFα, GM-CSFRα, IFNγ, and IL1 respectively and check their effect on HIF1α levels. Finally, pimonidazole is able to leave a mark in hypoxic regions inside a tissue; therefore it was used to check hypoxic regions in TA biopsies. Specifically, the following molecules were used:

Molecules	Company	Working concentration
Pimonidazole	Hypoxyprobe	60 µg/ml
$CoCl_2$	Sigma-Aldrich	200 µM
DMOG	Selleckchem	0.5/1 mM
IL6	R&D Systems	10 ng/ml
sIL6R	R&D Systems	100 ng/ml
IL1β	R&D Systems	20 ng/ml
IFNγ	R&D Systems	100 ng/ml
TNFα	R&D Systems	20 ng/ml
Tocilizumab	Roche	10 µg/ml
Infliximab	Janssen biologicals	10 µg/ml
IgG	Sigma	10 µg/ml
Anakinra	Sobi™	5 µg/ml
Anti-IFNγ (mab285)	R&D Systems	10 µg/ml
Placebo	Kiniksa	20 µg/ml
Mavrilimumab	Kiniksa	20 µg/ml
rhGM-CSF	R&D Systems	20 ng/ml

VSMC were also subjected to low oxygen concentration in a hypoxic chamber named as HPX condition which means 1,1% O_2. The rest of the experiments were performed in normal incubators at 21% O_2, also known as normoxia (NRX).

RNA extraction

RNA extraction was carried out using the chloroform-isopropanol method. Samples were recovered in 1ml of Tri-Reagent (MRC, Inc) which promotes cellular lysis and contributes to the preservation of samples, avoiding RNA degradation. For fresh artery samples, 20µm sections from fresh-frozen arteries in OCT were homogenized by

passing the sample 3 times through a 20G needle with a syringe. For cultured artery samples 2 or 3 sections were homogenized with Bullet Blender Tissue Homogenizer (Next Advance).

The RNA extraction samples were mixed with chloroform and then centrifuged to obtain different phases. RNA, which remains in the aqueous phase, was recovered in a new tube and precipitated using isopropanol. The pellets obtained after centrifugation were washed two times with ethanol at 75%. After the last wash, ethanol was removed, and samples were resuspended in 20µl of DEPC water (Ambion, Life Technologies). Finally, RNA concentration was quantified using Quawell Q3000 UV (Quawell Technology Inc., San Jose, USA) spectrophotometer.

Reverse transcription and quantitative real-time PCR

RNA retrotranscription to cDNA was carried out with 1µg of RNA per sample, to a final volume of 100µl. High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used for the reverse transcription. In each sample, the reaction was performed in 10µl of RT buffer, 4µl of dNTPs mix, 10µl of random primers, 5µl of Multiscribe® Reverse Transcriptase, 21µl of DEPC water (Ambion) and 1µg of RNA diluted in 50µl of DEPC water (Ambion). The reaction was performed in a thermal cycler (Applied Biosystems) programmed 10 minutes at 25ºC, 120 minutes at 37ºC and 5 seconds at 85ºC. Samples were used or stored at -20ºC until needed.

Gene expression was measured by quantitative real-time PCR. For the reaction, 1µl of cDNA was mixed with 6 µl of Taqman Universal Master Mix (Applied Biosystems), 0.6 µl of the pertinent probe (Applied Biosystems) and 9.25 µl of DEPC water (Ambion) in 384-well plates. Fluorescence was measured real time during the PCR reaction using Viia™7 Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific) and the results were analyzed with the QuantStudio Real-Time PCR Software v1.1 (Applied Biosystems). Gene expression was normalized to the expression of the endogenous control gene *GUSB* using comparative CT method.

The following probes (all from Thermo Fisher Scientific / Applied Biosystems) were used to assess the mRNA expression of the corresponding genes:

Gene name	Probe reference	Gene name	Probe reference
CCL2	Hs00234140_m1	GUSB	Hs99999908_m1
CCL4	Hs99999148_m1	HIF1A	Hs00153153_m1
CCL5	Hs00174575_m1	HLA-DRA	Hs00219575_m1
CD14	Hs00169122_g1	ICAM1	Hs99999152_m1
CD16	Hs04334165_m1	IFNG	Hs00174143_m1
CD163	Hs00174705_m1	IL10	Hs00961622_m1
CD20	Hs00544818_m1	IL17A	Hs00174383_m1
CD206	Hs00267207_m1	IL1B	Hs01555413_m1
CD34	Hs00990732_m1	IL23A	Hs00372324_m1
CD3E	Hs01062241_m1	IL6	Hs00985639_m1
CD68	Hs00154355_m1	ITGA5	Hs01547673_m1
CD83	Hs00188486_m1	ITGAV	Hs00233808_m1
COL1A1	Hs00164004_m1	ITGB1	Hs01127536_m1
COL3A1	Hs00164103_m1	MMP2	Hs00234422_m1
CX3CXL1	Hs00171086_m1	MMP9	Hs00234579_m1
CXCL10	Hs00171042_m1	NOS2 (iNOS)	Hs01075529_m1
CXCL11	Hs00171138_m1	PDGFA	Hs00234994_m1
CXCL12	Hs00164004_m1	PDGFB	Hs00234042_m1
CXCL8	Hs99999034_m1	PDGFRA	Hs00998018_m1
CXCL9	Hs00171065_m1	PDGFRB	Hs01019589_m1
EDN1	Hs00174961_m1	PECAM-1 (CD31)	hS00169777_m1
EDNRA	Hs00609865_m1	ROBO2	Hs00326067_m1
EDNRB	Hs00240747_m1	RORC	Hs01076112_m1
EGF	Hs0109999_m1	SLC2A1 (GLUT1)	Hs00892681_m1
EGFR	Hs01076090_m1	SLIT2	Hs01061407_m1
EGLN3 (PHD3)	Hs00222966_m1	SPI1 (PU.1)	Hs02786711_m1
ELN	Hs00355783_m1	TBX21 (T-bet)	Hs00894392_m1
FGF2	Hs00266645_m1	TIMP1	Hs00171558_m1
FLT1 (VEGFR1)	Hs01052961_m1	TNF	Hs00174128_m1
FN1	Hs01549976_m1	VCAM	Hs01003372_m1
GM-CSF	Hs00929873_m1	VEGFA	Hs00900055_m1
GM-CSFRA	Hs00538896_m1	VWF	Hs01109446_m1

Protein cell lysates

Total protein cell lysate

Cultured cells were lysed with RIPA (Radio-Immunoprecipitation Assay, Sigma-Aldrich, Ayrshire, UK) buffer supplemented with 1mM of PMSF (phenylmethylsulfonyl fluoride; Sigma), 1mM of 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (Roche), 2nM of orthovanadate (Sigma), 0.5µg/ml of leupeptin (Sigma), 2mM aprotinin (Thermo Scientific), 2mM of pepstatin (Sigma), 1mM of EDTA (Ethylene-diamine-tetra acetic acid, Sigma), 50mM of sodium fluoride (Sigma), and 1% of NP-40 detergent (Abcam), to obtain protein lysates.

Nuclear protein extraction

Nuclear and cytoplasm fractions were obtained from VSMC cultured on 6 well plates previously washed with cold PBS 1x to discard debris and albumin from the medium. Attached cells were obtained with scrapper and collected by centrifuging at 4ºC during 5 minutes at 1000rpm. Pellet obtained was resuspended with buffer I (1ml of 1M Hepes, pH8.0, 150µl of 1M $MgCl_2$, 1ml of 1M KCl, 100µl of 1M of dTT raised with ${}_dH_2O$ up to 100ml) supplemented with proteases and phosphatases inhibitors, as previously described. Samples were ice incubated for 15 minutes and NP40 1% was added before vortex briefly. Samples were then centrifuged at 12000rpm for 5 minutes at 4ºC and supernatants were collected as a cytoplasm fraction. Pellets were resuspended with buffer II (2ml of 1M Hepes pH8.0, 150µl of 1M $MgCl_2$, 25ml of glycerol, 42ml of 1M NaCl, 40µl of 0.5M EDTA, 100µl of 1M DTT raised with ${}_dH_2O$ up to 100ml) also supplemented with protease and phosphatase inhibitors.

Western blot

The same amount of protein per each condition was resolved on sodium dodecyl sulfate (SDS)-polyacrilamide (BioRad) electrophoresis gel at reducing conditions. 10% acrylamide gels were used unless otherwise indicated due to the molecular weight of

the analyzed protein. Samples were diluted with loading buffer NuPAGE® LDS Sample Buffer (Invitrogen) and β-mercaptoethanol (BioRad), used as a reducing agent, and they were heated for 7 minutes at 95ºC to completely denature proteins. Electrophoresis was carried out at 125 V with the running buffer 1x prepared from premixed 10x Tris/glycine/SDS running buffer to separate protein samples by SDS-PAGE during approximately 2h. After electrophoresis, the gels were blotted onto nitrocellulose membranes (Invitrogen) using the semidry iBlot Blotting System (Thermo Fisher). Blocking was performed by incubating the membranes at least one hour at room temperature with PBS 1x with 0.1% Tween-20 (PBST0.1) and a 5% of BSA (Bovine Serum Albumin, Sigma-Aldrich).

Immunodetection was performed by incubating primary antibodies diluted following the manufacturer's recommendations in blocking buffer at 4ºC, overnight. The day after, membranes were washed 3 times with PBST0.1 and incubated with the corresponding secondary antibodies diluted in blocking buffer for one hour at room temperature.

Chemiluminescence signal was measured with the ImageQuant LAS-4000 imaging system (GE Healthcare Life Science) while incubating the membranes with Supersignal West Femto, Dura or Pico Substrate kit (ThermoFisher). In some cases, membranes were treated for 30 minutes at room temperature with stripping buffer (100mM Glycine (Sigma) and 100mM of NaCl (Sigma) in water at pH 2.5) in order to reincubate the same membrane with different antibodies. Image analysis and quantification was done using FIJI (ImageJ) software. The antibodies and dilutions used were as follow:

Antibody	Company	Dilution	Host animal
Anti-β-actin	Sigma	1:5000	Mouse
HIF1α	BD Biosciences	1:500	Mouse
Lamin B	Santa Cruz	1:200	Mouse
Tubulin	Sigma-Aldrich	1:2000	Mouse
ICAM1	Cell Signalling	1:1000	Rabbit
STAT5	Cell Signalling	1:1000	Rabbit
pSTAT5	Cell Signalling	1:1000	Rabbit
Anti-rabbit-HRP	Cell Signalling	1:2000	Goat
Anti-mouse-HRP	BioRad	1:2000	Goat

ELISA (Enzyme-Linked ImmunoSorbent Assay)

ELISA technique allows the analysis of the concentration of a specific molecule in a variety of samples simultaneously. Each molecule is analyzed independently, thus the specific procedure depends on the commercial ELISA kit used. Basically, the different ELISA were carried out in a 96 well plate pre-coated with the specific antibody against the target molecule. After incubating the plate with the samples and proceed with different washes, each well was incubated with an enzyme-conjugated antibody that also recognize the molecule of interest. The substrate of the enzyme was then added to get a colorimetric signal proportional to the amount of the target molecule present in each sample. The reaction was finished with an acidic stop solution. The absorbance was measured at 450nm (and 580nm for background subtraction) in a plate reader (Multiskan Ascent, Thermo Scientific) and the concentration was extrapolated from a standard curve.

The following ELISA kits were used:

Molecule of interest	Company	Reference
ET-1	R&D Systems	DET100
CCL2 / MCP-1	R&D Systems	DCP00
CCL5 / RANTES	R&D Systems	DRN00B
CXCL8 / IL-8	R&D Systems	D8000C
VEGFA	Thermo Fisher Scientific	BMS277-2
TNFα	R&D Systems	HSTA00E
IL6	R&D Systems	D6050
IL1β	R&D Systems	DLB50
GM-CSF	R&D Systems	HSGM0
IFNγ	R&D Systems	DIF50
CXCL10	R&D Systems	DIP100
MMP-9	R&D Systems	F9M00
IL-10	R&D Systems	D1000B
sCD83	Antibodies online	abx151006
TIMP-1	R&D Systems	DTM100

Immunofluorescence and confocal microscopy

Fresh or cultured biopsies were fixed with paraformaldehyde (PFA) 4% diluted with PBS 1x for one hour and then cryopreserved with increasing concentrations of sucrose (15%-30%) before being embedded in Tissue-Tek OCT Compound (Sakura) and preserved at -80ºC until needed. Section of 7µm were obtained with a cryotome cryostat (Leica Microsystems) and stored at -20ºC until needed.

For pimonidazole or HIF1α, alone or double-stained with cell markers, the following protocol was used.

TA slides or VSMC grown in gelatin-coated coverslips were fixed with PFA 4% for 10 minutes at room temperature. After washing the samples three times with PBS 1x sections were blocked for peroxidase activity with 3% H_2O_2 from Alexa Fluor™ 488 Tyramide SuperBoost™ Kit (Invitrogen). Instructions from the kit were modified as follow. Peroxidase blocking was carried out for one hour at room temperature. After three washes with PBS 1x sections were blocked and permeabilized with blocking buffer (with 0.5% Triton™ X-100 (Sigma)). Samples were incubated with primary antibodies diluted in blocking buffer overnight at 4ºC. Negative controls used are the same samples that follows the exact same protocol but without primary antibody. After three washes with PBS 1x with 0.5% Triton™ X-100 (Sigma) (PBST0.5), a suitable secondary antibody was diluted in the secondary antibody solution (Poly-HRP-conjugated goat anti-rabbit secondary antibody) from the kit and incubated during 1h at room temperature. Then, samples were washed three times with PBST0.5 and incubated for 7 minutes with tyramide solution. Stop solution was added and washed two times with PBS and one last wash with distilled water. Finally, mounting medium with 4',6-diamidino-2-phenylindole (DAPI FluoromountG, Southern Biotech) was used to stain the nuclei and to preserve the preparations. Immunofluorescence samples were observed with an LSM 880 with Airyscan (Zeiss) at the advanced microscopy unit from the scientific and technological centers from the University of Barcelona. Raw data was then analyzed using the ImageJ software. The antibodies and working dilutions used were as follows:

Antibody	Company	Dilution	Reference
Rabbit-anti-pimonidazole	Hypoxyprobe	1/300	Pab2627
Mouse-anti-αSMA	Abcam	1/100	Ab54723
Rabbit-anti-HIF1α	Novus Biologics	1/300	NB100-449
Mouse-anti-CD31	DAKO	Not required	IR610
Mouse-anti-CD45	DAKO	Not required	GA751
Donkey-anti-mouse-555	Thermo Fisher Scientific	1/300	A31570
Tyramide SuperBoost™ Kit, goat anti-rabbit-488 IgG	Thermo Fisher Scientific	Not required	B40943

Stainings for the remaining proteins the following protocol was used. 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 the following table. 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.

Antibody	Company	Dilution	Reference
Mouse-anti-CD68	DAKO	Not required	KP1
Mouse-anti-CD3	Thermo Fisher Scientific	1/200	F7.2.38
Rabbit-anti-CD3ε	Raybiotech	1µg/ml	119-11933
Mouse-anti-CD16	Bio Rad	1/40	2H7
Mouse-anti-CD20	DAKO	Not required	L26
Mouse-anti-CD31	DAKO	Not required	JC70A
Mouse-anti-αSMA	Abcam	1/100	Ab54723
Rabbit-anti-GMCSF	Abcam	5µg/ml	Ab220888

Rabbit-anti-GMCSFRα	Biorbyt	10µg/ml	Orb157207
Mouse-anti-HLA-DRA	Beckton Dickinson	1/100	G46-6
Rabbit-anti-HNE	Bioss Antibodies	20µg/ml	Bs6313R
Rabbit-anti-pSTAT5	Cell Signalling	1/200	C71E5
Mouse-anti-CD34	Ventana	0.8µg/ml	QBEnd/10
Rabbit-anti-VEGFA	Santa Cruz Biotechnology	4µg/ml	Sc-152
Anti-mouse-555	Thermo Fisher Scientific	6.6µg/ml	A31570
Anti-rabbit-488	Thermo Fisher Scientific	6.6µg/ml	A21206

In-situ RNA hybridization

RNAScope (RS) (ACDbio, Abingdon, UK) in situ hybridization was performed at Kiniksa pharmaceuticals on purchased formalin-fixed paraffin-embedded (FFPE) sections of GCA and control biopsies to detect transcripts of specific genes, including GM-CSF, GM-CSFRα, CD83 and Spi1 (PU.1). After fixation and sectioning, tissue was permeabilized and probed with target-specific double Z probes specific to single target mRNA, and hybridization signals were further amplified for detection. Visualized with a microscope, each red dot represents a single target mRNA molecule. 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

Immunohistochemistry

Two micrometer thick temporal artery sections from purchased FFPE samples were used for immunohistochemistry. After 20-minute antigen retrieval with citrate buffer (pH 6), samples were immunostained with specific antibodies, using the Leica Microsystems' Bond-max automated immunostainer and the Bond Polymer Refine Detection System (Leica Microsystems), developed with diaminobenzidine and counterstained with hematoxylin (antibodies used, dilutions and optimized incubation times: see following table). Positive and negative control tissues for protocol optimization were selected from Human Protein Atlas (www.proteinatlas.org) and obtained from Institut d'Investigacions Biomèdiques August Pi i Sunyer Biobank were the whole protocol for immunohistochemistry was performed.

Antibody	Company	Dilution	Reference
Mouse-anti-CD83	BioRad	100 μg/ml	HB15-E
Rabbit-anti-GMCSFRα	Biorbit	10 μg/ml	Orb157207
Mouse-anti-GMCSF	My Biosource	10 μg/ml	7U1
Rabbit-anti-pJAK2	Abcam	2.5 μg/ml	Ab32101
Goat-anti-PU.1	My Biosource	2.5 μg/ml	MBS247864
Rabbit-anti-pSTAT5	Cell Signalling	1/150	C71E5

Statistical analysis

Mann-Whitney U test, Wilcoxon test or Student's t-test, when applicable, were used for quantitative independent or paired data. Calculations were performed with the Graphpad Prism v6. Due to the small number of cases in some experiments statistics are only indicative. p-values are indicated as follows: *≤0,05 **≤0,01 ***≤0,001 ****≤0,0001.

5

Results

HIF1α distribution in GCA lesions

Hypoxic regions in GCA temporal arteries

In order to evaluate hypoxia in GCA patients, TA-derived from GCA patients compared with controls were assessed by immunofluorescence. GCA lesions cultured with pimonidazole, a hypoxia marker, showed hypoxic regions in the temporal artery wall of GCA lesions in comparison to a control TAB by immunofluorescence (figure 1).

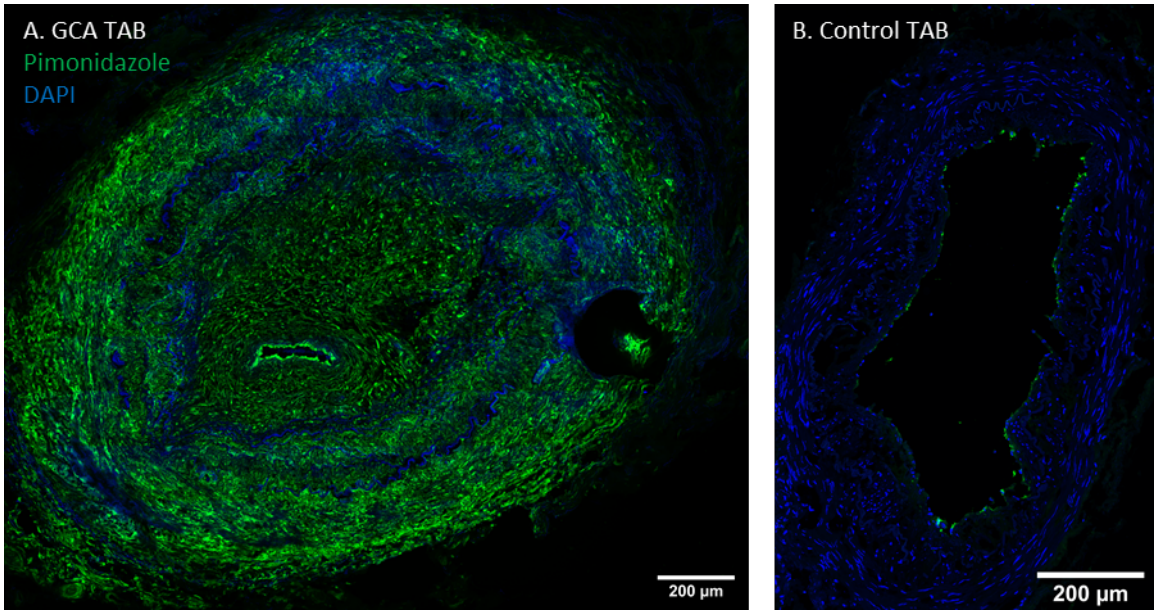


Figure 1. Hypoxic regions in GCA lesions. TA biopsies from GCA (A; n=3) and control patient (B; n=3) were cultured *ex vivo* with pimonidazole (hypoxia marker). The tissue was stained for pimonidazole (green) to show hypoxic areas and DAPI (blue) to show nuclei. Images are shown as mosaics of several photos. Scale bar 200µm

HIF1α in GCA lesions

HIF1A mRNA levels tested by quantitative real-time PCR in fresh TA were globally increased in GCA lesions even though HIF1A mRNA expression in several GCA samples were similar to controls, as shown in figure 2 A.

In order to investigate HIF1α at the protein level, GCA lesions were stained for HIF1α and cell markers of interest in 7µm slides followed by confocal microscopy analysis; TA biopsies from GCA invariably showed the presence of HIF1α in the media layer of the artery wall (figure 2 C), whereas control TA biopsies showed presence of HIF1α only in 2 out of 5 arteries.

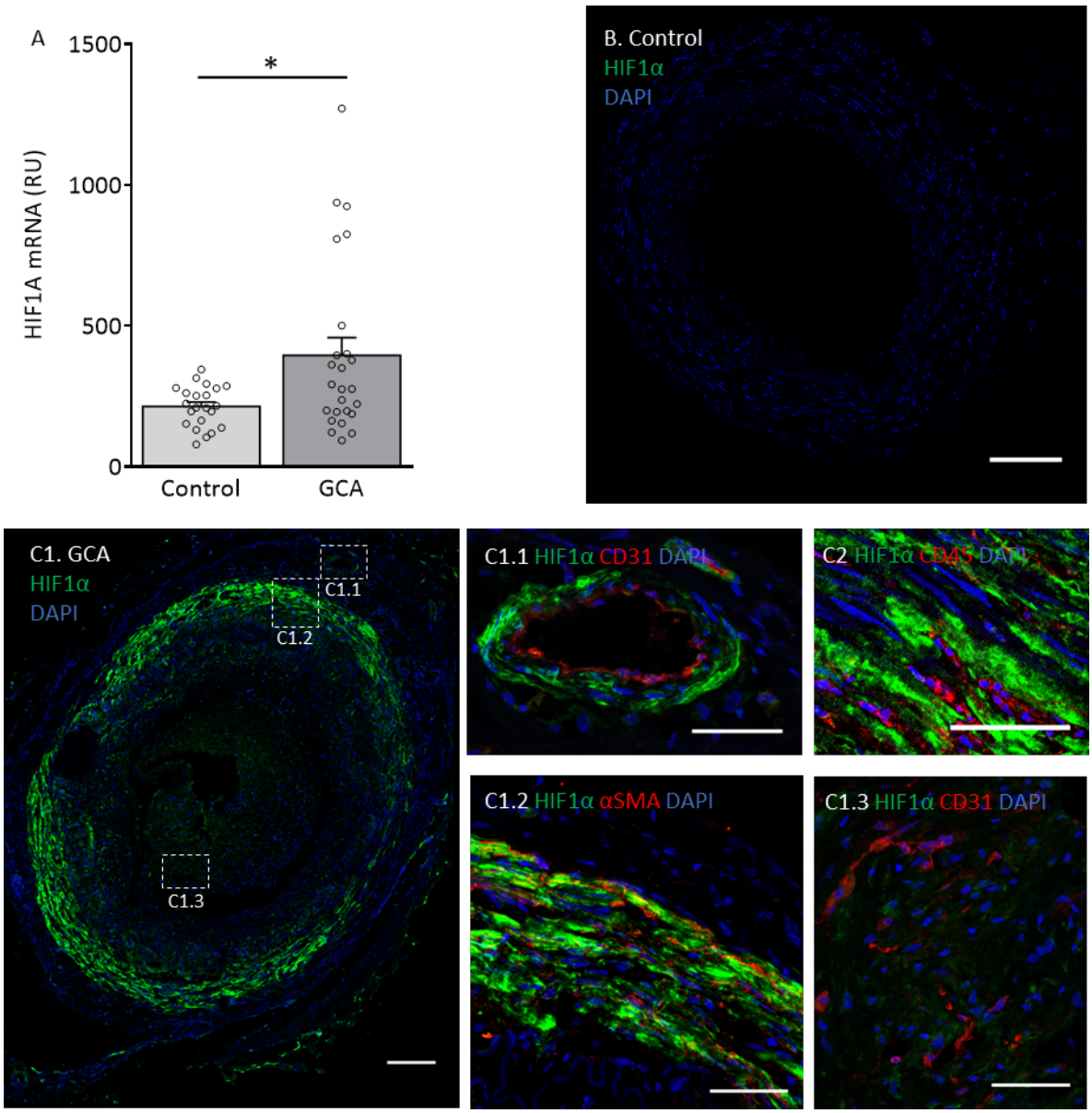


Figure 2. HIF1α expression in GCA lesions. (A) HIF1A mRNA levels in fresh-frozen GCA (n=25) and control (n=22) temporal arteries expressed in relative units using GUSB as housekeeping gene. (B) Fresh cryopreserved control temporal artery stained for HIF1α (green) and DAPI (blue). (C) Fresh cryopreserved GCA TA stained for HIF1α (green) and cell markers (red); C1.1 (CD31 for endothelial cells), C1.2 (αSMA for smooth muscle cells), C1.3 (CD31) and C2 (CD45 for immune cells). Scale bar: 200µm (B, C1; Images showed are mosaics of several photos); 40µm (C1.1, C1.2, C1.3, C2). Bars represent mean ± SEM. *p<0.05

As shown in figure 2, HIF1α showed a heterogeneous expression pattern in GCA lesions. It was mainly expressed in the media layer, identified by the presence of αSMA (figure 2 C1.2). Areas of the artery wall with presence of HIF1α also showed presence of leukocytes (CD45+) (figure 2 C2). *Vasa vasorum* of the GCA lesions identified with CD31 staining, showed HIF1α around these CD31+ cells, suggesting that pericytes expressed HIF1α (figure 2 C1.1).

Inflammatory microenvironment promotes nuclear HIF1α in VSMC

Intracellular HIF1α localization in co-culture *in vitro* model

Since VSMC were the main cell type expressing HIF1α, we explored the effect of inflammation on its expression, stabilization and nuclear translocation. In order to analyze the intracellular presence of HIF1α under an inflammatory microenvironment, co-culture of VSMC with PBMC (65), were established. This model allows the study of VSMC-PBMC interaction emulating, in a simplified experiment, the inflammatory context present in GCA patients. HIF1A mRNA levels were increased in VSMC under co-culture with PBMC in comparison with VSMC alone (figure 3A). These increased levels were also present at the protein level, tested by western blot of total protein extraction (figure 3B).

In order to determine the intracellular location of HIF1α inside the VSMC, nuclear protein extractions and immunofluorescence staining were performed. First, by nuclear protein extraction we detected increased levels of HIF1α in the nuclei of VSMC co-cultured with PBMC by western blot (figure 3C). Second, by immunofluorescence we also detected nuclear HIF1α in the co-cultured VSMC whereas there was almost no detection in VSMC alone (figure 3D).

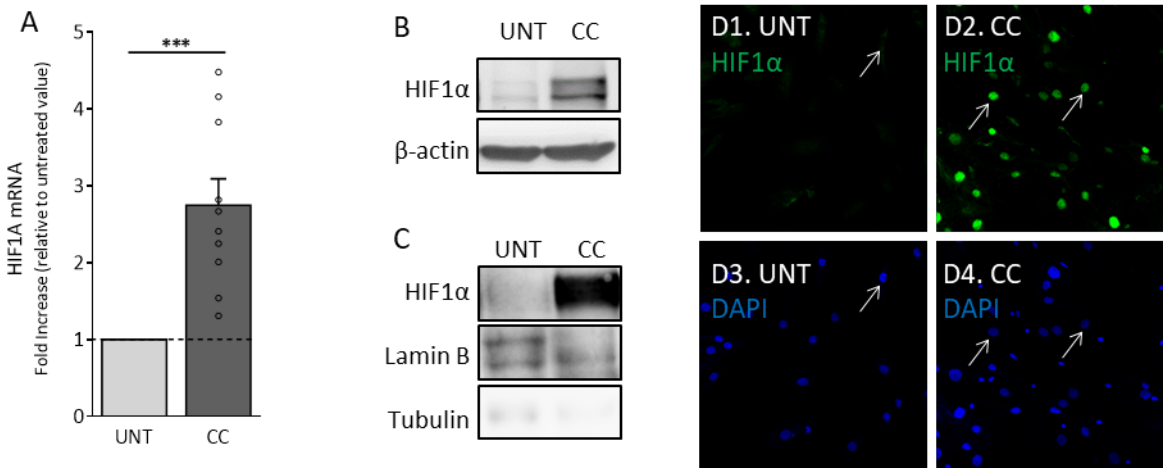


Figure 3. VSMC express HIF1α under an inflammatory microenvironment. (A) HIF1A mRNA levels in VSMC co-cultured with PBMC (CC) during 24h expressed in fold increase relative to VSMC alone (UNT) (n=10). (B) Total protein levels of VSMC alone (UNT) versus VSMC co-cultured with PBMC (CC) during 48h (n=3). (C) Nuclear protein levels of VSMC alone (UNT) versus VSMC co-cultured with PBMC (CC) during 48h (n=3). (D) Immunofluorescence of HIF1α (green) and DAPI (blue) of VSMC alone (UNT) versus VSMC co-cultured with PBMC (CC) during 48h; Arrows show the same nuclei in both photos (D1-D3 and D2-D4). Bars represent mean ± SEM. ***p≤0.001

Inflammatory molecules implicated in HIF1α stabilization

Significantly increased HIF1A mRNA levels were observed when VSMC were treated with IL6 and its soluble receptor (sIL6R) (figure 4 A). To a lesser extent, an increase was also observed when VSMC were treated with IL1β and IFNγ or IFNγ in combination with TNFα, but not with TNFα alone (figure 4 A). When VSMC were treated with IL6+sIL6R, IL1β or IFNγ+TNFα increased HIF1α protein was observed by western blot of total protein extraction in all treated conditions (figure 4 B). The same result was observed in nuclear protein extracts (figure 4 C). We also explored nuclear expression of HIF1α by immunofluorescence with similar results showing presence of HIF1α in the nuclei of VSMC treated with the indicated inflammatory molecules (figure 4 D). All cytokines used were present in the supernatant of VSMC-PBMC co-culture model as shown in supplementary figure 1.

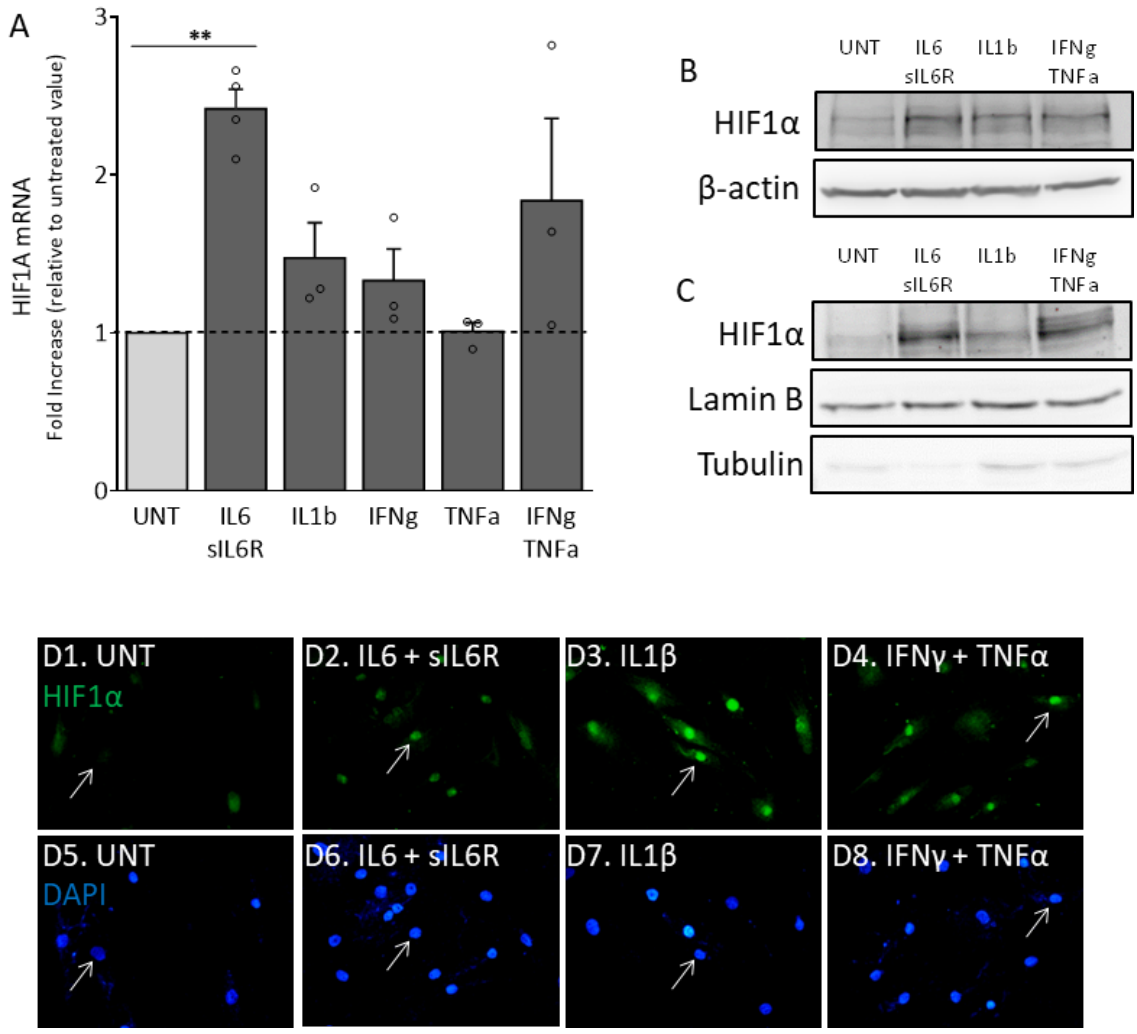


Figure 4. HIF1α expression in VSMC under different inflammatory stimuli. (A) HIF1A mRNA levels in VSMC treated with IL6+sIL6R, IL1β, IFNγ, TNFα or IFNγ+TNFα during 24h expressed in fold increase relative to untreated (UNT) value of three independent experiments. (B) Total protein levels of HIF1α in VSMC alone (UNT) in comparison with VSMC treated with IL6+sIL6R, IL1β or IFNγ+TNFα during 48h. (C) Nuclear protein levels of HIF1α in VSMC alone (UNT) in comparison with VSMC treated with IL6+sIL6R, IL1β or IFNγ+TNFα during 48h. (D) Immunofluorescence of HIF1α (green) and DAPI (blue) in VSMC alone (UNT) in comparison to VSMC treated with IL6+sIL6R, IL1β or IFNγ+TNFα during 48h. Bars represent mean ± SEM. **p≤0.001 (due to the small number of cases, statistics are only indicative).

HIF1α acts as a TF in VSMC

To further study if nuclear translocation of HIF1α was associated with expression of HIF-1α target genes, SLC2A1 (167) and EGLN3 (139) were evaluated in the VSMC-PBMC co-culture system and in VMSC treated with recombinant inflammatory molecules (IL6+sIL6R or IL1β or IFNγ+TNFα). SLC2A1 and EGLN3 mRNA levels showed a clear tendency to increase in all experimental conditions (figure 5).

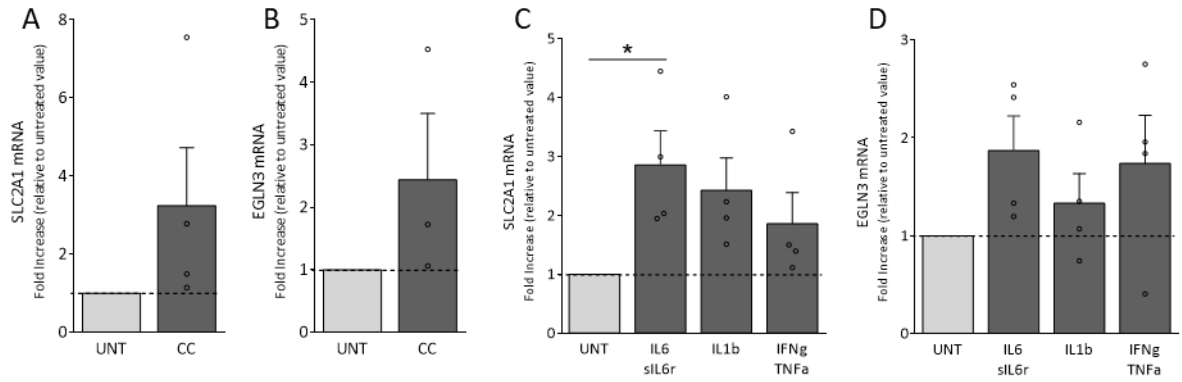


Figure 5. HIF1α is active under a complex inflammatory microenvironment or under specific inflammatory stimuli. (A) SLC2A1 mRNA levels in VSMC alone (UNT) or co-cultured with PBMC (CC) during 48h. (B) EGLN3 mRNA levels in VSMC alone (UNT) or co-cultured with PBMC (CC) during 48h. (C) SLC2A1 mRNA levels in VSMC untreated (UNT) or treated with IL6+sL6R, IL1β or IFNγ+TNFα during 48h. (D) EGLN3 mRNA levels in VSMC alone (UNT) or treated with IL6+sL6R, IL1β or IFNγ+TNFα during 48h. All data is represented in fold increase relative to VSMC-UNT. Bars represent mean ± SEM. *p<0.05 (due to the small number of cases, statistics are only indicative).

HIF1α stabilization effect in VSMC (gene expression analysis)

To investigate the HIF1α role in VSMC and its contribution to GCA pathogenesis, we inhibited the principal protein responsible for HIF1α hydroxylation and subsequent degradation, which is PHD. In order to hydroxylate, PHD needs O_2, Fe^{2+} and 2-oxoglutarate among other molecules. While hypoxia reduces O_2 levels, $CoCl_2$ competes with Fe^{2+} and DMOG competes for 2-oxoglutarate. Thus, we induced HIF1α stabilization using DMOG, $CoCl_2$ or hypoxic conditions. HIF1α protein was present in VSMC under the described conditions (supplementary figure 2). Then we isolate total RNA to analyze the expression of several genes involved in GCA pathogenesis (Table 1).

The molecules included extracellular matrix proteins and growth factors involved in vascular remodeling including angiogenesis, chemokines involved in leuckocyte chemotaxis , and other inflammatory molecules.

SLC2A1 a HIF-1α target gene, was increased indicating the functional activity of HIF1α stabilization under the specified treatments.

Among all tested genes, EDN1, PDGFA, PDGFB, PDGFRA, PDGFRB, CCL2, CCL5, CXCL12, CXCL8, ICAM1, VEGFA, IL6, IL1B, TNFA and CSF2 were modulated. These are important genes in vascular remodeling, cell migration, angiogenesis and inflammation. mRNA levels of CXCL8, ICAM1, VEGFA, IL6, IL1B, TNFA and CSF2 increased in VSMC when HIF1α is stabilized, whereas CCL2, CCL5, CXCL12, EDN1, PDGFA/B and their receptors decreased.

Genes	DMOG/Untreated				CoCl2/Untreated		Hypoxia/Normoxia	
	4h		8h		4h	24h	o/n	24h
	0,5 mM	1 mM	0,5 mM	1 mM	0,2 mM	0,2 mM	1,1% O2	1,1% O2
Vascular remodeling								
MMP2	1,05	0,96	1,06	1,11	1,07	1,19	0,99	0,96
MMP9	0,96	0,96	1,72	1,12	0,99	1,46	1,15	1,50
COL1A1	1,04	0,88	0,87	0,80	**0,63****	0,92	1,13	1,24
COL3A1	0,95	0,90	0,85	0,82	**0,65****	0,85	1,10	1,24
FN1	1,03	1,04	0,89	1,04	0,79	1,03	1,00	1,09
EDN1	**0,41***	0,94	**0,14******	0,47	0,86	**0,53***	0,85	0,99
EDNRA	1,49	1,32	1,73	1,90	1,13	0,87		
EDNRB	0,93	0,90	1,24	1,27	2,23	4,33		
ELN	1,01	0,96	0,78	0,78	0,82	0,66		
FGF2	0,80	**0,54***	0,76	0,57	1,14	0,76		
PDGFA	0,76	**0,47****	0,86	1,04	**0,37****	2,72		
PDGFB	0,75	**0,25***	6,56	7,36	**0,38****	0,97		
PDGFRA	1,00	**0,87***	1,13	0,96	0,87	1,58		
PDGFRB	1,09	1,01	1,08	1,33	0,76	**0,57***		
ITGA5	1,75	1,08	**2,02***	1,94	1,25	3,84		
ITGAV	1,00	0,81	1,01	0,73	0,99	1,27		
ITGB1	1,04	0,89	0,97	0,76	0,92	1,19		
Migration								
CCL2	**0,53***	0,57	0,47	0,76	0,88	**0,70***	**0,63****	0,72
CCL4	0,77	1,06	1,72	1,67	1,13	0,42	0,38	0,59
CCL5	1,19	1,17	1,11	1,40	**0,83****	1,02	1,00	1,11
CXCL9	0,95	0,96	1,16	1,27	0,87	0,8	1,57	1,48
CXCL10	1,14	2,39	1,12	1,90	1,14	0,48	0,57	1,25
CXCL11	0,79	1,12	0,77	1,05	0,68	0,80	0,80	1,36
CXCL12	0,96	0,91	0,91	1,02	**0,62****	**0,49***	0,93	**0,81***
CX3CL1	1,21	1,15	1,51	2,03	1,21	**0,29***	**0,80***	0,82
CXCL8	2,19	2,23	3,04	6,27	**5,22***	3,65		
ROBO2	0,81	**0,76***	1,05	1,34	0,81	**0,44***		
SLIT2	1,00	1,04	0,82	1,08	0,93	1,75		
Angiogenesis								
EGF	0,86	0,85	1,12	1,27	0,99	0,91	0,92	0,89
EGFR	**1,20***	0,95	1,57	1,42	1,20	1,27	1,05	0,98
VEGFA	5,36	3,50	16,30	**12,98***	2,38	**7,43***	4,64	3,68
FLT1	1,17	0,77	1,68	1,03	1,32	2,79	1,04	1,27
IL6	4,32	2,54	8,00	**4,82****	3,20	**2,22***	1,73	1,82
Other genes								
IL1B	1,47	0,58	3,90	2,44	2,16	3,99	1,77	1,7
TNF	1,50	6,69	2,85	7,30	1,58	1,18	0,88	1,64
ICAM1	1,68	1,05	3,91	**2,43****	**2,07***	**1,92***	1,25	1,15
VCAM	0,84	0,92	0,87	1,31	2,87	**0,25***	**0,51*****	0,64
SLC2A1	**10,64****	**7,94****	**24,22*****	**18,64****	**2,55****	**9,72***	9,92	9,42
CSF2	1,07	0,94	2,50	5,15	1,59	1,49	1,13	1,82

Table 1 (previous page). mRNA expression levels of genes implicated in GCA physiopathology in VSMC cultured under different PHD inhibition approaches to stabilize HIF1α. Values represent the mean of 4 independent experiments expressed in fold change relative to untreated value. p-value: *≤0,05 **≤0,01 ***≤0,001 ****≤0,0001 (due to the small number of cases, statistics are only indicative). We run out of sample to test some genes in hypoxia conditions shown as grey boxes.

HIF1α stabilization effect on vascular remodeling in VSMC

HIF1α stabilization in VSMC affect vascular remodeling involved factors

To validate the results obtained in Table 1 the protein expression of some HIF1α regulated genes was assessed in supernatants of VSMC treated with PHD inhibitors. While ET-1 protein concentration decreased in all treated conditions (figure 6 A-C), PDGFA/B protein levels were below the detection threshold in all situations (data not shown).

Previous results of the group reported ET-1 and PDGF as important molecules implicated in VSMC migration (76,84). Therefore, a scratch assay was performed in order to characterize the functional consequences of HIF1α stabilization. This experiment demonstrates that VSMC showed a decreased ability of closing the wound when they have increased levels of HIF1α.

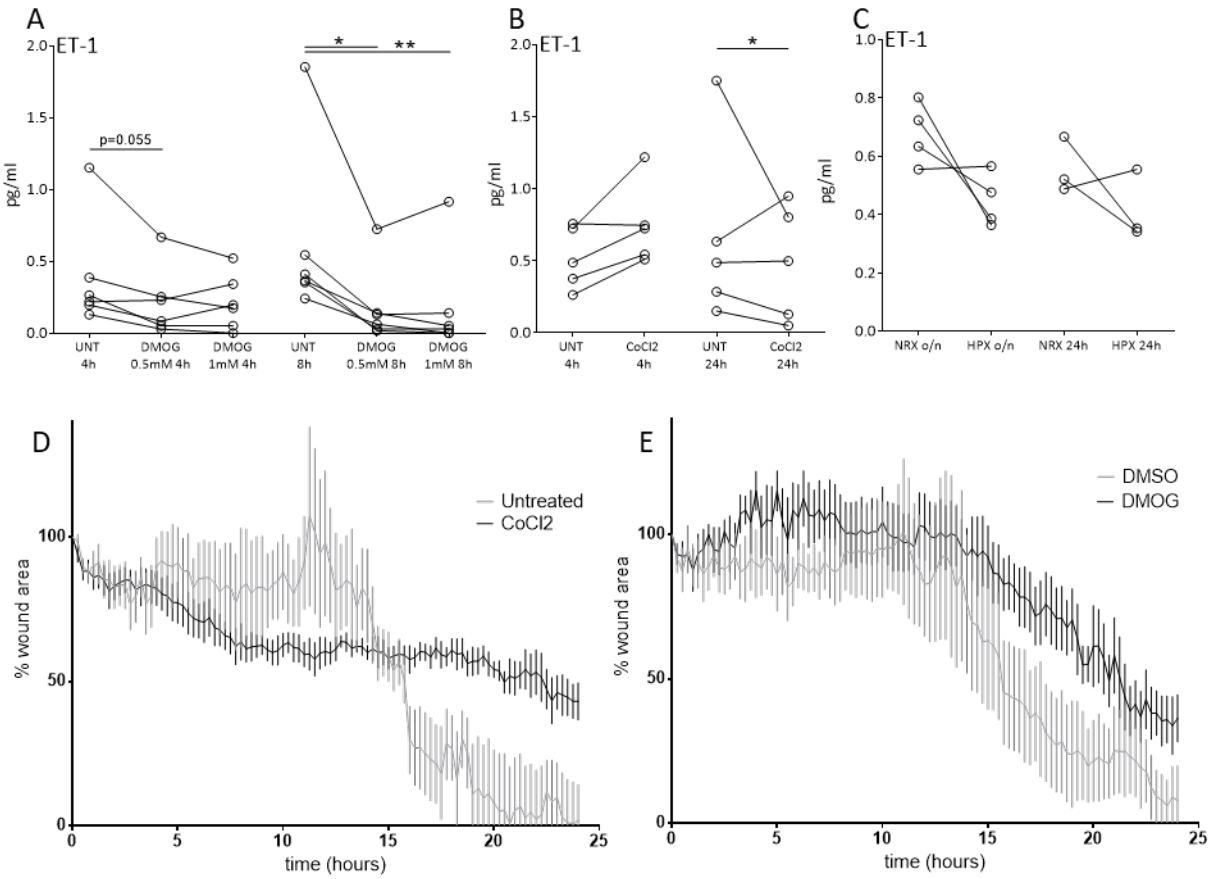


Figure 6. HIF1α stabilization in VSMC promotes ET-1 release and a decrease in wound healing capacities. (A-C) ET-1 protein levels tested by ELISA in supernatants of VSMC treated with DMOG (A), $CoCl_2$ (B) or hypoxia (HPX; C) expressed in pg/ml. (D and E) Wound area evolution over time (24h) after scratch generation in VSMC treated or not with $CoCl_2$ or DMOG, referred to initial scratch area. *p<0,05 **p≤0,01 (due to the small number of cases, statistics are only indicative).

HIF1α stabilization in VSMC affects cell migration molecules

Since some of the molecules modulated in the experiments summarized in table 1 were related to cell migration, cell supernatants of these experiments were tested to investigate concentrations of several chemokines

VSMC treated with inducers of HIF1α stabilization released less CCL2, less CCL5 but more CXCL8 (Figure 7) in accordance with the effects on the corresponding transcripts (Table 1). CXCL12 protein levels were also determined but protein concentrations were below the detection threshold in all situations (data not shown).

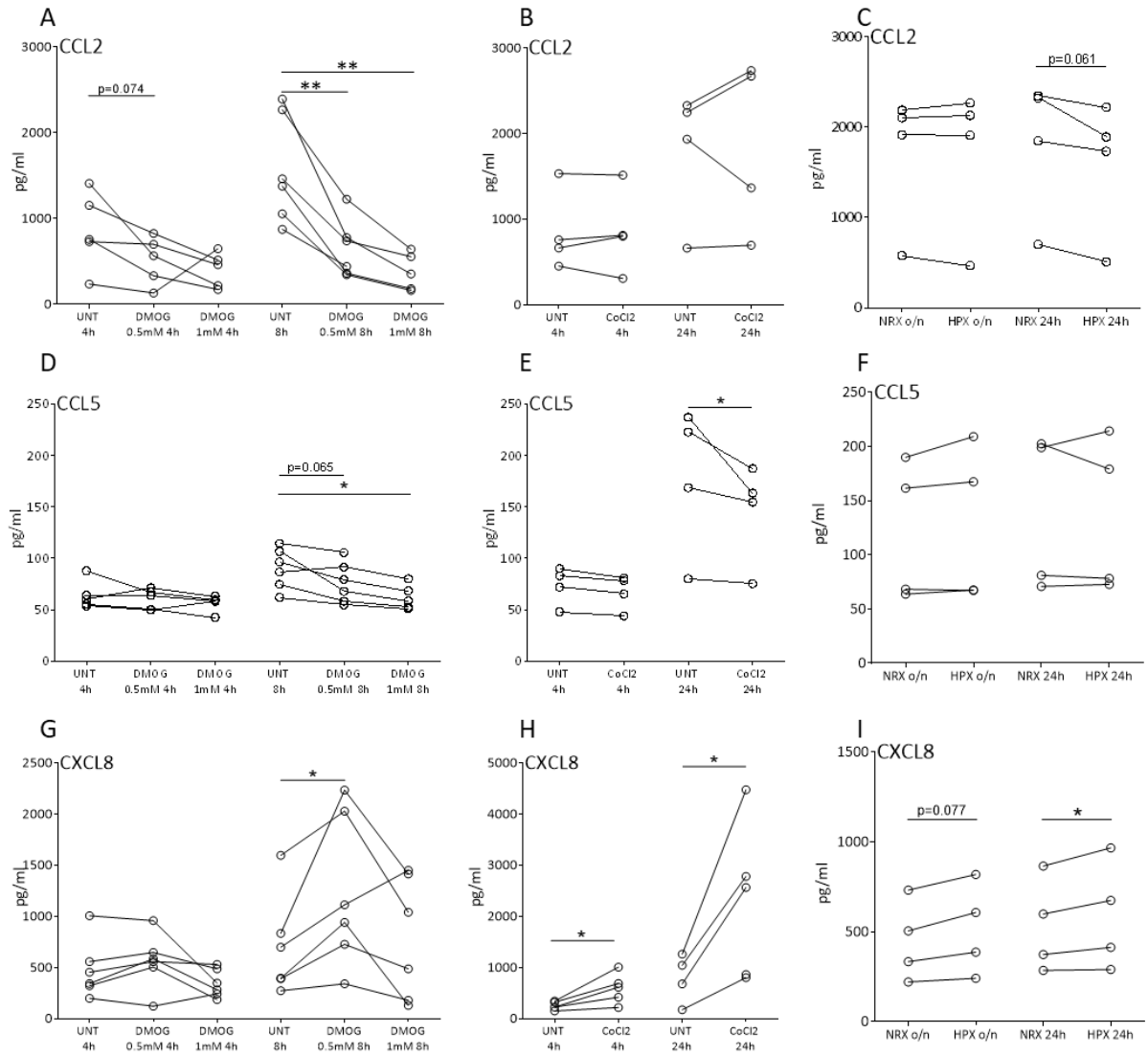


Figure 7. HIF1α stabilization in VSMC promote a decrease in CCL2 and CCL5 but an increase in CXCL8 release. (A-C) CCL2 protein levels tested by ELISA in supernatants of VSMC treated with DMOG (A), $CoCl_2$ (B) or hypoxia (HPX; C) expressed in pg/ml. (D-F) CCL5 protein levels tested by ELISA in supernatants of VSMC treated with DMOG (D), $CoCl_2$ (E) or hypoxia (HPX; F) expressed in pg/ml. (G-I) CXCL8 protein levels tested by ELISA in supernatants of VSMC treated with DMOG (D), $CoCl_2$ (E) or hypoxia (HPX; F) expressed in pg/ml. *p<0,05 **p≤0,01 (due to the small number of cases, statistics are only indicative).

Other HIF1α stabilization effects on VSMC related to angiogenesis and inflammation

Three different angiogenic-related molecules, VEGFA, IL6 and CXCL8 are increased at mRNA level in HIF1α-stabilized VSMC as shown in Table 1. We examined their protein expression in the corresponding supernatants and validated mRNA results at the prtein level (Figure 8 and 7 G-I).

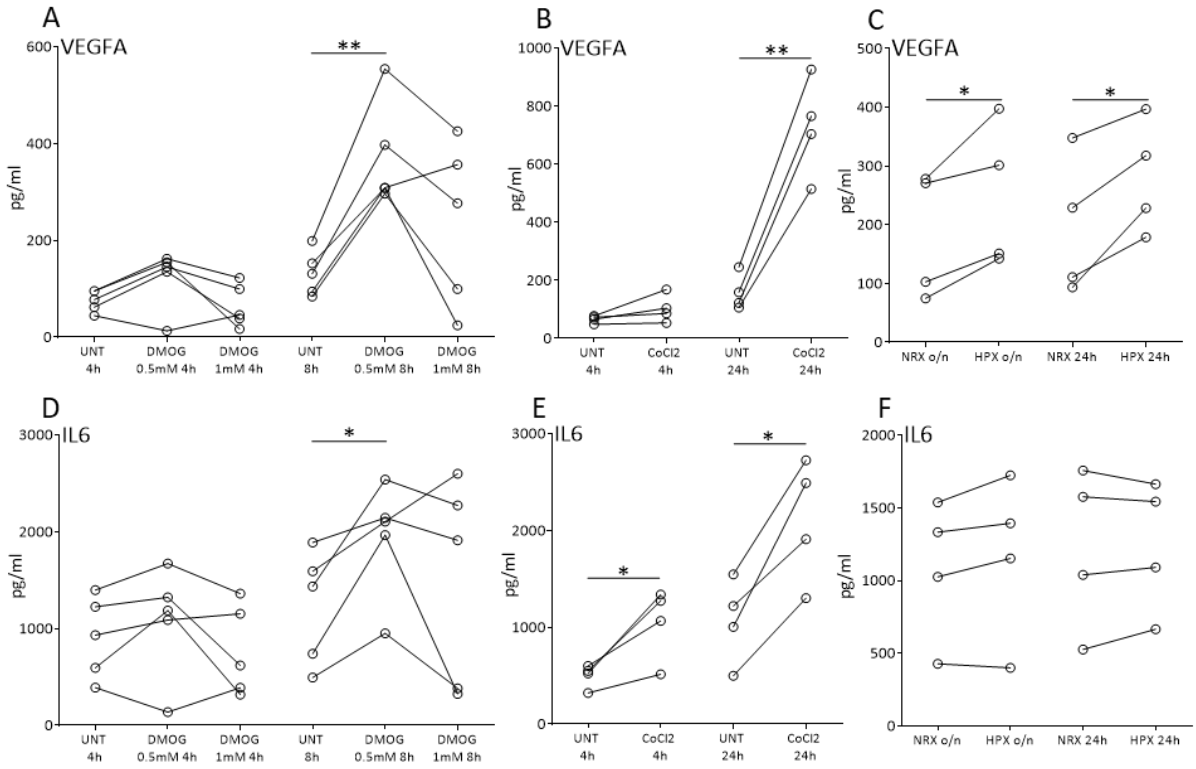


Figure 8. HIF1α stabilization in VSMC promote an increase in angiogenic factors. (A-C) VEGFA protein levels tested by ELISA in supernatants of VSMC treated with DMOG (A), $CoCl_2$ (B) or hypoxia (HPX; C) expressed in pg/ml. (D-F) IL6 protein levels tested by ELISA in supernatants of VSMC treated with DMOG (D), $CoCl_2$ (E) or hypoxia (HPX; F) expressed in pg/ml. *p<0,05 **p≤0,01 (due to the small number of cases, statistics are only indicative).

An adhesion related gene, ICAM1, also showed increased mRNA levels in VSMC with PHD inhibitors. However, when protein extracts from VSMC treated under the HIF1α stabilization conditions explained before were analyzed, no differences were observed among them (figure 9). The supernatants of these cells were also checked for soluble ICAM1, but the protein levels were below the sensitivity of the ELISA (data not shown).

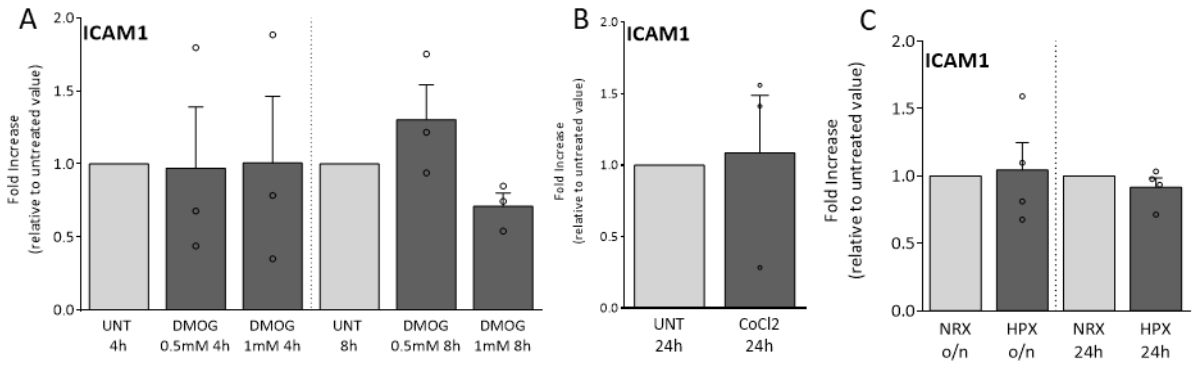


Figure 9. HIF1α stabilization in VSMC do not promote changes in adhesion molecules. (A-C) ICAM1 tested by western blot and normalized with β-actin levels represented in fold increase relative to untreated value in total protein extracts from VSMC treated with DMOG (A), $CoCl_2$ (B) or hypoxia (HPX; C).

Effects of blocking specific cytokines on HIF1α expression under an inflammatory microenvironment

In order to translate these results from bench to bedside, we sough to determine the effect of inhibiting specific cytokines for GCA, on HIF1α expression and stability in VSMC alone or co-cultured with PBMC as a model of vascular inflammation.

We tested the following neutralizing monoclonal antibodies: tocilizumab (anti-IL6R), infliximab (anti-TNFα), anakinra (a recombinant IL1 receptor antagonist), mab285 (anti-IFNγ) and mavrilimumab (anti-GM-CSFRα).

While HIF1A mRNA levels did not change under any treatment (figure 10A), HIF1α protein decreased in some situations (figure 10B). In the conditions used in our experiments, blocking IL-6 receptor with tocilizumab did not have any effect on HIF1α protein (figure 10 C). However, infliximab (figure 10 D), anakinra (figure 10 E) or mavrilimumab (figure 10 G) induced a significant decrease in HIF1α protein. Likewise mab-285 (figure 10 F) also led to a decrease in HIF1α protein but to a lesser extent.

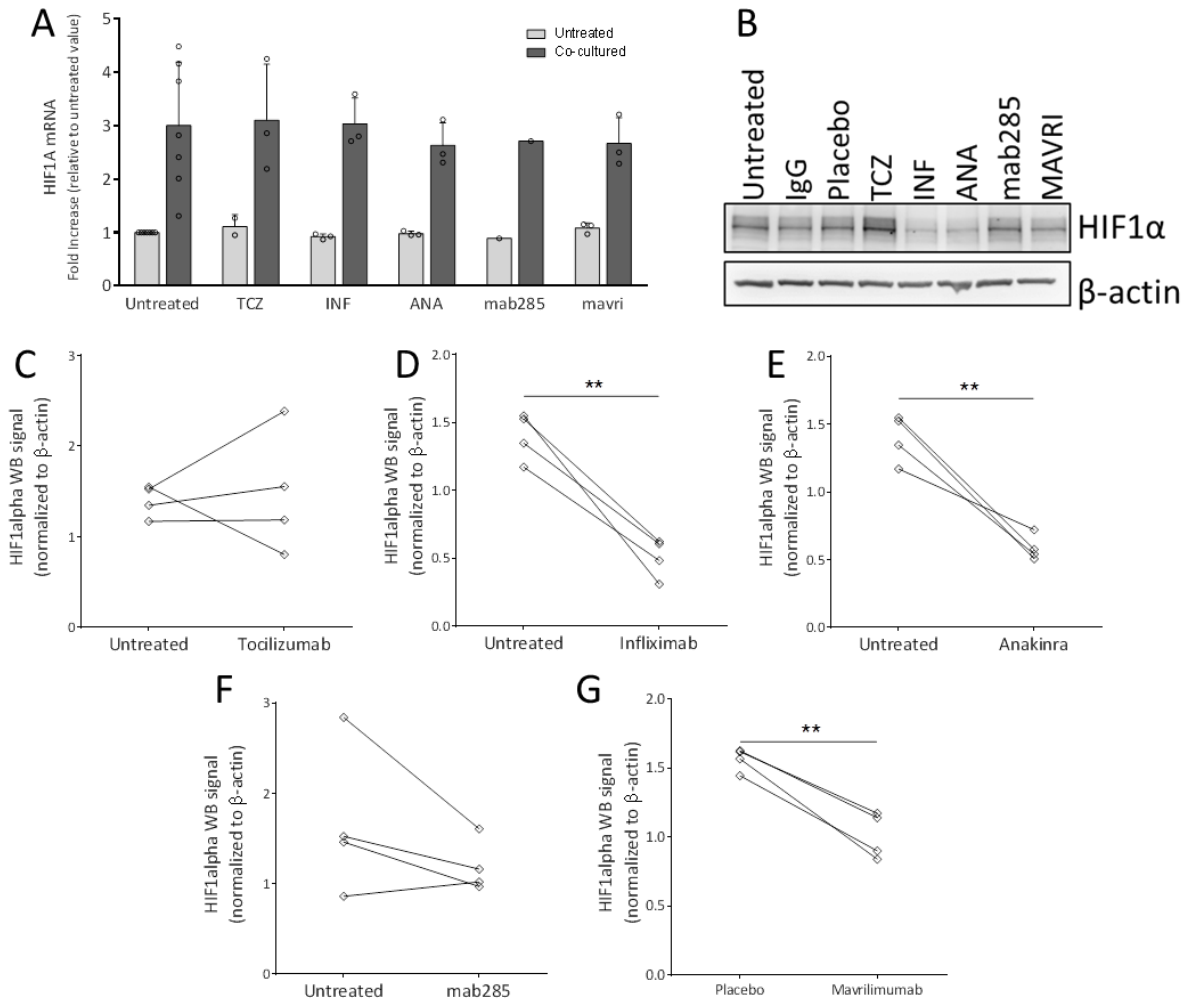


Figure 10. Effect of anti-cytokine targeted therapies on HIF1α stabilization. (A) HIF1A mRNA levels in VSMC alone (UNT; light grey) or co-cultured with PBMC (CC; dark grey) and untreated or treated with tocilizumab (TCZ), infliximab (INF), anakinra (ANA), mab285 (an anti-IFNγ monoclonal antibody) or mavrilimumab (mavri) during 48h. (B) HIF1α protein determined by western blot in VSMC co-cultured with PBMC and treated or not with, tocilizumab (TCZ), infliximab (INF), anakinra (ANA), mab 285 (an anti-IFNγ monoclonal antibody) and mavrilimumab (MAVRI) during 48h. (C-G) Quantification of HIF1α tested by western blot (B) and normalized with β-actin levels in total protein extracts from VSMC co-cultured with PBMC. **p≤0,01; (due to the small number of cases, statistics are only indicative).

GM-CSF and GM-CSFRα expression is increased in GCA lesions

Since inhibiting GM-CSF effect with mavrilimumab affects HIF1α protein levels and GM-CSF is a key player in inflammation we decided to further explore this line of research. Moreover, recently a phase II clinical trial with mavrilimumab in GCA patients has been completed with satisfactory results (112), and GM-CSF is gaining interest in GCA pathogenesis (168,169). Therefore, GM-CSF effect on GCA pathogenesis and its implication with hypoxia pathway were further characterized.

RT-PCR analysis of GM-CSF and GM-CSFRα showed that these transcripts were increased in homogenized temporal artery biopsies from patients with GCA, whereas GM-CSF mRNA was virtually undetectable, and GM-CSFRα expression was very low in control arteries (figure 11 A,B). Likewise, GM-CSF or GM-CSFRα mRNA were clearly detectable by in situ RNA hybridization in all arterial layers of GCA biopsies, whereas virtually no signal for either gene product was detectable in control arteries (figure 11 C–E). Finally, immunostaining confirmed the presence of GM-CSF and GM-CSFRα protein on infiltrating inflammatory cells and endothelial cells in GCA arteries. In contrast, no GM-CSF protein and only low levels of GM-CSFRα protein were detected in control arteries (figure 11 F,G).

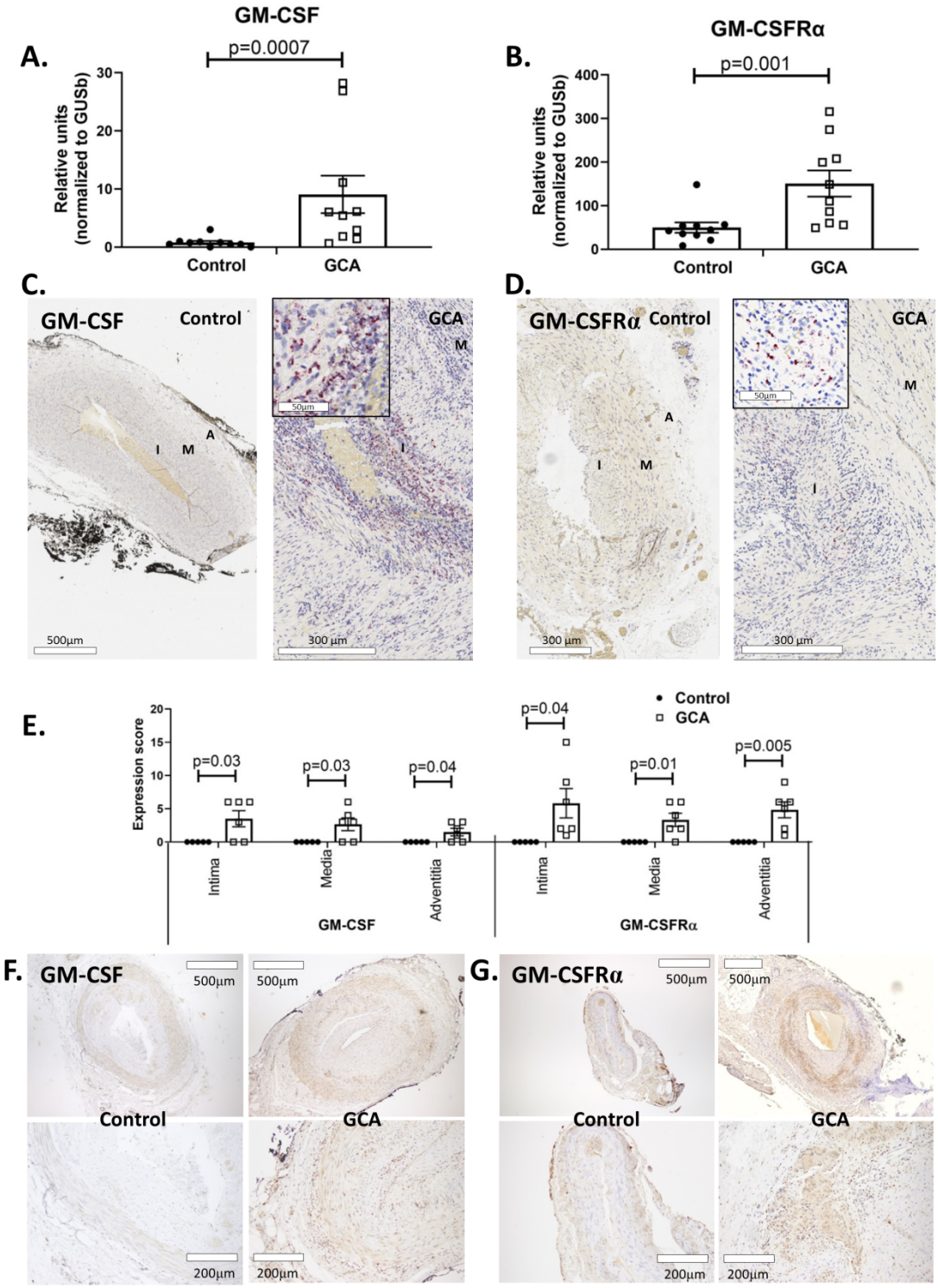
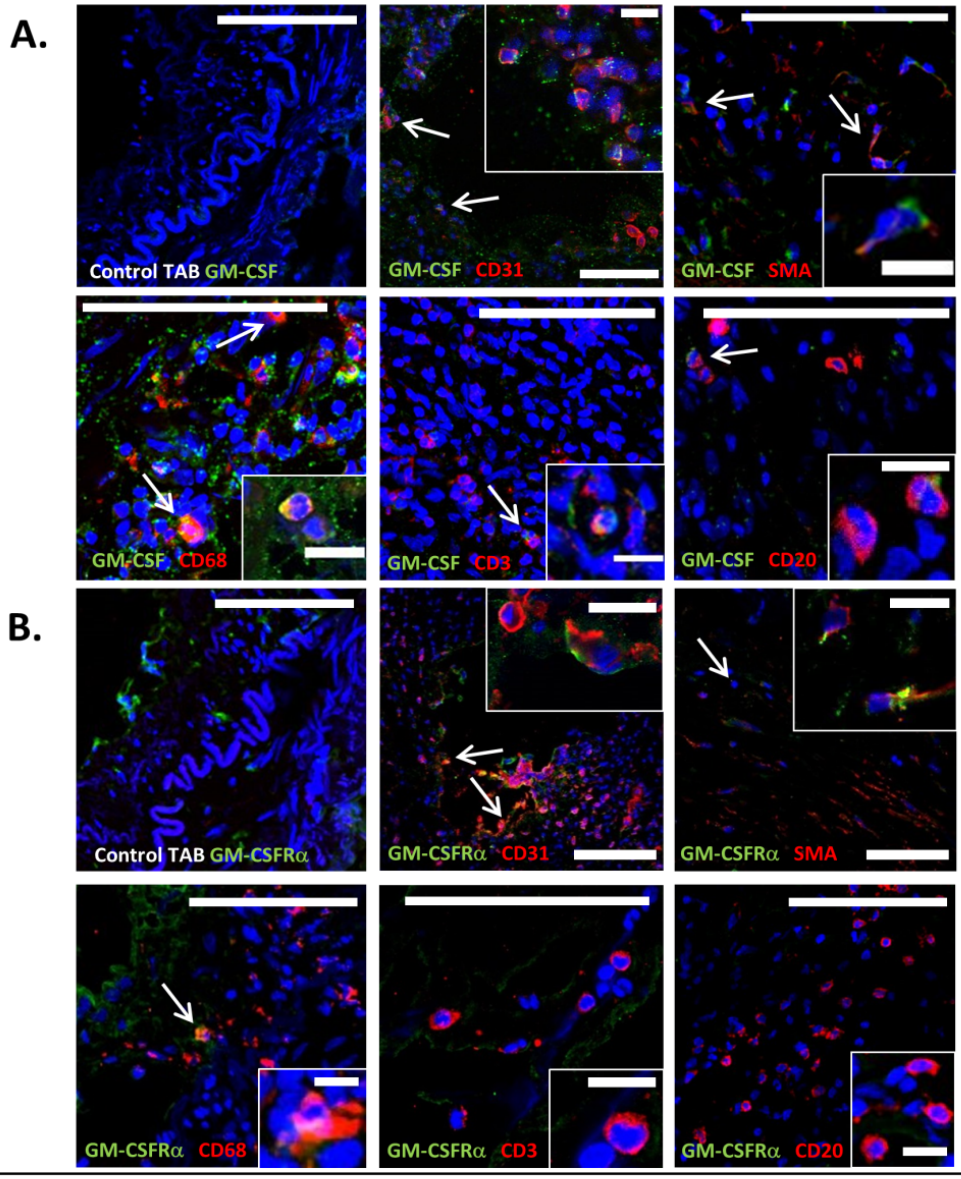


Figure 11. GMCSF and GM-CSFRα expression in GCA lesions. Concentrations of GMCSF (A) and GM-CSFRα mRNA (B) in fresh-frozen histologically negative arteries (controls) (n=10) versus GCA-positive arteries (n=10). GM-CSF (C) and GM-CSF-Rα (D) RNA hybridization signals (red dots) on control temporal arteries and GCA-involved arteries. I: Intima layer; M: Media layer; A: Adventitia layer. (E) Quantitation of RNAscope signal (expression score) in different arterial layers in 6 GCA-involved and 5 control arteries. Immunostaining with anti-GM-CSF (F) and anti-GM-CSF-Rα (G) antibodies (brown colour) of FFPE normal or GCA-involved arteries (representative of 5 controls and 12 GCA arteries).

Next, cell subsets potentially expressing GM-CSF and GM-CSFRα in GCA lesions were explored. As illustrated by immunofluorescence in figure 12, GM-CSF was mainly observed in macrophages and luminal endothelial cells and, to a lesser extent, in T cells, intimal myofibroblasts, and endothelial cells from vasa vasorum and neovessels. GM-CSFRα was detected mainly in macrophages, giant cells, endothelial cells and intimal myofibroblasts.

Serum GM-CSF concentration at diagnosis was 0.061±0.02 pg/mL (average ±SEM) in patients with GCA and 0.035±0.02 pg/mL in controls (p=0.889).



C.

	Control		GCA	
	GM-CSF	GM-CSFRα	GM-CSF	GM-CSFRα
Luminal endothelium	-	+	+++	++
Neovessels / Adventitial endothelium	-	-	+	+
VSMC	-	-	+	+/-
Neointimal myofibroblasts	-	-	++	++
Macrophages	-	-	+++	++
B cells	-	-	+	+/-
T cells	-	-	++	-

Figure 12 (previous page). GM-CSF and GM-CSF-Rα expression by immune and resident cells. Merged double immunofluorescence staining with anti-GM-CSF (A) or anti-GM-CSF-Rα (B) antibodies (both in green) and anti-cell markers CD68 (macrophages), CD31 (endothelial cells), CD3 (T lymphocytes), CD20 (B lymphocytes), SMA (smooth muscle actin identifying vascular smooth muscle cells and myofibroblasts), (all in red) of fresh-frozen temporal arteries from patients with GCA or controls (first panel). Nuclei are stained with DAPI (blue). Co-expression (orange/yellow) is pointed with arrows and insets show magnified double-positive cells (Scale bars in figures measure 100μm and 15μm for insets). (C) Summary panel of GMCSF and GM-CSF-Rα expression by different cell types in 3 GCA-involved temporal arteries detected by immunofluorescence as in A and B. (+++): 50-100% positive cells; (++): 20-40% positive cells; (+): less than 20% cells; (+/-): scattered cells; (-): negative.

GM-CSF receptor-driven signaling pathways are activated in GCA lesions, and expression of molecules regulated by this pathway is increased

After observing higher expression of GM-CSF and GM-CSFRα in GCA-involved arteries, signaling molecules downstream of GM-CSFR were examined. As shown in figure 13 A,B and supplementary figure 3, JAK2 and STAT5A, the main signaling proteins activated by GM-CSFR engagement, were phosphorylated in GCA lesions, and transcripts regulated by STAT5, such as Spi1 (PU.1) and CD83, were significantly increased in GCA arteries (figure 13 C–G). CD83 and PU.1 protein, absent in controls, were clearly expressed in GCA arteries (figure 13 H,I). PU.1 was detected in the nuclei, consistent with its function as transcription factor and suggestive of nuclear translocation on activation of upstream signaling. CD83 staining was more diffuse, possibly due to detection of its soluble form in addition to the membrane molecule.

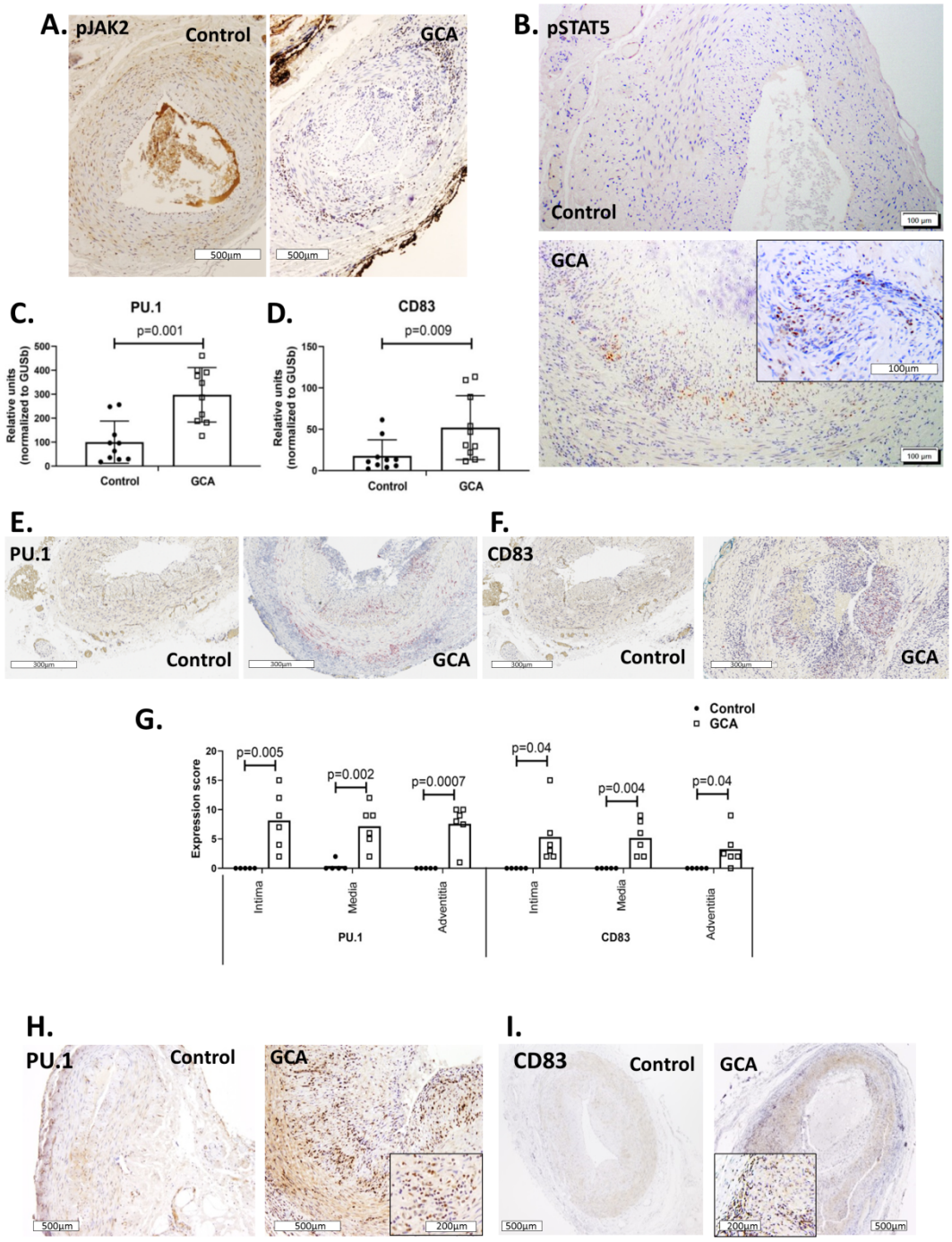


Figure 13. Activation of GM-CSF R-driven signaling pathways and target gene expression in GCA lesions. (A-B) Immunostaining of histologically negative temporal artery biopsies (control) and GCA-involved arteries with anti-phospho-JAK2 (A) or anti-phospho-STAT5 (B) antibody (brown colour). Representative of 12 GCA and 5 control arteries. (C) mRNA concentrations of PU.1 (C) and CD83 (D), in fresh-frozen control and GCA arteries (n=10 each group). (E-G) PU.1 (E) and CD83 (F) RNAscope images with positive red staining on control (N=5) and GCA temporal arteries (N=6), with their corresponding quantitation (G) in the intima, media, and adventitia layers of the artery wall. (H-I) Immunohistochemistry with anti-PU.1 (H) and anti-CD83 (I) antibodies on FFPE control and GCA arteries (brown). Representative of 12 GCA arteries and 5 controls. Magnification of each figure is indicated individually.

GM-CSFRα inhibiting monoclonal antibody, mavrilimumab, effects on GCA lesions

Mavrilimumab reduces lymphocyte and myeloid cell markers in *ex-vivo* cultured arteries from patients with GCA

To determine the contribution of GM-CSF to the above results and to assess the effects of GM-CSF pathway blockade on vascular inflammation, GCA arteries were cultured with anti-GM-CSFRα mavrilimumab for 5 days. Compared with placebo, treatment with mavrilimumab resulted in reduced phospho-STAT5 in lesions (figure 14 A,B) and in lower mRNA expression of Spi1 (PU.1), a transcription factor that, along with STAT5, mediates GM-CSF effects (figure 14C). Furthermore, treatment with mavrilimumab resulted in significantly lower mRNA levels for T cell marker CD3ε, B cell marker CD20, monocyte marker CD14 and myeloid cell marker CD16 mRNAs (figure 14D). By contrast, no consistent changes were observed with transcripts for the macrophage marker CD68. Accordingly, fewer CD16+ and CD3ε+ infiltrating cells and no change in CD68+ cells were observed by immunofluorescence (figure 14F). The reduction in CD20 transcripts, however, did not result from decreased numbers of B cells in tissue during the duration of mavrilimumab exposure (figure 14 E,F).

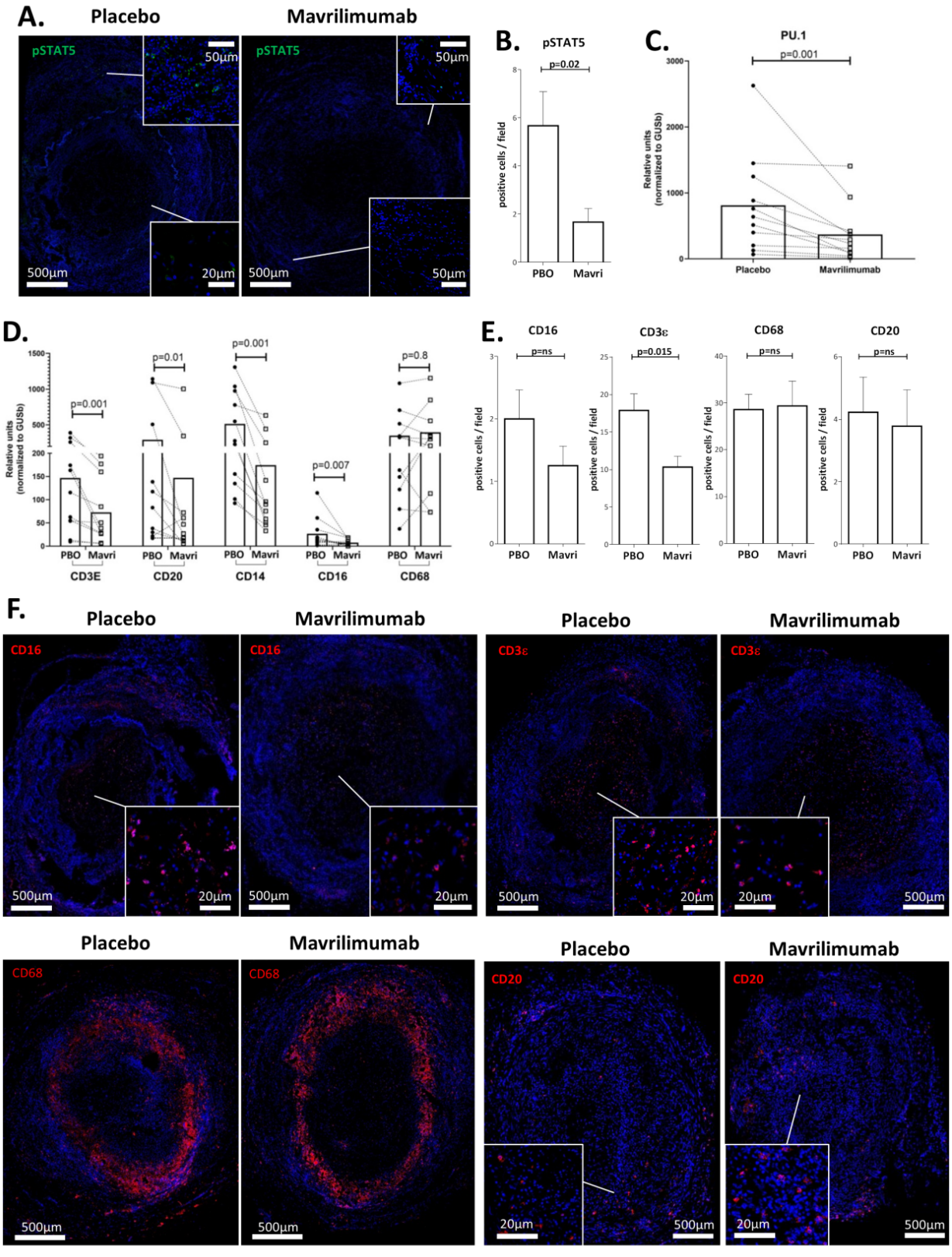

A.
Placebo
Mavrilimumab
pSTAT5
50µm
500µm
20µm
B.
pSTAT5
p=0.02
positive cells / field
PBO
Mavri
C.
PU.1
p=0.001
Relative units (normalized to GUSb)
Placebo
Mavrilimumab
D.
p=0.001
p=0.01
p=0.001
p=0.007
p=0.8
Relative units (normalized to GUSb)
PBO Mavri
CD3E
CD20
CD14
CD16
CD68
E.
CD16
CD3ε
CD68
CD20
p=ns
p=0.015
positive cells / field
PBO
Mavri
F.
Placebo
Mavrilimumab
CD16
CD3ε
CD68
CD20
500µm
20µm

Figure 14 (previous page). Effect of mavrilimumab on inflammatory infiltrates in ex vivo cultured arteries from patients with GCA. (A) Immunofluorescence staining with anti-phospho-STAT5 antibody (green) of a GCA artery cultured with placebo or mavrilimumab. (B) Quantification of positive cells per field A; this experiment was performed three times with similar results. (C) mRNA SpI1/ PU.1 transcripts in 11 cultured GCA affected temporal arteries in the presence of placebo or mavrilimumab. (D) Transcripts of cell markers CD3ε, CD20, CD14, CD16 & CD68 in 11 cultured GCA-involved temporal arteries exposed to placebo or mavrilimumab. (E-F) Immunofluorescence staining (F) of cultured GCA-involved arteries in the presence of placebo or mavrilimumab with anti-CD16, anti-CD3ε, anti-CD68, anti-CD20 (red color) and DAPI (blue); Quantification (E) of positive cells per field F. Representative of 3 GCA cultured arteries.

Mavrilimumab reduces expression of molecules involved in T cell activation and related to the Th1 differentiation pathway in *ex-vivo* cultured arteries from patients with GCA

To further delineate the effects of mavrilimumab, expression of HLA-DR and CD83, relevant molecules to antigen presentation and T cell activation, was examined. Mavrilimumab significantly reduced HLA-DR and CD83 transcripts (figure 15A). Interestingly, concentration of the soluble, shed form of CD83, with counter-regulatory functions, did not decrease in the supernatant (figure 15A). HLA-DR reduction was also observed at the protein level (figure 15A).

To determine whether these effects resulted in decreased differentiation of T cells towards the Th1 or Th17 lineage, select markers were explored. Transcripts of master regulators of Th1 and Th17 differentiation, TBX21 (T-bet) and RORC (RORγ), respectively, trended lower. Cytokines/chemokines related to Th1 differentiation pathway (interferon-γ (IFNγ) and CXCL10) trended lower (mRNA level) or were significantly lower (protein level) (figure 15B). IL-17A mRNA was virtually undetected in cultured arteries, and IL-23p19 had disparate response among donors (figure 15C).

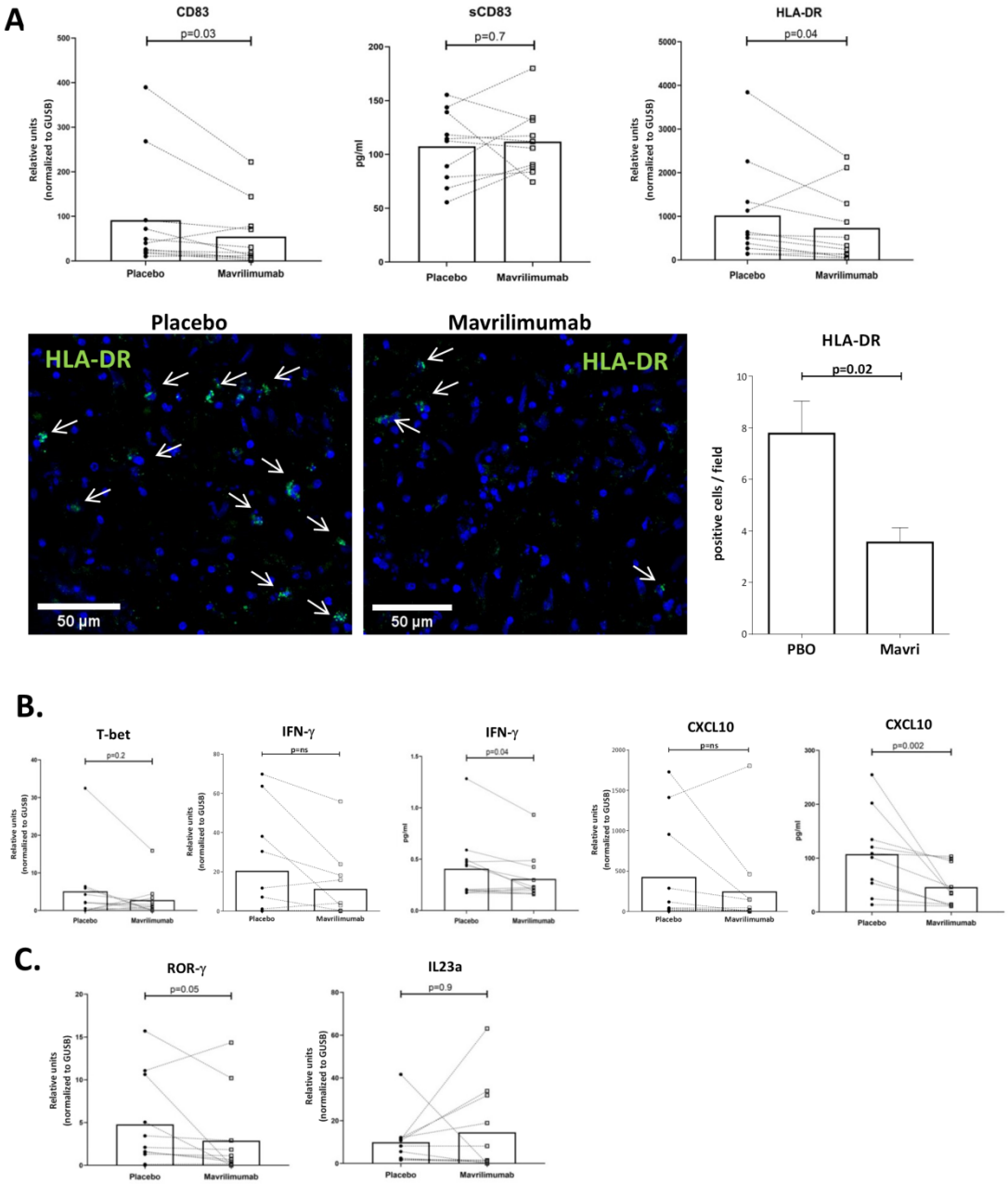


Figure 15. Mavrilimumab decreases molecules related to T lymphocyte activation and differentiation. (A) mRNA transcripts of CD83 (left) and HLA-DR (right) in GCA positive temporal arteries (n=11) cultured with placebo or mavrilimumab. Soluble CD83 (sCD83) measured (pg/ml) in supernatants of 9 GCA cultured arteries exposed to placebo or mavrilimumab (central panel). Image below shows HLA-DR expression by immunofluorescence in a GCA artery cultured with placebo or mavrilimumab. Images show a detailed amplification with arrows indicating green HLA-DR positive cells. Nuclei are stained with DAPI (blue). The graph on the right show the number of HLA-DR positive cells per field in 9 fields per section. Immunofluorescence was performed in 2 GCA cultured arteries, with consistent results. (B) mRNA Transcripts of TBX21 (T-bet), IFNG (IFN-γ), and CXCL10 in GCA arteries cultured with placebo or mavrilimumab (n=11). IFN-γ and CXCL-10 proteins were also measured in artery culture supernatants of the same specimens. (C) RORC (ROR-γ) and IL-23a mRNA measurement in cultured GCA arteries treated with placebo or mavrilimumab.

Mavrilimumab decreases pro-inflammatory cytokines in *ex-vivo* cultured arteries from patients with GCA

Mavrilimumab elicited a significant reduction in the production and release of pro-inflammatory cytokines IL-6, TNFα and IL-1β, mostly but not exclusively produced by macrophages (figure 16A). Mavrilimumab also decreased markers of M2-like phenotype, including the mannose receptor CD206 and the scavenger receptor CD163 (figure 16B). A trend towards an increase in the anti-inflammatory cytokine IL-10 (mRNA and protein) was also observed (figure 16B).

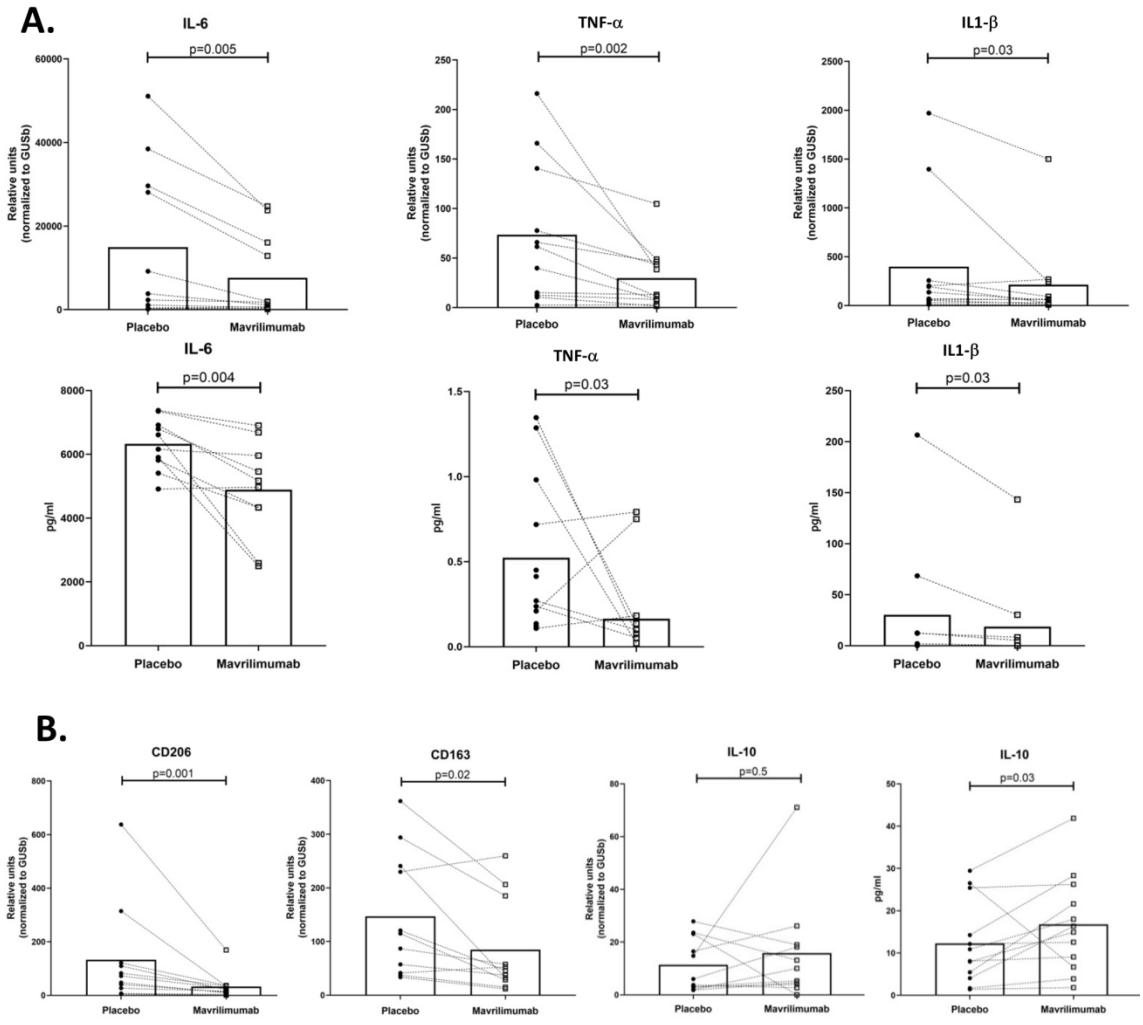


Figure 16. Mavrilimumab impacts macrophage functions. (A) Transcripts of IL6 (left), TNFA (central) and IL-1B (right) in GCA positive arteries (n=11) exposed to placebo or mavrilimumab (mRNA, relative units). IL-6, TNFα and IL-1β proteins (pg/mL) were also measured in GCA artery-culture supernatants of the same samples. (B) CD206, CD163 and IL-10 mRNA transcripts in the same GCA arteries exposed to mavrilimumab or placebo. IL-10 protein (pg/mL) was also detected in the supernatant (right panel).

Mavrilimumab decreases mediators of vascular injury in ex vivo cultured arteries from patients with GCA

Mavrilimumab decreased transcript and protein concentrations of the elastinolytic MMP-9, whereas mRNA and protein of its natural inhibitor TIMP-1 remained unchanged, resulting in a significant decrease in proteolytic MMP-9/TIMP-1 balance (figure 17 A,B). Mavrilimumab also reduced oxidative damage, as demonstrated by decreased presence of lipid peroxidation products (4-hydrynonenal (HNE) protein adducts) in cultured arteries exposed to mavrilimumab as compared with placebo (figure 17C). NOS2 (iNOS) mRNA expression also trended lower (figure 17D).

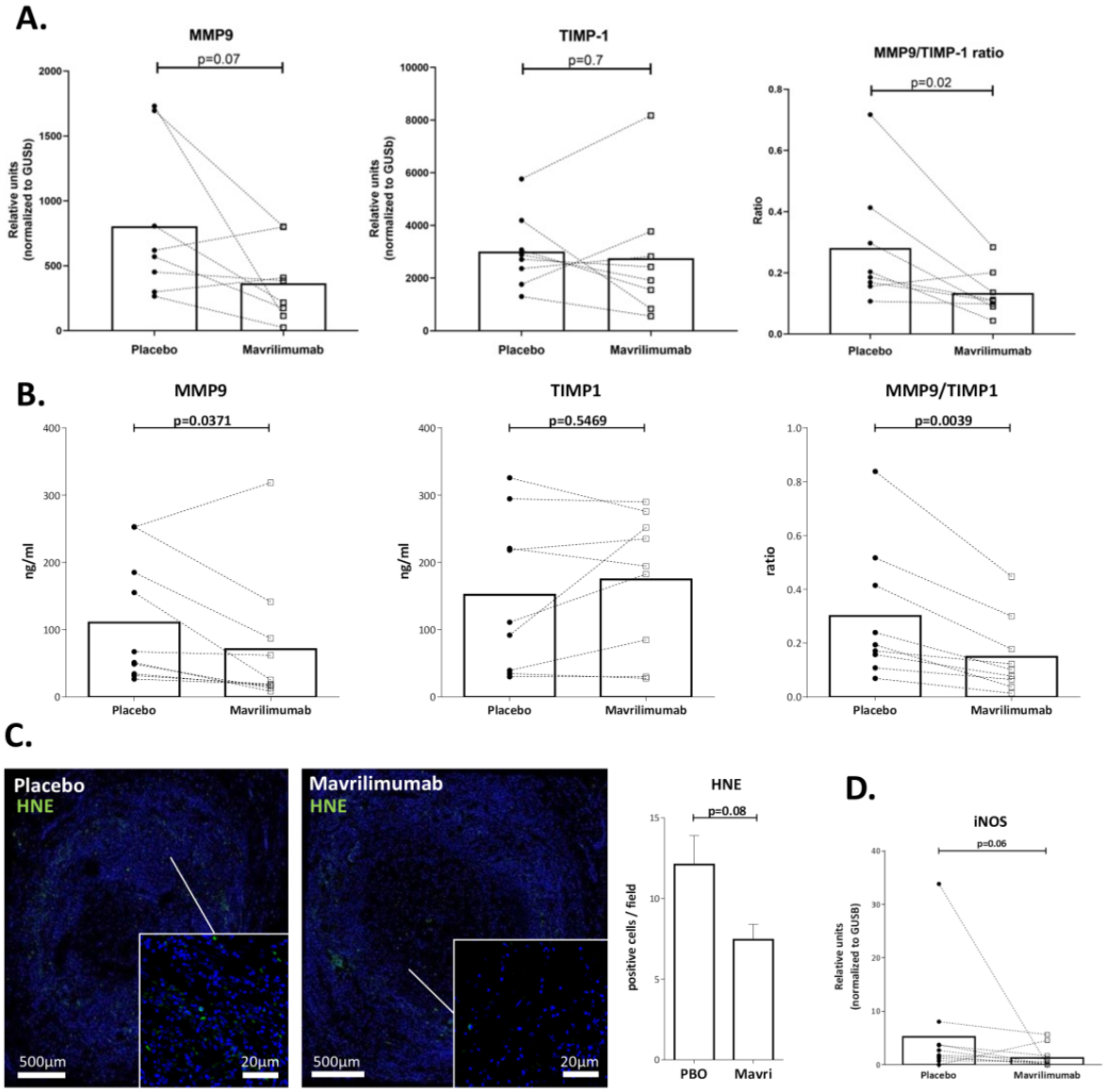


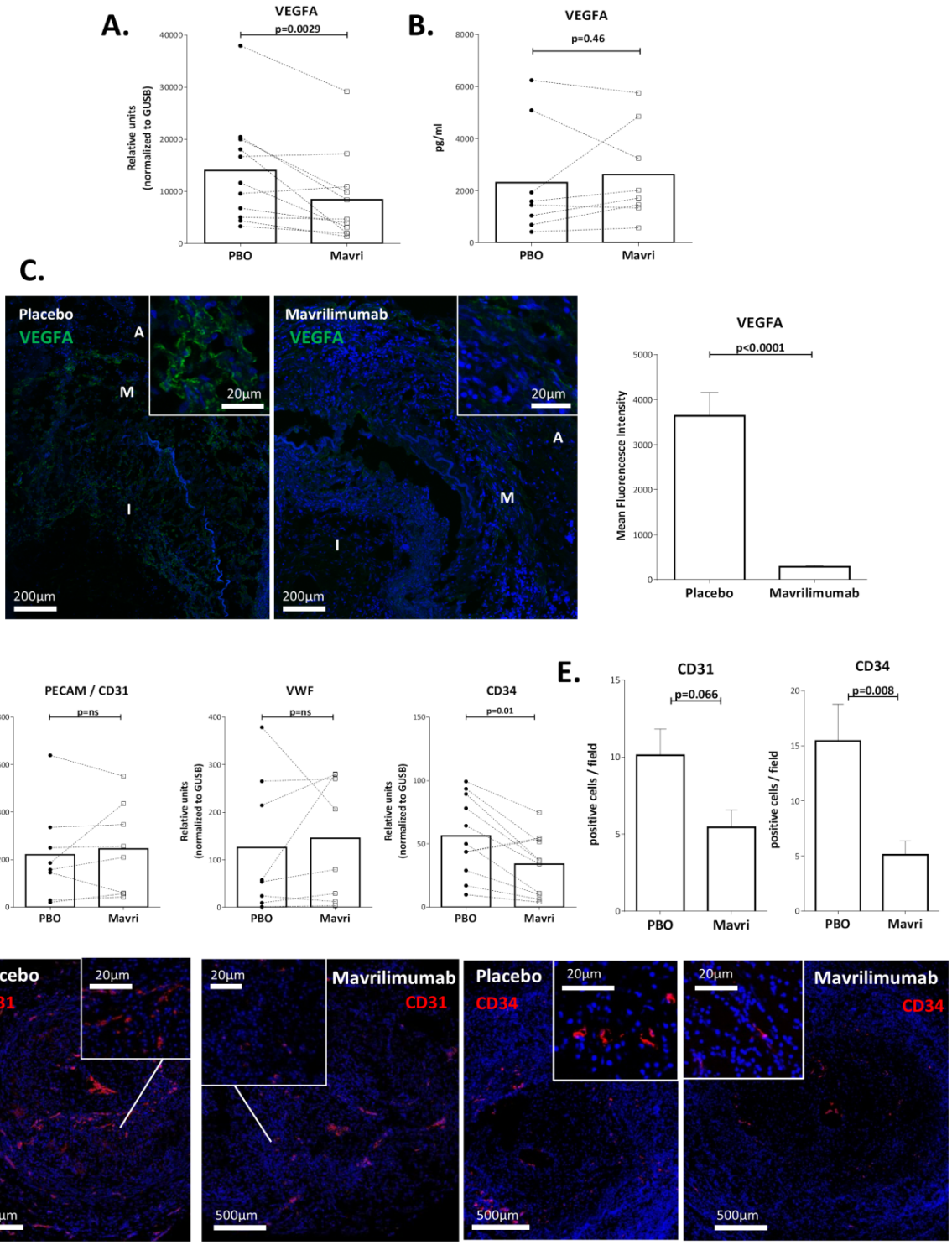
Figure 17. Effect of mavrilimumab on molecules related to vascular injury. (A) Transcripts of MMP-9, TIMP-1 and MMP9/TIMP1 RNA-ratio in 8 GCA positive temporal arteries cultured with placebo or mavrilimumab. (B) MMP9, TIMP1 protein concentration and MMP9/TIMP1 protein-ratio in the corresponding supernatants (ng/mL). (C) Immunofluorescence staining of HNE (green) with nuclei (in blue) in a GCA-involved artery cultured with placebo or mavrilimumab, and its quantitation (right panel); Immunofluorescence was performed in 2 GCA cultured arteries, with consistent results. (D) NOS2 transcripts in 11 cultured GCA arteries exposed to placebo or mavrilimumab.

Mavrilimumab reduces tissue angiogenesis in ex-vivo cultured arteries from patients with GCA

Mavrilimumab reduced VEGFA mRNA in cultured arteries and VEGFA protein expression in tissue by immunofluorescence (figure 18A–C). However, no changes in VEGFA protein in the supernatant was observed, possibly due to its matrix-binding capacity and its autocrine/paracrine function (170). Based on the reduction of this important angiogenic factor, we explored the effects of mavrilimumab on endothelial cell markers and angiogenesis. Mavrilimumab did not elicit changes in constitutive endothelial cell marker vWF or CD31 mRNAs but a decrease in CD34 mRNA, expressed by neovessels and haematopoietic stem cells (HSC) was observed (figure 18D). Immunofluorescence showed a reduction in CD31+ and CD34+ neovessels within inflammatory lesions on exposure to mavrilimumab (figure 18 E,F). Scattered CD34+ cells not aligned around a lumen were also observed in lesions and were reduced by mavrilimumab.

To corroborate these and the previous results obtained with mavrilimumab in the *ex vivo* model we cultured one GCA temporal artery biopsy with rhGM-CSF and we obtained opposite results (supplementary figure 4).

Figure 18. Mavrilimumab effect on angiogenesis (following page). (A) Detection of VEGFA transcripts in 11 GCA positive temporal arteries cultured with placebo or mavrilimumab. (B) Detection of VEGFA protein (pg/mL) in supernatants of 8 respective cultured arteries. (C) Immunofluorescence with anti-VEGFA antibody of a GCA artery cultured with placebo or mavrilimumab (I:intima, M:media, A:advetitia). The graph on the right shows quantification of mean fluorescence intensity of the entire artery wall. (D) Measurement of PECAM-1 (n=8), VWF (n=8) and CD34 (n=11) transcripts in GCA cultured temporal arteries treated with placebo or mavrilimumab. (E) Quantification (positive cells per field) of immunofluorescence. Immunofluorescence was performed on 2 cultured biopsies with consistent results. (F) Immunofluorescence with anti-CD31 or anti-CD34 antibody of a GCA artery cultured with placebo or mavrilimumab.


A.
VEGFA
p=0.0029
Relative units (normalized to GUSB)
PBO
Mavri
B.
VEGFA
p=0.46
pg/ml
PBO
Mavri
C.
Placebo
VEGFA
A
M
I
20µm
200µm
Mavrilimumab
VEGFA
VEGFA
p<0.0001
Mean Fluorencesce Intensity
Placebo
Mavrilimumab
D.
PECAM / CD31
p=ns
VWF
p=ns
CD34
p=0.01
Relative units (normalized to GUSB)
E.
CD31
p=0.066
CD34
p=0.008
positive cells / field
F.
Placebo
CD31
Mavrilimumab
CD31
Placebo
CD34
Mavrilimumab
CD34
500µm

HIF1α and GM-CSF have inter-related mechanisms in GCA lesions

Blocking GM-CSF signaling pathway through the inhibition of its specific receptor GM-CSRα, with mavrilimumab, showed effects on HIF1α itself and some of its downstream target genes. More in detail, in figure 10G we show the effect of GM-CSF blockade decreasing in HIF1α protein levels in VSMC exposed to an inflammatory microenvironment. In the *ex-vivo* model of GCA mavrilimumab decreases HIF1α target genes such as IL6, IL1β, and TNFα as shown in figure 16 A.

To further support the interplay between HIF1α and GM-CSF it is important to highlight that several HIF1α inflammatory inducers are decreased in GCA lesions under the effect of mavrilimumab such as IL6, IL1β, TNFα and IFNγ (figure 16A and figure 15B).

6

Discussion

In the present study we have analyzed the contribution of hypoxia inducible factor, HIF1α, to GCA pathophysiology and the effect of GM-CSFR blockade in its stabilization and downstream effects. In accordance with other similar autoimmune diseases like rheumatoid arthritis (150,171), hypoxia was detected in GCA lesions and may play an important role in vascular inflammation and remodeling.

HIF1A is increased at mRNA level in temporal artery lesions from patients with GCA compared with normal arteries. Even though this difference is statistically significant there are an important number of patients with HIF1A mRNA levels similar to controls. In order to unravel the reason could be interesting to correlate HIF1A mRNA levels with some histopathological data such as angiogenesis or intimal hyperplasia which may compensate or increase hypoxia in the vessel wall, respectively. However, the number of biopsies included in the present work is too small to study possible correlations between this result and patient or sample characteristics. It is important to take into account that the control temporal artery biopsies are not from healthy donors, they are from patients with suspected GCA but without inflammation in the tissue and with alternative diagnosis, frequently with a systemic inflammatory reaction. This is a limitation of the study and affects all experiments where temporal artery biopsies are used. Even though our control cohort was not from healthy controls, we were able to see differences between GCA and non-GCA tissue. While 40% of control temporal arteries show presence of HIF1α in the tissue, all GCA samples expressed it. The biopsy procedure includes ischemia/reperfusion phenomena derived from clamping and ligation of collateral branches that may affect the levels of hypoxia and/or HIF1α itself, showing an aberrant presence in control arteries. HIF1α pattern in the lesions is heterogeneous throughout the artery wall. HIF1α is mainly present in the media layer of the GCA lesions as shown by co-staining with αSMA. Since part of the angiogenesis is localized in the neointima layer, there is where HIF1α protein expression was also expected but not detected. However, the presence of HIF1α in the media layer together with CD45+ cells point to these inflammatory cells and their inflammatory products as the main cause of HIF1α expression in the VSMC, the cells within the media layer.

In order to verify that inflammatory cells present in the artery wall are responsible for HIF1α expression in the media layer, VSMC were co-cultured *in-vitro* with or without

PBMC. In this thesis, we have validated that this co-culture system constitutes a valid *in-vitro* model of vascular inflammation because VSMC acquire a pro-inflammatory phenotype (65). When VSMC were co-cultured with PBMC mimicking an inflammatory microenvironment increased levels of HIF1α in VSMC were observed. Considering that HIF1α is part of an important transcription factor (HIF1) its intracellular location is crucial, we then evaluated whether HIF1α was translocated to the nuclei where it could be functional. By nuclear protein extraction and western blot we were able to demonstrate HIF1α presence in the nuclei of the VSMC co-cultured with PBMC. This result was also demonstrated by immunofluorescence that allowed us to show that this higher HIF1α expression occurs in the nuclei of VSMC, validating the activation of this TF. Although in these experiments PBMC and VSMC were not from the same patient, we have previously demonstrated that autologous co-cultures yielded comparable results (76).

It is important to highlight that all experiments (except those indicated otherwise) were performed in normoxic conditions. This demonstrates the outstanding role of an inflammatory microenvironment, and not necessarily hypoxia, in HIF1α stabilization. Although in GCA lesions hypoxia is present, probably inflammation boosters HIF1α stabilization.

In order to better characterize the interactions causing the increment in HIF1α in VSMC under an inflammatory microenvironment, VSMC were cultured under specific inflammatory stimuli. Some of inflammatory molecules known to be present in GCA lesions such as IL1β (172), IL6 (166,172,173), IFNγ (65) and TNFα (172) were tested *in vitro* with VSMC to investigate whether they were able to stabilize HIF1α. IFNγ and TNFα were also tested together due to their synergic known effects (174). Results from the present study prove that IL6 (together with its soluble receptor, sIL6R), IL1β and IFNγ together with TNFα are capable to increase HIF1α in VSMCs. Activation of HIF1α as a transcription factor was underlined by detection of its target genes SLC2A1 and EGLN3 increase in VSMC under inflammatory microenvironment, since both molecules are characteristic target genes driven by HIF1 (139,167,175).

To explore the HIF1 role in GCA pathogenesis we examined changes in gene expression induced by stabilization of HIF1α stabilized in VSMC (from now on, HIF1α+ VSMC). Three different experimental approaches were done to induce HIF1α stabilization

($CoCl_2$, DMOG and hypoxia). Even though all three experiments lead to PHD inhibition by different mechanisms, using different approaches we have been able to reduce unspecific effects of individual approaches to obtain more robust results. All three approaches increased GLUT1 mRNA (SLC2A1) demonstrating not only HIF1α stabilization but also its transcriptional activity validating the methodology.

Surprisingly, although ET-1 expression is increased in GCA lesion, mainly produced by inflammatory cells (76), it was decreased in HIF1α+ VSMC which can be explained by the cell-specific target gene regulation (176). Accordingly, VSMC migration decreased. Similarly, while CCL2 and CCL5 are increased in GCA compared to control arteries (65,67,68), HIF1α+ VSMC show decreased levels of both. However, VSMC are not the main cell type responsible for CCL5 expression in GCA, where immune cells also produce it (65). Regarding CXCL8 there has been some contradictory results, as independent studies show either decrease (68) or increase (65,177) in GCA lesions. HIF1α+ VSMC show increased CXCL8 confirming CXCL8 as a HIF1α target gene (178). It would be interesting to check functional chemotaxis towards different immune cell populations since monocyte-recruiting chemokines (CCL2 and CCL5) are decreased and CXCL8 which recruits T cells, neutrophils, and endothelial cells but not monocytes is increased.

CXCL8 is known as a chemotactic factor, but it is also a proangiogenic factor (179). Therefore, regarding angiogenesis, HIF1α+ VSMC have increased levels of three different molecules implicated in this pathological process, VEGFA, IL6 and CXCL8. These results reinforce the well-known role of HIF1α in neovessel formation (180). Since angiogenesis is present in the arterial wall of GCA lesions (47), HIF1α may well be an important factor involved in this process. To further study HIF1α implication in GCA angiogenesis an appropriate approach would be to functionally study angiogenesis with the supernatants of HIF1α+ VSMC, such as a tube assay with human umbilical venous endothelial cells (HUVEC) cultured with Matrigel.

Curiously, VSMC cultured in hypoxia did not show any special effect in comparison to DMOG or $CoCl_2$ treatment. It should be pointed out that PHD inhibition through DMOG or $CoCl_2$ is far more specific for HIF1α stabilization than incubating cells at 1,1% O_2. A hypoxic environment has many others effects beyond HIF1α, such as regulation of

transcriptional repression, that may preclude the observation of HIF1α stabilization (181).

In order to facilitate bench to bedside translation, the next aim of the present work was to analyze if the inhibition of relevant cytokines was able to modulate HIF1α stability in a GCA context and further analyze the effect of potential therapies. To accomplish this objective, first, the co-culture *in vitro* model of GCA was used and levels of HIF1α in VSMC were increased in comparison with VSMC cultured alone.

In this experimental system, inhibition of TNFα, IL1 and GM-CSF with infliximab, anakinra or mavrilimumab respectively, drastically decreases HIF1α protein levels. However, IL-6 inhibition with tocilizumab shows a heterogeneous effect on HIF1α stabilization, as in the GiACTA trial where 40% of patients were non-responders (102,107).

Since GM-CSF is recently gaining interest in GCA pathophysiology (59,112,168,182,183) and a phase II clinical trial with mavrilimumab for the treatment of GCA has been recently completed with satisfactory results (115) we focused on this cytokine.

We have demonstrated the expression of GM-CSFRα and confirm the expression of GM-CSF, within the lesions of GCA-affected arteries previously reported (64,168). Macrophages were the main cell type expressing GM-CSF and GM-CSFRα in inflamed arteries. Luminal endothelial cells and, to a lesser extent, intimal myofibroblasts and endothelial cells from vasa vasorum and neovessels also expressed GM-CSF along with a small subset of T cells (163). GM-CSFRα was expressed mainly by macrophages, endothelial cells, and intimal myofibroblasts, suggesting that these cell types would be the most responsive to GM-CSF.

Contrary to a report in granulomatosis with polyangiitis (184), but similar to findings in other inflammatory conditions (161–163), GM-CSF was barely detectable in serum from patients with GCA, with no differences from healthy individuals. This supports a paracrine function of GM-CSF in the inflammatory microenvironment and limits the utility of serum GM-CSF as biomarker of disease activity.

Detection of JAK2 and STAT5A phosphorylation in GCA lesions, along with increased expression of paradigmatic STAT5-regulated molecules, such as CD83 and transcription factor Spi1/PU.1 (185), suggested activation of GM-CSF receptor driven signaling pathways. Increased expression of additional relevant STAT5 or PU.1 regulated

molecules, including MHC class II molecule HLA-DR, adhesion molecules ICAM-1 or VCAM-1, macrophage marker CD163, pro-inflammatory cytokines such as IL-1 and TNFα, and metalloproteases such as MMP-9 has been previously demonstrated in GCA (51,54,72,186,187). Although these pathways can be activated by other cytokines, these data suggest active GM-CSF signaling in GCA arteries and a contribution of GM-CSF to the increased expression of key molecules involved in the pathogenesis of GCA.

To confirm the participation of GM-CSFR-mediated signaling in the activation of these and additional relevant molecules and inflammatory cell markers, temporal arteries from patients with histopathologically-proven GCA were exposed to mavrilimumab. Treatment with mavrilimumab resulted in significantly decreased transcripts of lymphoid markers, including B lymphocyte surface molecule CD20 and T lymphocyte surface glycoprotein CD3ε. A significant decrease in classical monocyte marker CD14 and myeloid cell marker CD16 mRNAs was also observed. In contrast, there was no consistent change in the expression of CD68, a scavenger receptor widely expressed by macrophages.

Mavrilimumab decreased expression of molecules produced by dendritic cells and B cells which are essential for antigen-presenting function/T cell activation, such as CD83 and HLA-DR(188,189). This likely resulted in decreased Th1 differentiation, as indicated by reduced expression of Th1 related molecules, including IFNγ, TNFα and IFNγ-induced molecules such as CXCL10. Molecules related to Th17 differentiation, IL-1β and IL-6, were also decreased, but a more direct impact on IL-17 production could not be assessed. Although we and others have previously shown increased IL-17 expression in affected temporal arteries from patients with GCA (59,60,62,63), baseline expression of IL-17 was very low in cultured arteries, possibly related to previous glucocorticoid treatment in the majority of patients(60) or to the possible impact of culture on certain molecules(68).

Mavrilimumab had a significant impact on pro-inflammatory functions of macrophages and endothelial cells including expression of IL-1, TNFα and IL-6, and expression of adhesion molecules for leukocytes. It also tended to increase expression and release of the anti-inflammatory cytokine IL-10, produced by regulatory T and B cells and M2-type macrophages(190). Mavrilimumab reduced MMP-9 expression with no change in expression of its natural inhibitor TIMP-1, thereby suggesting a shift in the MMP-9

proteolytic balance(72). Proteolytic enzyme MMP-9 has elastinolytic activity and may contribute to elastin degradation since it is expressed and activated in GCA lesions and in aortic tissue (12). MMP-9 may also contribute to GM-CSF-induced aneurysm formation, as shown in an animal model (191). Macrophages present in GCA lesions have oxidative capacity as indicated by the presence of lipid peroxidation products (HNE) in GCA lesions (66). Treatment with mavrilimumab decreased HNE presence in cultured arteries indicating that mavrilimumab decreases oxidative damage in inflamed arteries.

The tuning in macrophage function induced by mavrilimumab does not parallel classical M1 (pro-inflammatory) or M2 (anti-inflammatory, reparative) phenotypes. Mavrilimumab reduced M1 markers including HLA-DR and iNOS and tended to increase M2 cytokine IL-10. However, mavrilimumab also reduced CD206 and CD163 which have been considered markers of M2 phenotype (192). It is important to remark that this distinction has been established mostly in in vitro differentiated macrophages or in murine models. In humans, plasticity of macrophages is far more complex (192). For example, macrophages co-expressing CD206 and MMP-9 have been observed in GCA lesions (168) and a population of proinflammatory CD14+ HLA-DR high CD206+ macrophages has been identified in human viral hepatitis (193). Overall, mavrilimumab decreased the inflammatory and destructive potential of macrophages.

GM-CSF also influences endothelial cell behavior and stimulates angiogenesis in experimental systems (194). Accordingly, mavrilimumab reduced microvessel density in GCA lesions. In addition to its potential direct effects, our results indicate that GM-CSF regulates VEGFA production. Since CD34 is expressed not only by endothelial cells from neovessels but also by hematopoietic stem cells (HSC), which have recently been identified in chronic inflammatory lesions and promoted by GM-CSF (195), we cannot exclude the possibility that some detected CD34+ cells were ectopic HSC. Mavrilimumab reduction of ectopic HSC may be a potential new relevant effect. Since neoangiogenesis is prominent in GCA lesions, and newly formed capillaries express adhesion molecules and recruit inflammatory leukocytes into arteries (54,196), mavrilimumab could indirectly reduce leukocyte recruitment by decreasing neoangiogenesis in addition to its direct effects on myeloid and other cells.

This GM-CSFR/GM-CSF study has some limitations: First, the relatively small number of cases investigated, inherent to the low incidence of GCA and the need of viable fresh tissue. Second, our model explores changes induced by mavrilimumab in a target organ isolated from a functional immune system. However, the effects of mavrilimumab observed were consistent with the known functions of GM-CSF obtained in a variety of experimental systems. Third, due to the small amount of available tissue, our experiments were limited to a single time-point and we cannot exclude that effects could be more prominent at other time points. Finally, most arteries were obtained from patients who had previously received glucocorticoid treatment, as currently advised by international guidelines upon GCA suspicion (197). Previous glucocorticoid exposure reduces baseline expression of a variety of molecules including GM-CSF (68,198). It would be possible that using treatment-naive samples, changes would have been more prominent. However, this setting better reflects the real world and mavrilimumab still adds to potential glucocorticoid effects on key inflammatory molecules.

GM-CSFRα blockade in a GCA context decrease several pro-inflammatory cytokines like IL6, IL1β, IFNγ and TNFα. In this thesis, it has been demonstrated that this molecules are able to stabilize HIF1α protein. Moreover, stabilization of HIF1α on VSMC increases GM-CSF transcripts together with IL6, IL1β, TNFα, and VEGFA, which are decreased under mavrilimumab treatment. In fact, GM-CSF and HIF1α have been previously reported together regarding angiogenesis and apoptosis (199–201). The interplay between GM-CSF and HIF1α is summarized in figure 19.

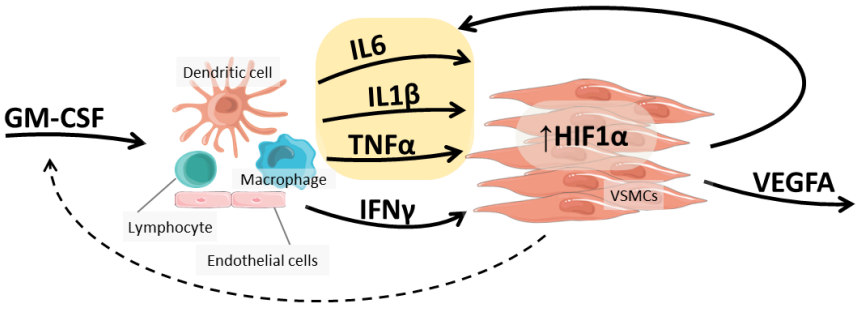


Figure 19. GM-CSF and HIF1α interplay in GCA pathogenesis. GM-CSF activates dendritic cells, macrophages, lymphocytes and endothelial cells, which then produce more IL6, IL1β, TNFα and IFNγ through. These cytokines present in the microenvironment are able to stabilize HIF1α in VSMC, which promotes production of additional IL6, IL1β, TNFα, and GM-CSF in a lesser extent. VEGFA is also released by these VSMC with HIF1α stabilized and this is markedly reduced in the whole artery by blocking GM-CSF with mavrilimumab.

Putting all these data together we demonstrate that both HIF1α and GM-CSF play an important role in the regulation of pathways considered relevant to the pathogenesis of GCA and we hypothesize that GM-CSF and HIF1α are closely related in this pathophysiology and may be involved in feed-forward mechanism of the disease.

7

Conclusions

The conclusions derived from this thesis are as follows:

1. Temporal artery biopsies from giant cell arteritis (GCA) patients show hypoxic regions through the artery wall and the key transcription factor activated by hypoxia, hypoxia inducible factor 1 alpha (HIF1α), is increased in GCA lesions. HIF1α is present in the media layer of GCA lesions.
2. Under a normoxic inflammatory microenvironment VSMC have increased levels of HIF1α in the nuclei of the cells. Moreover, HIF1α functional activity is also increased under this condition, as indicated by increased expression of target genes.
3. Stabilizing HIF1α in VSMC modify its transcriptome affecting several important pathways in GCA pathophysiology:
    a. VSMC show decreased ET-1 levels and also a reduced migratory capacity as disclosed by a delay in scratch-wound closure when HIF1α is stabilized which may have an impact on vascular remodeling that occurs in GCA lesions.
    b. VSMC increase production of angiogenic factors, VEGFA, IL6 and CXCL8, when HIF1α is stabilized.
    c. VSMC decrease production of chemokines CCL2, CCL5 and increase CXCL8 synthesis when HIF1α is stabilized possibly influencing chemotactic recruitment of different inflammatory cells subsets into the affected vascular wall.
    d. VSMC increase production of transcripts of cytokines related to inflammation (IL6, IL1B, TNFA and CSF2) when HIF1α is stabilized.
4. Several anti-cytokine therapies, infliximab (anti-TNFα), anakinra (IL1 receptor antagonist), and mavrilimumab (anti-GMCSFRα) are able to decrease HIF1α protein under an inflammatory microenvironment.
5. GM-CSF and GM-CSFRα expression are increased in GCA lesions, the downstream signaling pathways (JAK2 and STAT5) are activated, and consequently the expression of molecules regulated by this pathway (PU.1 and CD83) is increased.

6. GM-CSFRα inhibition with the blocking monoclonal antibody, mavrilimumab, induced the following effects on GCA lesions:
    a. Mavrilimumab reduces lymphocyte and myeloid cell markers in ex-vivo cultured arteries from patients with GCA
    b. Mavrilimumab reduces expression of molecules involved in T cell activation and related to the Th1 differentiation pathway in ex-vivo cultured arteries from patients with GCA
    c. Mavrilimumab decreases pro-inflammatory cytokines in ex-vivo cultured arteries from patients with GCA
    d. Mavrilimumab decreases mediators of vascular injury in ex vivo cultured arteries from patients with GCA
    e. Mavrilimumab reduces tissue angiogenesis in ex-vivo cultured arteries from patients with GCA
7. Blocking GM-CSF through inhibition of its specific receptor with mavrilimumab reduces some HIF1α targets (IL6, IL1β, and TNFα) which, including IFNγ, are also identified as stimulus for HIF1α that accumulated in VSMC, supporting the relationship between HIF1α and GM-CSF in GCA pathogenesis.

8

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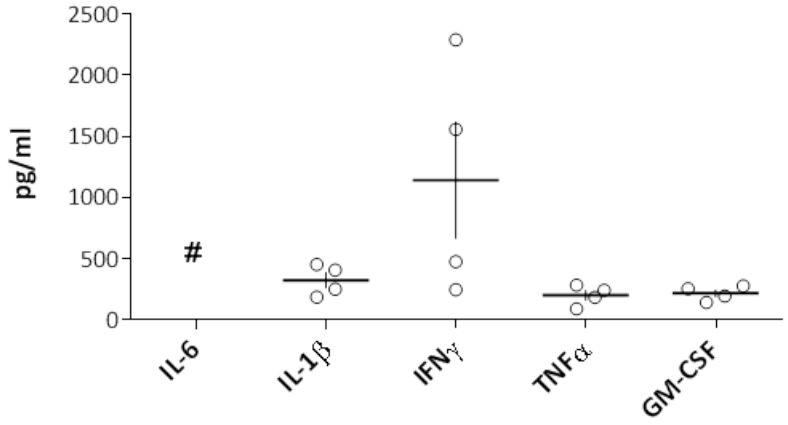
Additional data

Presence of pro-inflammatory molecules in the co-culture model supernatant

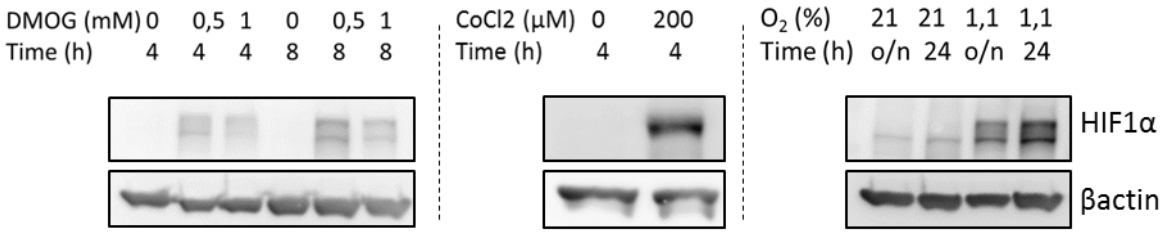
In this thesis several pro-inflammatory molecules were targeted by different pharmacological approaches (IL-6, IL-1β, IFNγ, TNFα, and GM-CSF). These molecules were detected in the supernatants of co-cultured VSMC with PBMC after 48h.



Supplementary figure 1. Presence of pro-inflammatory molecules in the co-culture supernatant. Quantification (pg/ml) of several proteins performed in the supernatant fluid of 4 co-culture experiments (VSMC + PBMC) at 48h. # All samples were above upper detection limit for IL6 (5850pg/ml).

HIF1α stabilization by DMOG, $CoCl_2$, or hypoxia

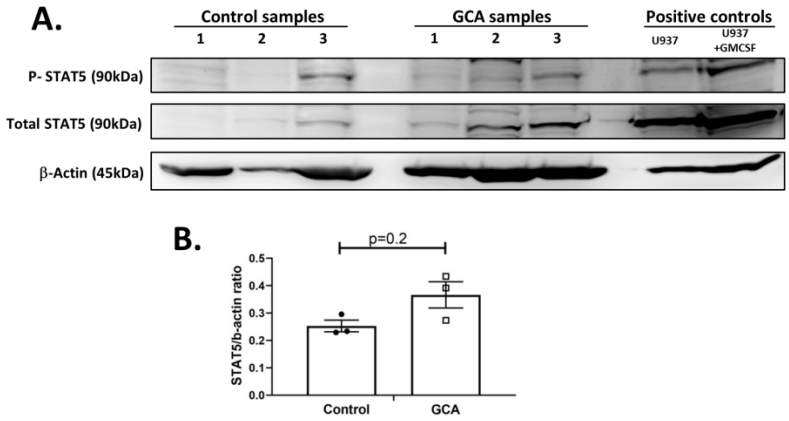
In this thesis we use DMOG, $CoCl_2$, and hypoxia to stabilize HIF1α in VSMC. To corroborate this phenomenon we performed protein analysis by western blot. VSMC treated with DMOG or $CoCl_2$ or incubated under hypoxic condition show elevated HIF1α protein levels.



Supplementary figure 2. HIF1α stabilization in VSMC through 3 different approaches. HIF1α protein checked by western blot in VSMC cultured with DMOG, $CoCl_2$ or under hypoxic conditions.

STAT5 activation in GCA lesions

Since phosphorylation of STAT5 is downstream GM-CSFRα activation, we wanted to check STAT5 and its phosphorilation directly in GCA involved temporal arteries. These results show presence of STAT5 and p-STAT5 in GCA involved tissue.

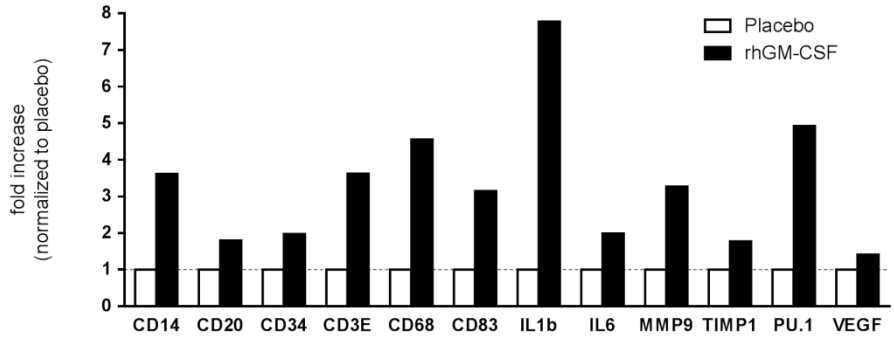


Supplementary figure 3. STAT5 activation in GCA lesions. Immunoblot (A) and its corresponding densitometric quantitation (B) of total STAT5 or STAT5 phosphorylated at the tyrosine 694 residue, in lysates of fresh temporal arteries from 3 controls and 3 GCA patients, with β-actin expression as control for loading. Lysates of U937 cell line with or without exposure to recombinant GM-CSF at 20 ng/ml were used as a positive control for STAT5 expression and phosphorylation.

GM-CSF effect on GCA lesions

To further support the effect of inhibiting GM-CSF with mavrilimumab, recombinant human

GM-CSF showed increased expression of the main transcripts decreased by mavrilimumab in an *ex-vivo* cultured artery from a GCA patient.



Supplementary figure 4. GM-CSF effect on GCA lesions. Transcript measurement of relevant pro-inflammatory cytokines and cell markers from a GCA positive temporal artery biopsy cultured in presence of placebo or recombinant human GMCSF (rhGM-CSF) at 20ng/ml, given as fold increase, normalized to placebo.

Annex

OPEN ACCESS

TRANSLATIONAL SCIENCE

Blocking GM-CSF receptor α 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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ABSTRACT

Background Effective and safe therapies are needed for the treatment of patients with giant cell arteritis (GCA). Emerging as a key cytokine in inflammation, granulocyte-macrophage colony stimulating factor (GM-CSF) may play a role in promoting inflammation in GCA.

Objectives To investigate expression of GM-CSF and its receptor in arterial lesions from patients with GCA. To analyse activation of GM-CSF receptor-associated signalling pathways and expression of target genes. To evaluate the effects of blocking GM-CSF receptor α with mavrilimumab in ex vivo cultured arteries from patients with GCA.

Methods Quantitative real time PCR, in situ RNA hybridisation, immunohistochemistry, immunofluorescence and confocal microscopy, immunoassay, western blot and ex vivo temporal artery culture.

Results GM-CSF and GM-CSF receptor α mRNA and protein were increased in GCA lesions; enhanced JAK2/STAT5A expression/phosphorylation as well as increased expression of target genes CD83 and Spi1/PU.1 were observed. Treatment of ex vivo cultured GCA arteries with mavrilimumab resulted in decreased transcripts of CD3ε, CD20, CD14 and CD16 cell markers, and reduction of infiltrating CD16 and CD3ε cells was observed by immunofluorescence. Mavrilimumab reduced expression of molecules relevant to T cell activation (human leukocyte antigen-DR [HLA-DR]) and Th1 differentiation (interferon-γ), the pro-inflammatory cytokines: interleukin 6 (IL-6), tumour necrosis factor α (TNFα) and IL-1β, as well as molecules related to vascular injury (matrix metalloprotease 9, lipid peroxidation products and inducible nitric oxide synthase [iNOS]). Mavrilimumab reduced CD34 + cells and neoangiogenesis in GCA lesions.

Conclusion The inhibitory effects of mavrilimumab on multiple steps in the GCA pathogenesis cascade in vitro are consistent with the clinical observation of reduced GCA flares in a phase 2 trial and support its development as a therapeutic option for patients with GCA.

Key messages

What is already known about this subject?

- GM-CSF transcripts were detected in temporal arteries from patients with giant cell arteritis (GCA) more than two decades ago.
- More recently, GM-CSF protein has been shown to be produced and secreted by peripheral blood mononuclear cells from patients with active GCA and detected in GCA-involved temporal arteries by immunohistochemistry.
- Expression of GM-CSF receptor and its functional role in GCA lesions has not been previously explored.

What does this study add?

- The study demonstrates expression of GM-CSF and its receptor in distinct cell subsets in GCA lesions.
- Moreover, GM-CSF receptor signalling is activated, and expression of typical target genes is increased.
- Exposure of ex-vivo cultured arteries to mavrilimumab reduces CD16 and CD3ε cell infiltration and reduces key molecules involved in T cell activation and differentiation, expression of pro-inflammatory cytokines, markers of vascular injury and neoangiogenesis.
- Taken together, these data point towards a relevant role of GM-CSF in the development of vascular inflammation and injury in GCA.

How might this impact on clinical practice or future developments?

- The clear impact of mavrilimumab on key steps in the pathogenesis of GCA supports its further development as a therapeutic option for patients with GCA.

INTRODUCTION

Giant cell arteritis (GCA) is a chronic inflammatory condition affecting large and medium arteries in individuals older than 50 years. Common manifestations include headache, scalp tenderness, polymyalgia rheumatica and systemic symptoms.[1 2] Inflammation-induced vascular remodelling results in ischaemic complications or aneurysms.[3]

High-dose glucocorticoids (GCs) dramatically improve symptoms of GCA, but relapses occur in 34%–75% of patients when GCs are tapered,[4–6]

leading to prolonged treatment and frequent GC-associated side effects.[7 8] Blocking the interleukin 6 (IL-6) receptor with tocilizumab (TCZ) demonstrated efficacy in reducing relapses, sparing GC,[9 10] and improving quality of life.[11] However, more than 40% of patients treated with TCZ are unable to maintain GC-free remission and about 60% of responders relapse on discontinuation,[12] indicating heterogeneity in response and underlining the need for alternative therapeutic options. TCZ also inhibits synthesis of acute-phase reactants, even without full suppression of disease activity, rendering their use unreliable for monitoring of disease flare.[13 14]

The search for additional therapeutic targets in GCA is hampered by the limited understanding of pathogenesis. Studies indicate that genetics, ageing and immune responses against unknown antigen(s) likely play a major role.[15 16] Dendritic cells activated by innate immune mechanisms may drive adaptive immunity by stimulating T lymphocytes and promoting their differentiation into Th1 and Th17 effector cells.[17–24] Concomitant and subsequent activation of macrophages amplifies inflammatory loops, leading to vascular injury and remodelling.[25–27]

GM-CSF is a pro-inflammatory cytokine produced by fibroblasts, epithelial, endothelial, myeloid and T cells on stimulation with other cytokines or pathogen-associated molecular pattern molecules.[28–30] GM-CSF has a seminal role in disease progression in animal models of inflammatory conditions.[28–30] GM-CSF receptor is composed of an alpha-chain conferring specificity and a signalling beta-chain shared with other cytokine receptors (IL-3, IL-5 and IL-34).[28–30] On GM-CSF binding, the receptor beta-chain predominantly signals through JAK2–STAT5 pathway. GM-CSF acts primarily on myeloid cells, promoting activation of dendritic cells and macrophages and differentiation of monocytes into dendritic cells, but other cell types may also respond.[28–30] GM-CSF mRNA has been detected in arterial lesions of GCA, and GM-CSF protein production by circulating peripheral blood mononuclear cells from GCA patients is increased compared with healthy controls.[22 24] According to its known biological functions, GM-CSF may have a role in promoting and amplifying vascular inflammation and injury in GCA.

Mavrilimumab is a fully human IgG4 monoclonal antibody able to neutralise GM-CSF effects by binding to the GM-CSF receptor alpha chain (GM-CSFRα).[31] In a phase 2b trial in patients with rheumatoid arthritis, mavrilimumab showed comparable efficacy to anti-TNFα blocker golimumab and superior efficacy compared with placebo, as well as a good safety profile.[32–34] The putative role of GM-CSF in critical steps of GCA pathogenesis suggests therapeutic potential for mavrilimumab in this disease, supported by a recent phase 2 trial.[35]

This study aimed to investigate the expression of GM-CSF and GM-CSFRα in inflamed arteries from patients with GCA, to detect activation of GM-CSFR-related signalling pathways and modulation of downstream gene expression, and to investigate the impact of GM-CSFRα blockade with mavrilimumab on inflammation in ex vivo cultured arteries from patients with GCA.

PATIENTS AND METHODS

Patients

The study investigated samples from four different patient groups according to the processing of their biospecimens (clinical characteristics of patients, controls and their samples: online supplemental table S1).

Temporal artery culture

Details have been previously described[36] and are available in online supplemental methods.

In situ RNA hybridisation

RNAScope (RS) (ACDbio, Abingdon, UK) in situ hybridisation was performed on formalin-fixed paraffin-embedded (FFPE) sections of GCA and control temporal artery biopsies to detect transcripts of specific genes, including GM-CSF, GM-CSFRα, CD83 and Spi1 (PU.1). After fixation and sectioning, tissue was permeabilised and probed with target-specific double Z probes specific to single target mRNA, and hybridisation signals were further amplified for detection. Visualised with a microscope, each red dot represents a single target mRNA molecule. Expression score was calculated as RS score (dots/cell) multiplied by positivity score (% cells positive with >1 dot/cell) (online supplemental table S2).

Candidate gene expression analysis

Candidate genes relevant to the immunopathogenesis of GCA were selected according to the current pathogenesis model[15 16] and known effects of GM-CSF in experimental systems.[28 29] Details of RNA extraction, reverse transcription and fluorescence quantification are provided in the online supplemental methods (online supplemental table S3).

Immunohistochemistry

Two micrometre thick temporal artery sections from FFPE samples were used for immunohistochemistry. After 20-minute antigen retrieval with citrate buffer (pH 6), samples were immunostained with specific antibodies, using the Leica Microsystems' Bond-max automated immunostainer and the Bond Polymer Refine Detection System (Leica Microsystems), developed with diaminobenzidine and counterstained with haematoxylin (antibodies used, dilutions and optimised incubation times: online supplemental table S4). Positive and negative control tissues for protocol optimisation were selected from Human Protein Atlas (www.proteinatlas.org) and obtained from Institut d'Investigacions Biomèdiques August Pi i Sunyer Biobank.

Immunofluorescence

Immunofluorescence staining and imaging were performed with fresh-frozen or cultured temporal artery sections (online supplemental methods and online supplemental table S4).

Protein detection by western blot

Fresh-frozen temporal artery biopsies (TABs) from three patients with GCA and three controls were processed as described in online supplemental table S4-B.

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

Cytokines, chemokines or membrane-bound molecules released into artery culture supernatants were detected by immunoassay (online supplemental table S5).

Statistical analysis

Non-parametric Mann-Whitney U test and Wilcoxon matched-pairs signed rank test were used for unpaired and paired data analysis, respectively, using Graphpad Prism software.

RESULTS

GM-CSF and GM-CSFRα expression is increased in GCA lesions

GM-CSF and GM-CSFRα transcripts were increased in homogenised temporal artery biopsies from patients with GCA, whereas GM-CSF mRNA was virtually undetectable, and GM-CSFRα expression was very low in control arteries (figure 1A,B). Transcripts for GM-CSF or GM-CSFRα mRNA were clearly detectable by in situ RNA hybridisation in all arterial layers of GCA biopsies, whereas virtually no signal for either gene product was detectable in control arteries (figure 1C–E).

Immunostaining confirmed the presence of GM-CSF and GM-CSFRα protein on infiltrating inflammatory cells and endothelial cells in GCA arteries. In contrast, no GM-CSF protein and only low levels of GM-CSFRα protein were detected in control arteries (figure 1F,G).

Cell subsets potentially expressing GM-CSF and GM-CSFRα in GCA lesions were explored. As illustrated by immunofluorescence in figure 2, GM-CSF was mainly observed in macrophages and luminal endothelial cells and, to a lesser extent, in T cells, intimal myofibroblasts, and endothelial cells from vasa vasorum and neovessels. GM-CSFRα was detected mainly in macrophages, giant cells, endothelial cells and intimal myofibroblasts.

Serum GM-CSF concentration at diagnosis was 0.061±0.02 pg/mL (average±SEM) in patients with GCA and 0.035±0.02 pg/mL in controls (p=0.889).

GM-CSF receptor-driven signalling pathways are activated in GCA lesions, and expression of molecules regulated by this pathway is increased

After observing higher expression of GM-CSF and GM-CSFRα in GCA-involved arteries, signalling molecules downstream of GM-CSFR were examined. As shown in figure 3A,B and online supplemental figure S1, JAK2 and STAT5A, the main signalling proteins activated by GM-CSFR engagement, were phosphorylated in GCA lesions, and transcripts regulated by STAT5, such as Spi1 (PU.1) and CD83, were significantly increased in GCA arteries (figure 3C–G). CD83 and PU.1 protein, absent in controls, were clearly expressed in GCA arteries (figure 3H,I). PU.1 was detected in the nuclei, consistent with its function as transcription factor and suggestive of nuclear translocation on activation of upstream signalling. CD83 staining was more diffuse, possibly due to detection of its soluble form in addition to the membrane molecule.

GM-CSFRα inhibiting monoclonal antibody mavrilimumab reduces lymphocyte and myeloid cell markers in ex vivo cultured arteries from patients with GCA

To determine the contribution of GM-CSF to the above results and to assess the effects of GM-CSF pathway blockade on vascular inflammation, GCA arteries were cultured with anti-GM-CSFRα, mavrilimumab, for 5 days. Compared with placebo, treatment with mavrilimumab resulted in reduced phospho-STAT5 in lesions (figure 4A,B) and in lower mRNA expression of Spi1 (PU.1), a transcription factor that, along with STAT5, mediates GM-CSF effects (figure 4C).[28–30] Furthermore, treatment with mavrilimumab resulted in significantly lower mRNA levels for T cell marker CD3ε, B cell marker CD20, monocyte marker CD14 and myeloid cell marker CD16 mRNAs (figure 4D). By contrast, no consistent changes were observed with transcripts for the macrophage marker CD68. Accordingly, fewer CD16 + and CD3ε + infiltrating cells and no change in CD68 + cells were observed by immunofluorescence (figure 4F). The reduction in CD20 transcripts, however, did not result from decreased numbers of B cells in tissue during the duration of mavrilimumab exposure (figure 4E,F).

Mavrilimumab reduces expression of molecules involved in T cell activation and related to the Th1 differentiation pathway in ex vivo cultured arteries from patients with GCA

To further delineate the effects of mavrilimumab, expression of human leukocyte antigen-DR (HLA-DR) and CD83, relevant molecules to antigen presentation and T cell activation, was examined. Mavrilimumab significantly reduced HLA-DR and CD83 transcripts (figure 5A). Interestingly, concentration of the soluble, shed form of CD83, with counter-regulatory functions, did not decrease in the supernatant (figure 5A). HLA-DR reduction was also observed at the protein level (figure 5A).

To determine whether these effects resulted in decreased differentiation of T cells towards the Th1 or Th17 lineage, select markers were explored. Transcripts of master regulators of Th1 and Th17 differentiation, *TBX21* (T-bet) and *RORC* (RORγ), respectively, trended lower (figure 5B,C). Cytokines/chemokines related to Th1 differentiation pathway (interferon-γ (IFNγ) and CXCL10) trended lower (mRNA level) or were significantly lower (protein level) (figure 5B). IL-17A mRNA was virtually undetected in cultured arteries (data not shown), and IL-23p19 had disparate response among donors (figure 5C).

Mavrilimumab decreases pro-inflammatory cytokines in ex vivo cultured arteries from patients with GCA

Mavrilimumab elicited a significant reduction in the production and release of pro-inflammatory cytokines IL-6, TNFα and IL-1β, mostly but not exclusively produced by macrophages (figure 6A). Mavrilimumab also decreased markers of M2-like phenotype, including the mannose receptor CD206 and the scavenger receptor CD163 (figure 6B). A trend towards an increase in the anti-inflammatory cytokine IL-10 (mRNA and protein) was also observed (figure 6B).

Further supporting these results, recombinant human GM-CSF increased expression of the main transcripts decreased by mavrilimumab (online supplemental figure S2)

Mavrilimumab decreases mediators of vascular injury in ex vivo cultured arteries from patients with GCA

Mavrilimumab decreased transcript and protein concentrations of the elastinolytic matrix metalloprotease 9 (MMP-9), whereas mRNA and protein of its natural inhibitor tissue inhibitor of metalloproteinases 1 (TIMP-1) remained unchanged, resulting in a significant decrease in proteolytic MMP-9/TIMP-1 balance (figure 7A,B). Mavrilimumab also reduced oxidative damage, as demonstrated by decreased presence of lipid peroxidation products (4-hydrynonenal (HNE) protein adducts) in cultured arteries exposed to mavrilimumab as compared with placebo (figure 7C). *NOS2* (inducible nitric oxide synthase [iNOS]) mRNA expression also trended lower (figure 7D).

Mavrilimumab reduces tissue angiogenesis in ex vivo cultured arteries from patients with GCA

Mavrilimumab reduced vascular endothelial growth factor A (VEGFA) mRNA in cultured arteries and VEGFA protein expression in tissue by immunofluorescence (figure 8A–C). However, no changes in VEGFA protein in the supernatant was observed, possibly due to its matrix-binding capacity and its autocrine/paracrine function.[37] Based on the reduction of this important angiogenic factor, we explored the effects of mavrilimumab on endothelial cell markers and angiogenesis. Mavrilimumab did

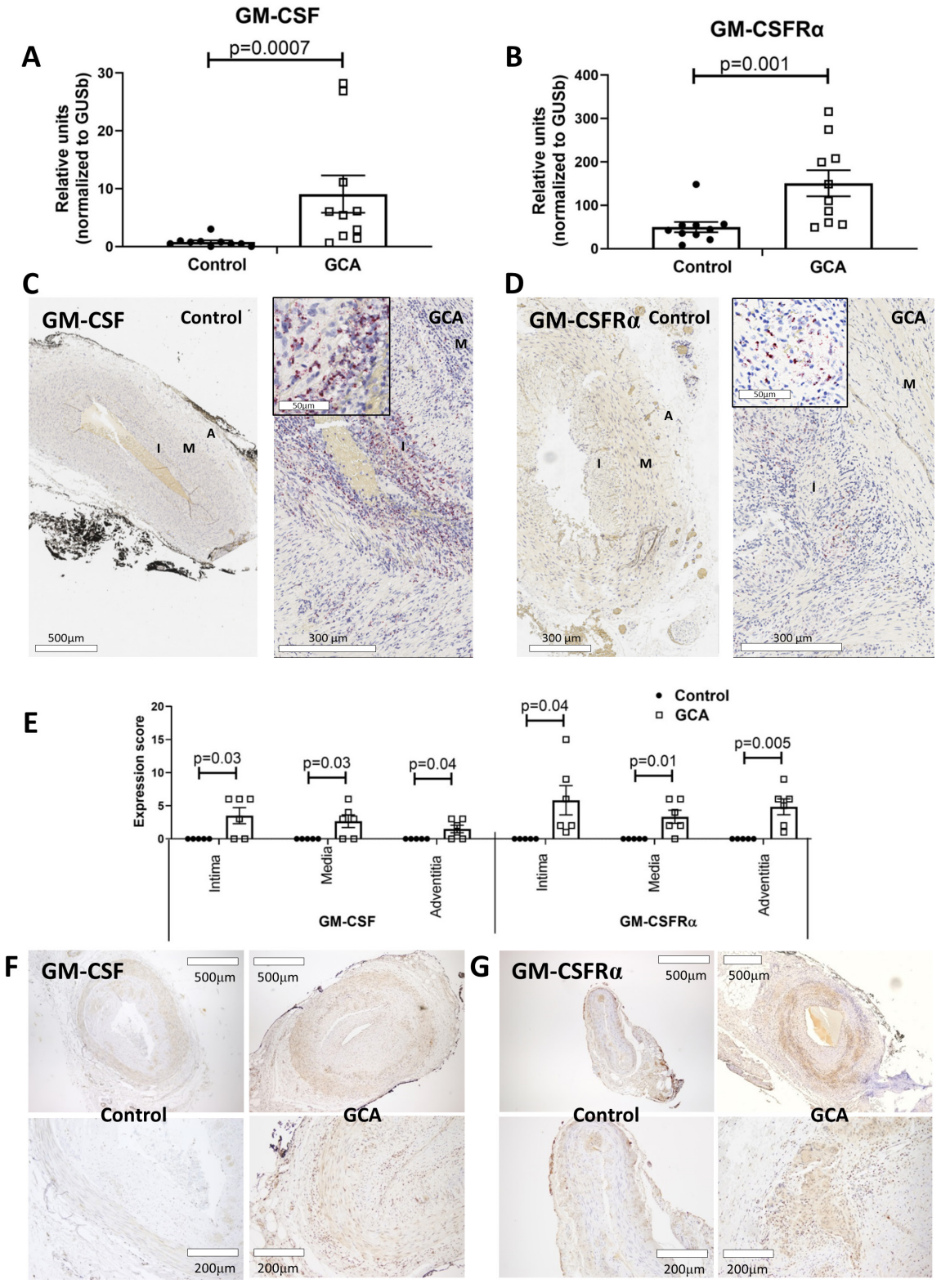
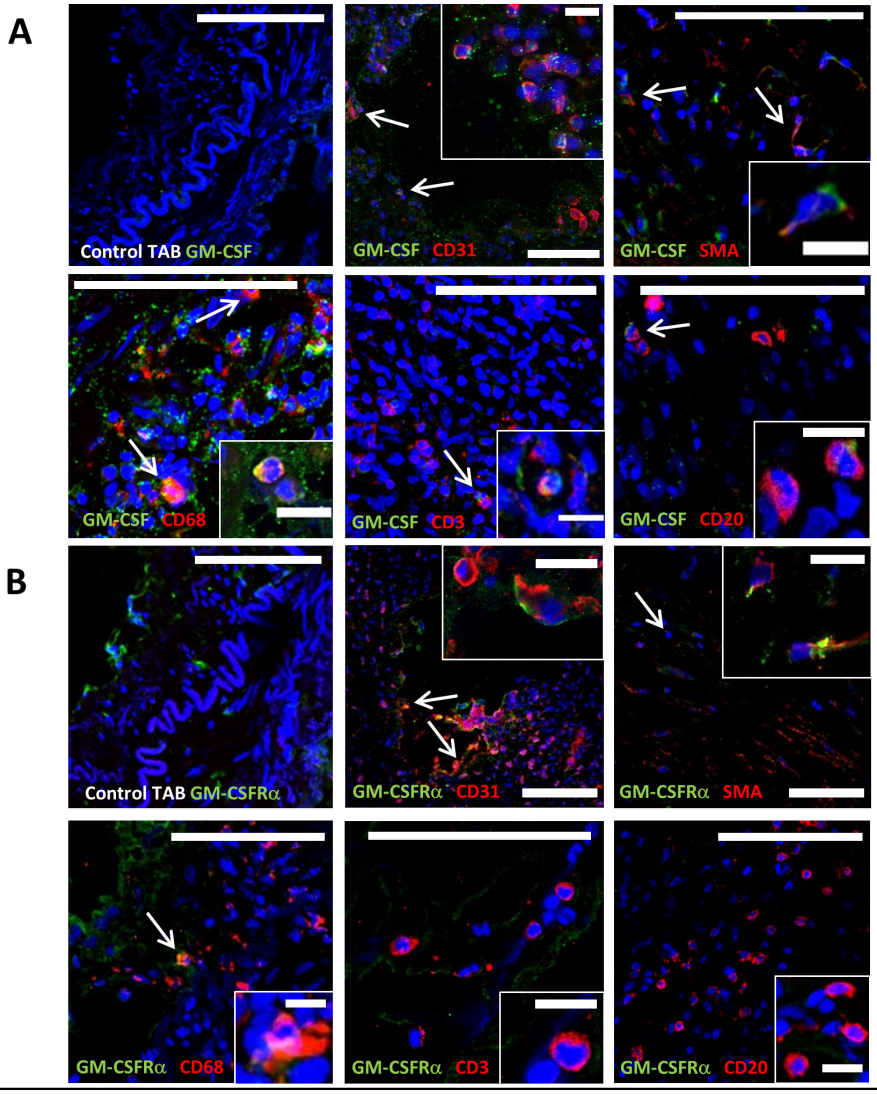


Figure 1 Granulocyte-macrophage colony stimulating factor (GM-CSF) and GM-CSFRα expression in GCA lesions. Concentrations of GM-CSF (A) and GM-CSFRα mRNA (B) measured by qRT-PCR in fresh-frozen histologically negative arteries (controls) (n=10) vs GCA-positive arteries (n=10). Results are expressed in relative units normalised to the housekeeping transcript *GUSB*. GM-CSF (C) and GM-CSFRα (D) RNA hybridisation signals (red dots) on control temporal arteries and GCA-involved arteries. (E) Quantitation of RS signal (expression score) in different arterial layers in 6 GCA-involved and 5 control arteries. Immunostaining with anti-GM-CSF (F) and anti-GM-CSFRα (G) antibodies (brown colour) of FFPE normal or GCA-involved arteries (representative of 5 controls and 12 GCA arteries). A, adventitia layer; FFPE, formalin-fixed paraffin-embedded; GCA, giant cell arteritis; GM-CSFRα, GM-CSF receptor alpha chain; I, intima layer; M, media layer; qRT-PCR, quantitative real-time PCR; RS, RNAScope.



C

	Control		GCA	
	GM-CSF	GM-CSFRα	GM-CSF	GM-CSFRα
Luminal endothelium	-	+	+++	++
Neovessels / Adventitial endothelium	-	-	+	+
VSMC	-	-	+	+/-
Neointimal myofibroblasts	-	-	++	++
Macrophages	-	-	+++	++
B cells	-	-	+	+/-
T cells	-	-	++	-

Figure 2 GM-CSF and GM-CSFRα expression by immune and resident cells. Merged double immunofluorescence staining with anti-GM-CSF (A) or anti-GM-CSFRα (B) antibodies (both in green) and cell surface markers CD68 (macrophages), CD31 (endothelial cells), CD3 (T lymphocytes), CD20 (B lymphocytes) and SMA (identifying vascular smooth muscle cells and myofibroblasts) (all in red) of fresh-frozen temporal arteries from patients with GCA or controls (first panel). Nuclei are stained with DAPI (blue). Co-expression (orange/yellow) is pointed with arrows and insets show magnified double-positive cells (scale bars in figures measure 100 µm and 15 µm for insets). (C) Summary panel of GM-CSF and GM-CSFRα expression by different cell types in three GCA-involved temporal arteries detected by immunofluorescence as in A and B. +++: 50%–100% positive cells; ++: 20%–40% positive cells; +: less than 20% positive cells; +/–: scattered cells; –: negative. DAPI, 4′,6-diamidino-2-phenylindole; GCA, giant cell arteritis; GM-CSF, granulocyte-macrophage colony stimulating factor; GM-CSFRα, GM-CSF receptor alpha chain; SMA, smooth muscle actin; TAB, temporal artery biopsy; VSMC, vascular smooth muscle cells.

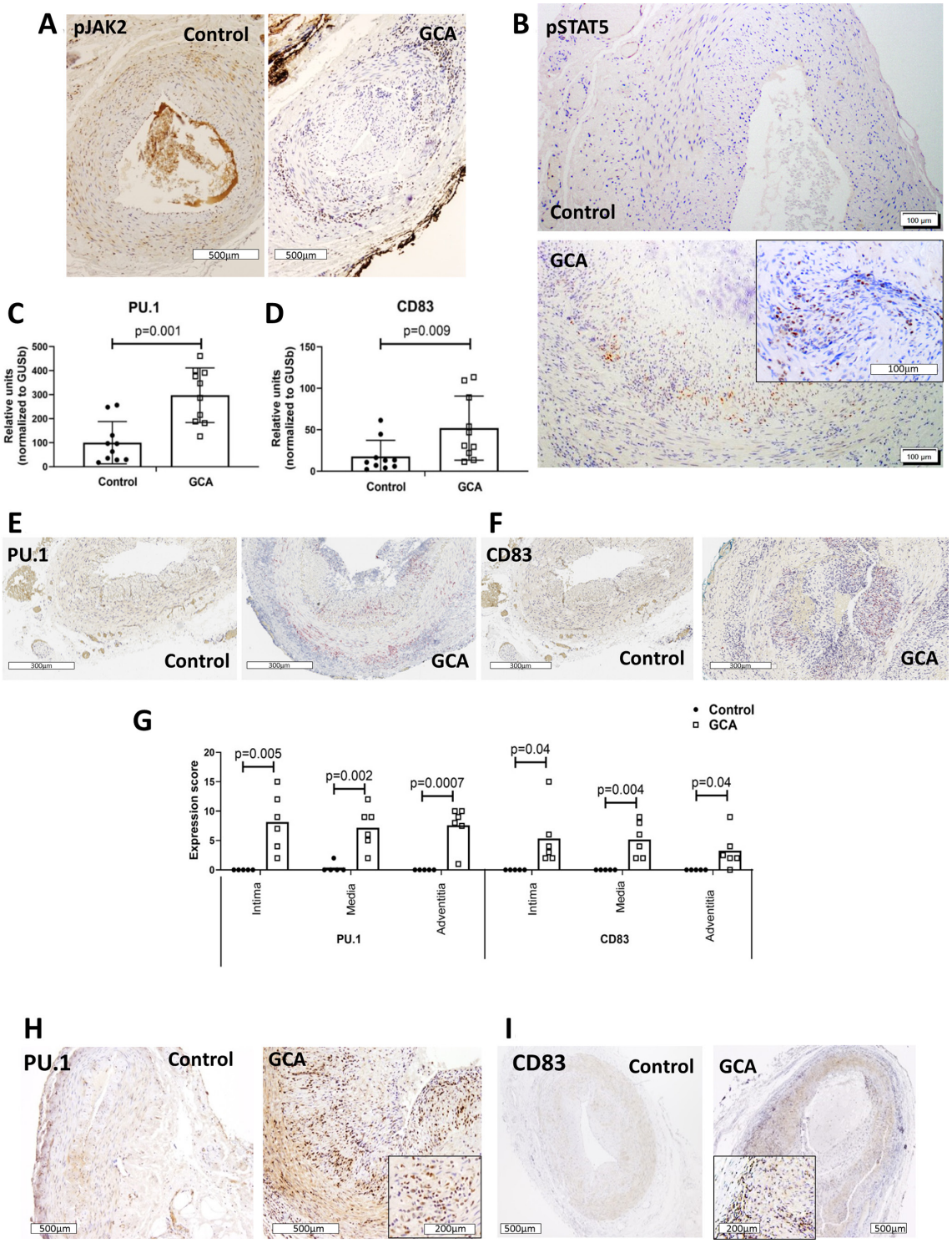


Figure 3 Activation of GM-CSFR-driven signalling pathways and target gene expression in GCA lesions. Immunostaining of histologically negative temporal artery biopsies (control) and GCA-involved arteries with anti-phospho-JAK2 (A) or anti-phospho-STAT5 (B) antibody (brown colour). Representative of 12 GCA and 5 control arteries. mRNA concentrations of PU.1 (C) and CD83 (D), in fresh-frozen control and GCA arteries (n=10 each group). PU.1 (E) and CD83 (F) RS images with positive red staining on control (n=5) and GCA temporal arteries (n=6), with their corresponding quantitation (G) in the intima, media and adventitia layers of the artery wall. Immunohistochemistry with anti-PU.1 (H) and anti-CD83 (I) antibodies on FFPE control and GCA arteries (brown). Representative of 12 GCA arteries and 5 controls. Magnification of each figure is indicated individually. FFPE, formalin-fixed paraffin-embedded; GCA, giant cell arteritis; GM-CSF, granulocyte-macrophage colony stimulating factor; GM-CSFRα, GM-CSF receptor alpha chain; RS, RNAScope.

not elicit changes in constitutive endothelial cell marker vWF or CD31 mRNAs but a decrease in CD34 mRNA, expressed by neovessels and haematopoietic stem cells (HSC) was observed (figure 8D).[38 39] Immunofluorescence showed a reduction in CD31 + and CD34+ neovessels within inflammatory lesions on exposure to mavrilimumab (figure 8E,F). Scattered CD34 +

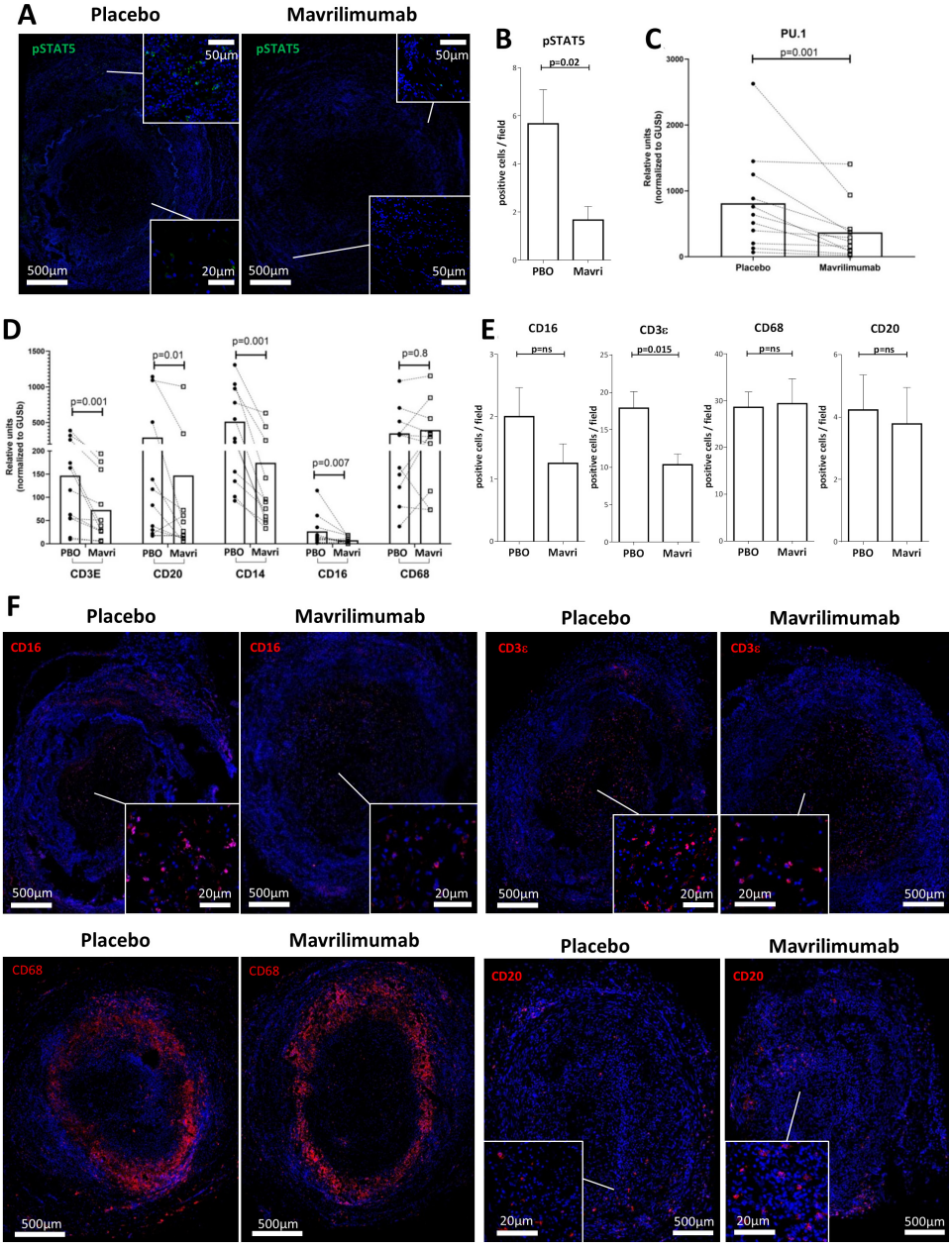


Figure 4 Effect of mavrilimumab on inflammatory infiltrates in ex vivo cultured arteries from patients with GCA. (A) Immunofluorescence staining with anti-phospho-STAT5 antibody (green) of a GCA artery cultured with placebo or mavrilimumab. (B) Quantification of positive cells per field A; this experiment was performed three times with similar results. (C) mRNA Spl1/PU.1 transcripts in 11 cultured GCA-affected temporal arteries in the presence of placebo or mavrilimumab. (D) Transcript levels for cell markers CD3ε, CD20, CD14, CD16 and CD68 in 11 cultured GCA-involved temporal arteries exposed to placebo or mavrilimumab. (E) Quantification of cells per field that are positive for anti-CD16, anti-CD3Ɛ, anti-CD68, and anti-CD20. (F) Immunofluorescence staining of cultured GCA-involved arteries in the presence of placebo or mavrilimumab with anti-CD16, anti-CD3Ɛ, anti-CD68, and anti-CD20 (red colour) and DAPI (blue). Representative of 3 GCA cultured arteries. Panel E is the quantification of panel F. DAPI, 4′,6-diamidino-2-phenylindole; GCA, giant cell arteritis.

cells not aligned around a lumen were also observed in lesions and were reduced by mavrilimumab.

DISCUSSION

This study demonstrates expression of GM-CSFRα, the target of mavrilimumab, within the lesions of GCA-affected arteries and confirms the increased production of GM-CSF previously reported.[24 40 41] Macrophages were the main cell type immunostained for GM-CSF and GM-CSFRα in inflamed arteries. Luminal endothelial cells and, to a lesser extent, intimal myofibroblasts and endothelial cells from vasa vasorum and neovessels also expressed GM-CSF along with a small subset of T cells, presumably ThGM-CSF cells.[30] GM-CSFRα was expressed mainly by macrophages, endothelial cells and intimal myofibroblasts, suggesting that these cell types would be the most responsive to GM-CSF.

Contrary to a report in granulomatosis with polyangiitis,[42] but similar to findings in other inflammatory conditions,[28–30] GM-CSF was barely detectable in serum from patients with GCA, with no differences from healthy individuals. This supports a paracrine function of GM-CSF in the inflammatory microenvironment and limits the utility of serum GM-CSF as a biomarker of disease activity.

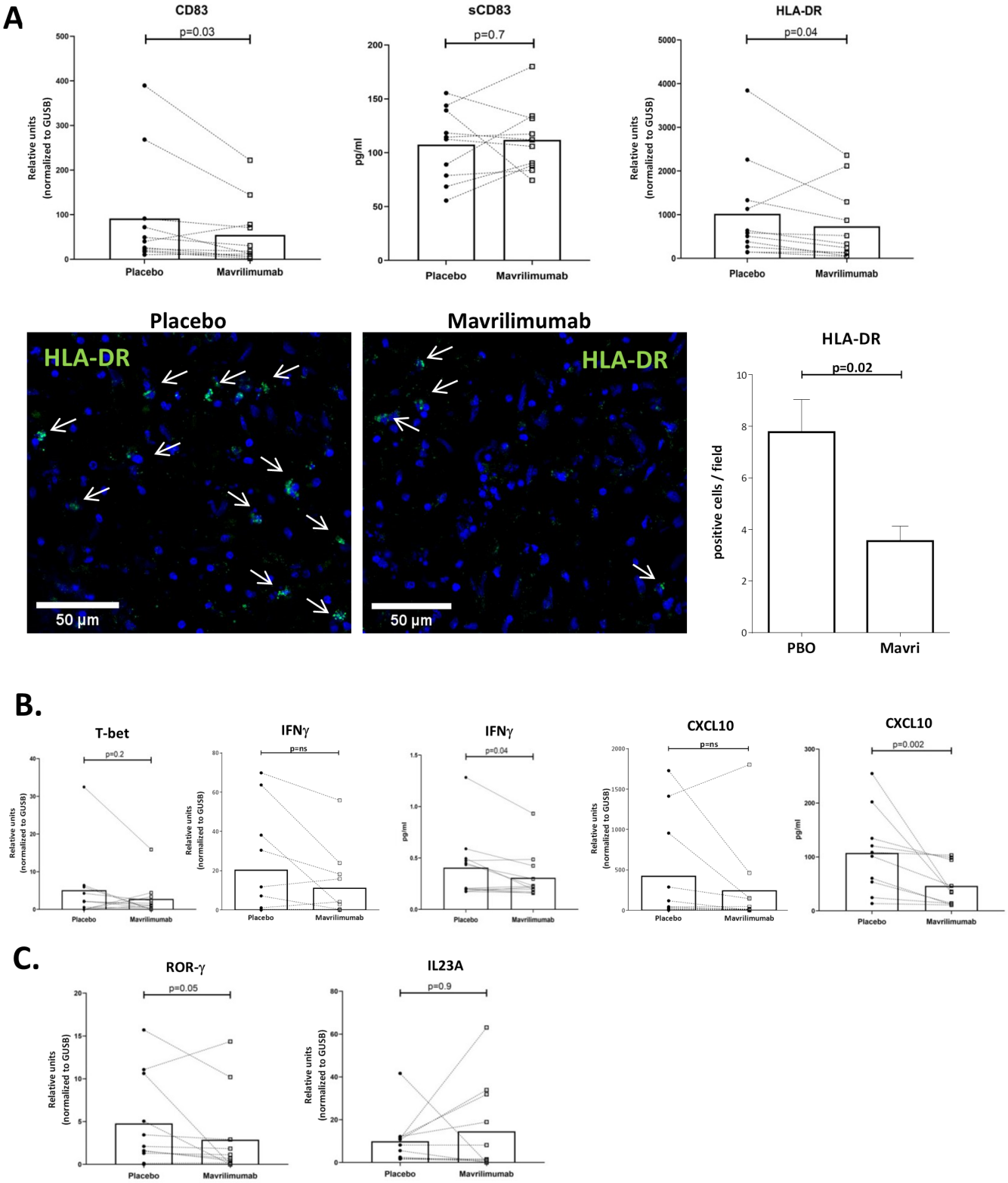


Figure 5 Mavrilimumab decreases molecules related to T lymphocyte activation and differentiation. (A) mRNA transcripts of CD83 (left) and HLA-DR (right) expressed in relative units and normalised to housekeeping gene *GUSB* in GCA-positive temporal arteries (n=11) cultured with placebo or mavrilimumab. Soluble CD83 measured (pg/mL) in supernatants of nine GCA cultured arteries exposed to placebo or mavrilimumab (central panel). Image shows HLA-DR expression by immunofluorescence in a GCA artery cultured with placebo or mavrilimumab. Images show detailed zoom amplification by confocal microscope with arrows indicating green HLA-DR-positive cells. Nuclei are stained with DAPI (blue). The graph on the right show the number of HLA-DR-positive cells per field in nine fields per section. Immunofluorescence was performed in two GCA cultured arteries, with consistent results. (B) mRNA transcripts of *TBX21* (T-bet), *IFNG* (IFNγ) and CXCL10 in GCA arteries cultured with placebo or mavrilimumab (n=11). IFN-γ and CXCL-10 proteins were also measured in artery culture supernatants of the same specimens. Results are expressed in pg/mL. (C) *RORC* (ROR-γ) and *IL-23A* mRNA measurement in cultured GCA arteries treated with placebo or mavrilimumab. DAPI, 4′,6-diamidino-2-phenylindole; GCA, giant cell arteritis; HLA-DR, human leukocyte antigen-DR; IFN, interferon.

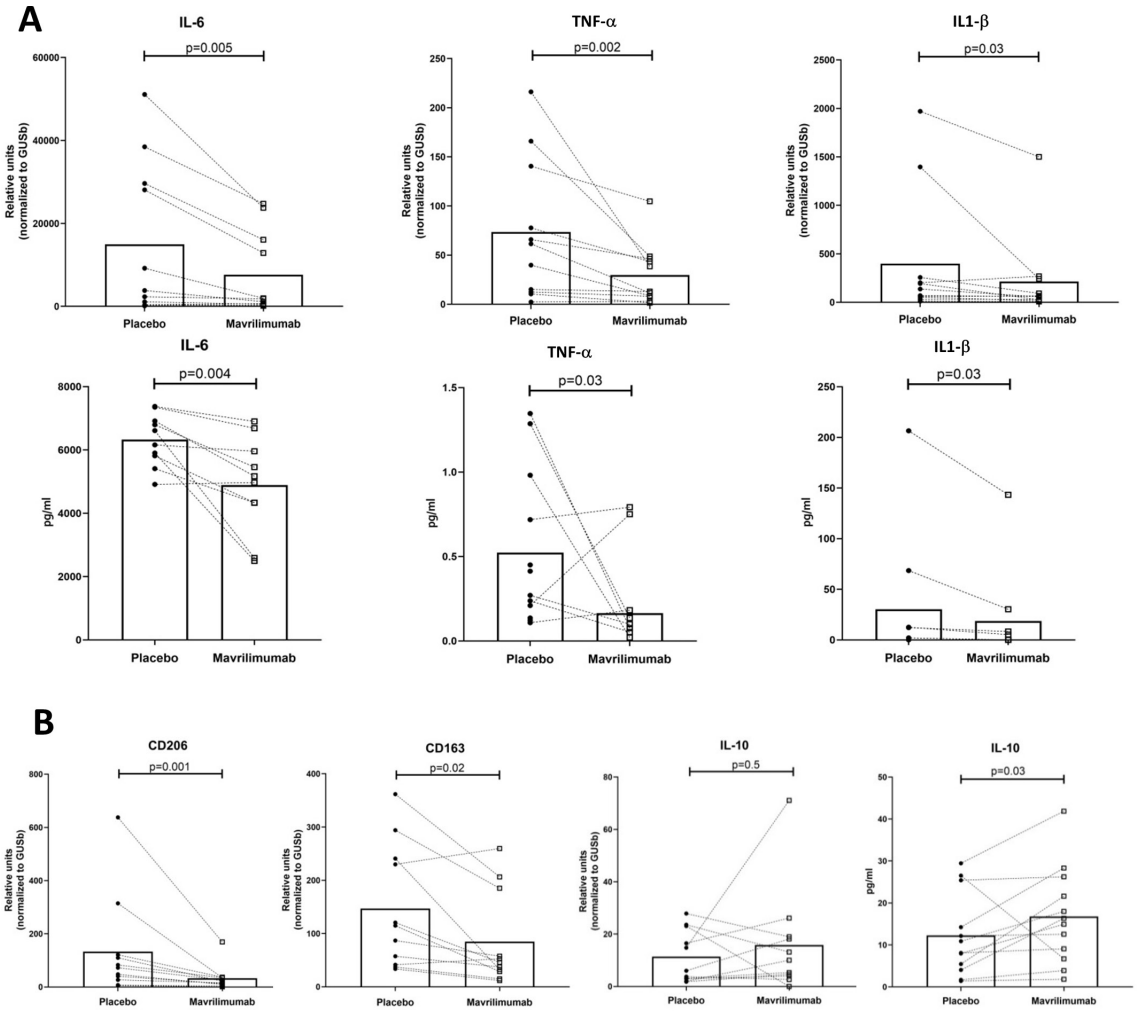


Figure 6 Mavrilimumab impacts macrophage functions. (A) Transcript levels of IL-6 (left), TNFα (central) and IL-1β (right) in GCA-positive arteries (n=11) exposed to placebo or mavrilimumab (mRNA, relative units). IL-6, tumour necrosis factor α (TNFα) and IL-1β proteins (pg/mL) were also measured in GCA artery culture supernatants of the same samples. (B) CD206, CD163 and IL-10 mRNA transcript levels in the same GCA arteries exposed to mavrilimumab or placebo. IL-10 protein (pg/mL) was also detected in the supernatant (right panel). GCA, giant cell arteritis; IL, interleukin.

Detection of JAK2 and STAT5A phosphorylation in GCA lesions, along with increased expression of paradigmatic STAT5-regulated molecules, such as CD83 and transcription factor Spi1/PU.1,[43] suggested activation of GM-CSF receptor-driven signalling pathways. Increased expression of additional relevant STAT5 or PU.1 regulated molecules, including major histocompatibility complex (MHC) class II molecule HLA-DR, adhesion molecules intercellular adhesion molecule 1 (ICAM-1) or vascular cell adhesion molecule 1 (VCAM-1), macrophage marker CD163, pro-inflammatory cytokines, such as IL-1 and TNFα, and metalloproteases such as MMP-9, has been previously demonstrated in GCA.[44–48] Although these pathways can be activated by other cytokines, these data suggest active GM-CSF signalling in GCA arteries and a contribution of GM-CSF to the increased expression of key molecules involved in the pathogenesis of GCA.

To confirm the participation of GM-CSFR-mediated signalling in the increased expression of these and additional relevant molecules and inflammatory cell markers, cultured temporal arteries from patients with histopathologically proven GCA were exposed to mavrilimumab. Treatment with mavrilimumab resulted in significantly decreased transcripts of lymphoid markers, including B lymphocyte surface molecule CD20 and T lymphocyte surface glycoprotein CD3ε. A significant decrease in classical monocyte marker CD14 and myeloid cell marker CD16 mRNAs was also observed. In contrast, there was no consistent change in the expression of CD68, a scavenger receptor widely expressed by macrophages.

Mavrilimumab decreased expression of molecules produced by dendritic cells and B cells, which are essential for antigen-presenting function/T cell activation, such as CD83 and HLA-DR.[49 50] This likely resulted in decreased Th1 differentiation, as indicated by reduced expression of Th1-related molecules, including IFNγ, TNFα and IFNγ-induced molecules such as CXCL10. Molecules related to Th17 differentiation, IL-1β and IL-6 were also decreased, but a more direct impact on IL-17 production could not be assessed. Although we and others have previously shown increased IL-17 expression in affected temporal arteries from patients with GCA,[18 21–23] baseline expression of IL-17 was very low in cultured arteries, possibly related to previous GC treatment in the majority of patients[18] or to the possible impact of culture on certain molecules.[36]

Mavrilimumab had a significant impact on pro-inflammatory functions of macrophages and endothelial cells, including expression of IL-1β, TNFα and IL-6, and expression of adhesion molecules for leucocytes. It also tended to increase expression and release of the anti-inflammatory cytokine IL-10, produced by regulatory T cells and B cells and M2-type macrophages.[51] Mavrilimumab reduced MMP-9 expression with no change in

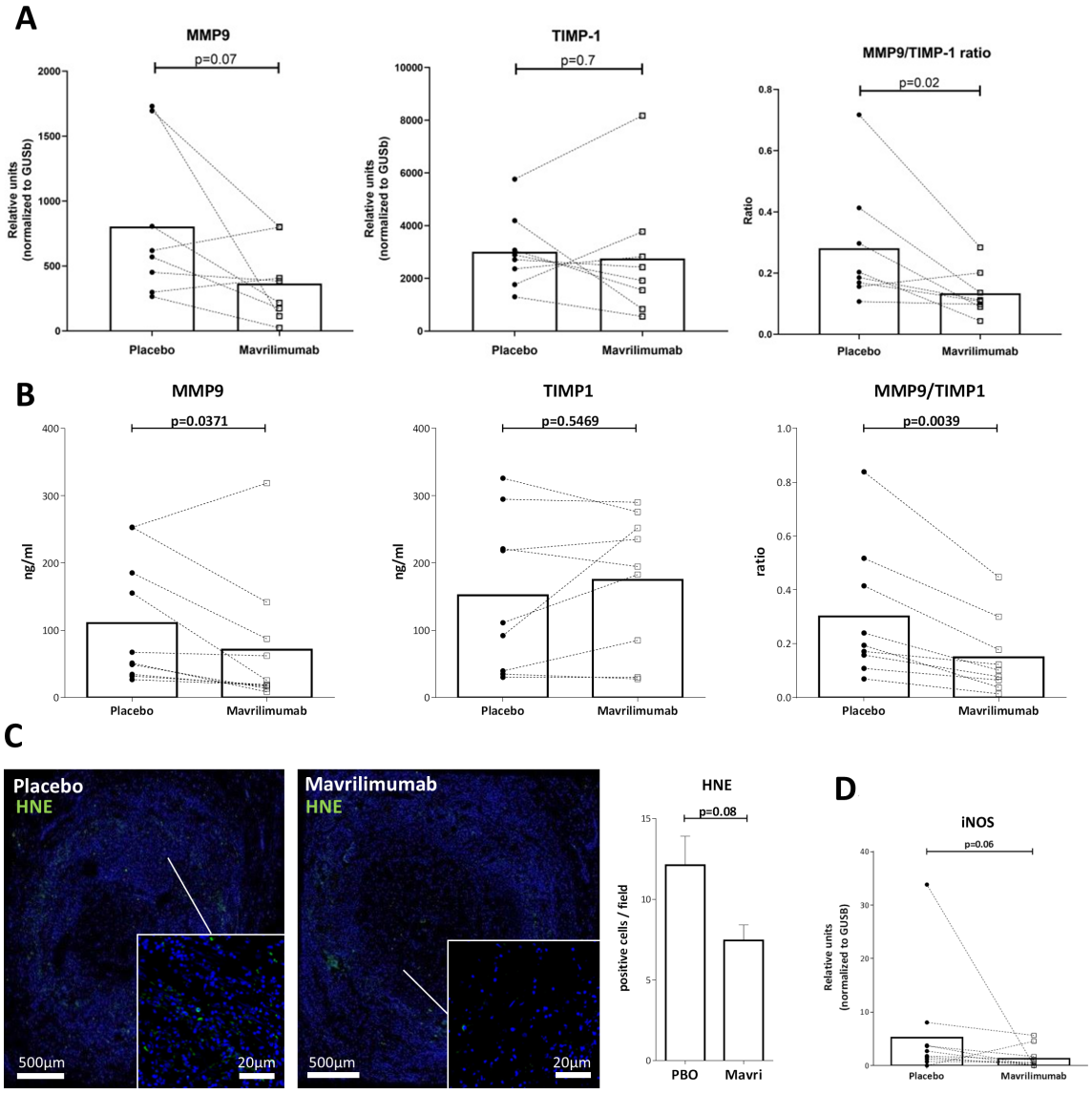


Figure 7 Effect of mavrilimumab on molecules related to vascular injury. (A) Transcripts of MMP-9, tissue inhibitor of metalloproteinases 1 (TIMP-1) and MMP-9/TIMP-1 mRNA ratio in 8 GCA-positive temporal arteries cultured with placebo or mavrilimumab. (B) MMP-9, TIMP-1 protein concentration and MMP-9/TIMP-1 protein ratio in the corresponding supernatants (ng/mL). (C) Immunofluorescence staining of HNE (green) with nuclei (in blue) in a GCA-involved artery cultured with placebo or mavrilimumab, and its quantitation (right panel). Immunofluorescence was performed in two GCA cultured arteries, with consistent results. (D) *NOS2* transcripts in 11 cultured GCA arteries exposed to placebo or mavrilimumab. GCA, giant cell arteritis; HNE, 4-hydrynonenal; MMP-9, matrix metalloprotease 9.

expression of its natural inhibitor TIMP-1, thereby suggesting a shift in the MMP-9 proteolytic balance.[47] Proteolytic enzyme MMP-9 has elastinolytic activity and may contribute to elastin degradation since it is expressed and activated in GCA lesions and in aortic tissue.[52] MMP-9 may also contribute to GM-CSF-induced aneurysm formation, shown in an animal model.[53] Macrophages present in GCA lesions have oxidative capacity as indicated by the presence of lipid peroxidation products (HNE) in GCA lesions.[27] Treatment with mavrilimumab decreased HNE presence in cultured arteries indicating that mavrilimumab decreases oxidative damage in inflamed arteries.

The tuning in macrophage function induced by mavrilimumab does not parallel classical M1 (pro-inflammatory) or M2 (anti-inflammatory, reparative) phenotypes. Mavrilimumab reduced M1 markers, including HLA-DR and iNOS, and tended to increase M2 cytokine IL-10. However, mavrilimumab also reduced CD206 and CD163, which have been considered markers of M2 phenotype.[54] It is important to remark that this distinction has been established mostly in in vitro differentiated macrophages or in murine models. In humans, plasticity of macrophages is far more complex.[54] For example, macrophages co-expressing CD206 and MMP-9 have been observed in GCA lesions[41] and a population of pro-inflammatory CD14 HLA-DR^{high} CD206^{+} macrophages has been identified in human viral hepatitis.[55] Overall, mavrilimumab decreased the inflammatory and destructive potential of macrophages.

GM-CSF influences endothelial cell behaviour and stimulates angiogenesis in experimental systems.[56] Accordingly, mavrilimumab reduced microvessel density in GCA lesions. In addition to its potential direct effects, our results indicate that GM-CSF regulates VEGFA production. Since CD34 is expressed not only by endothelial cells from neovessels but also by HSC, which have recently been identified in chronic inflammatory lesions and promoted by GM-CSF,[57 58] we cannot exclude the possibility

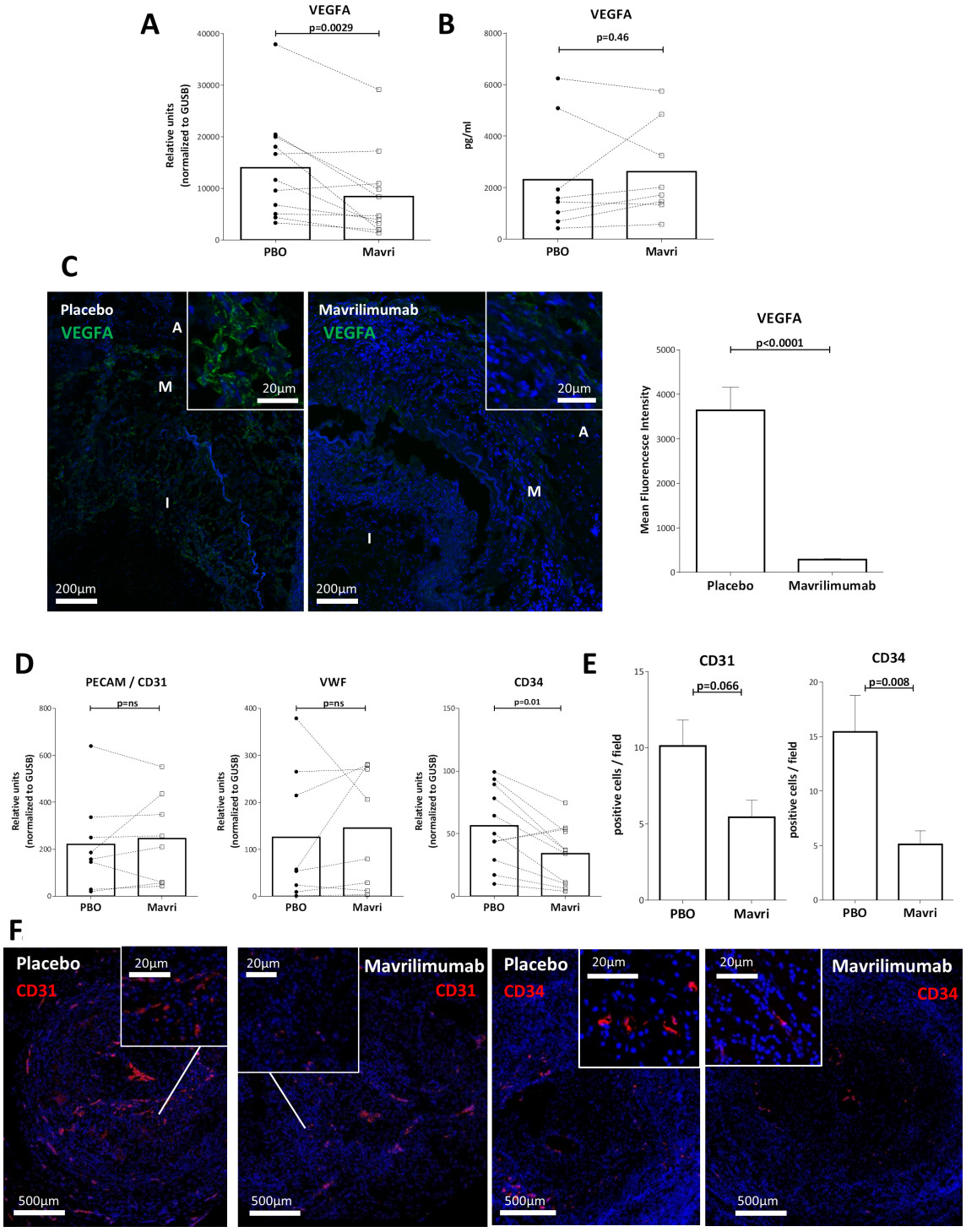


Figure 8 Mavrilimumab effect on angiogenesis. (A) Detection of vascular endothelial growth factor A (VEGFA) transcripts in 11 GCA-positive temporal arteries cultured with placebo or mavrilimumab. (B) Detection of VEGFA protein (pg/mL) in supernatants of eight respective arteries cultured with placebo or mavrilimumab. (C) Immunofluorescence with anti-VEGFA antibody of a GCA artery cultured with placebo or mavrilimumab (I, intima; M, media; A, adventitia). The graph on the right shows quantification of mean fluorescence intensity of the entire artery wall. (D) Measurement of PECAM-1 (n=8), vWF (n=8) and CD34 (n=11) transcripts in GCA cultured temporal arteries treated with placebo or mavrilimumab (relative units, normalised to housekeeping *GUSB*). (E) Quantification (positive cells per field) of immunofluorescence. Immunofluorescence was performed on two cultured biopsies with consistent results. (F) Immunofluorescence with anti-CD31 or anti-CD34 antibody of a GCA artery cultured with placebo or mavrilimumab. Inset images show zoom amplifications of positive (red) cells in areas of interest across the neointimal layer. Panel E is the quantification of panel F. GCA, giant cell arteritis.

that some detected CD34 + cells were ectopic HSC. Mavrilimumab reduction of ectopic HSC may be a potential new relevant effect of mavrilimumab. Since neoangiogenesis is prominent in GCA lesions, and newly formed capillaries express adhesion molecules and recruit inflammatory leucocytes into arteries,[45 57] mavrilimumab could indirectly reduce leucocyte recruitment by decreasing neoangiogenesis in addition to its direct effects on myeloid and other cells

Our study has limitations, including the relatively small number of cases investigated, inherent to the low incidence of GCA and the need of viable fresh tissue. In addition, our model explores changes induced by mavrilimumab in a target organ isolated from a functional immune system. However, the effects of mavrilimumab observed were consistent with the known functions of GM-CSF obtained in a variety of experimental systems. Furthermore, due to the small amount of available tissue, our experiments were limited to a single time-point. We cannot exclude that effects could be more prominent at other time points. Finally, most arteries were obtained from patients who had previously received GC treatment, as currently advised by international guidelines on GCA suspicion.[59] Previous GC exposure reduces baseline expression of a variety of molecules, including GM-CSF.[36 60] It would be possible that using treatment-naïve samples, changes would have been more prominent. However, this setting better reflects the real world and mavrilimumab still adds to potential GC effects on key inflammatory molecules.

In summary, this study reveals for the first time, functional changes induced by mavrilimumab in a classical target tissue of GCA. Mavrilimumab impacts inflammatory pathways considered relevant to the pathogenesis of vascular inflammation and injury, and the results from a recent phase 2 trial in which mavrilimumab was superior to placebo (both with 26-week prednisone taper) in reducing the risk of GCA flare and maintaining sustained remission[35] validated the role of GM-CSF in GCA.

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Contributors MCC and JFP are responsible for the overall content as the guarantor. MCC and JFP designed and supervised the work. MC-B, RA-R, FK and AD performed the experiments. GE-F, RR-G and JM-H performed accurate patient selection and collected biological samples. SM, AJ, AD'A and KB supervised experiments. MCC, MC-B and RA-R made the initial manuscript draft. All authors contributed intellectual input, revised data and revised and approved the manuscript.MCC dedicates her contribution to the Department of Oncology, Hospital Clínic, Barcelona, particularly to the Oncology specialists Montserrat Muñoz, Meritxell Molla and Immaculada Alonso for their excellent professional care and encouragement throughout the development of this study. Without their support, her contribution would have not been possible.

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Competing interests SM, AJ and AD'A report employment by Kiniksa Pharmaceuticals Corp. during development of the manuscript. KB and JFP report current employment by Kiniksa Pharmaceuticals Corp. MCC reports consulting fees from GSK, Abbvie, Vifor and Janssen, and a research grant from Kiniksa Pharmaceuticals Corp. GE-F reports consulting fees from Janssen.

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Data availability statement Data are available upon reasonable request. The individual anonymised data supporting the analyses contained in the manuscript will be made available upon reasonable written request from researchers whose proposed use of the data for a specific purpose has been approved. Data will not be provided to requesters with potential or actual conflicts of interest, including individuals requesting access for commercial, competitive or legal purposes. Data access may be precluded for studies in which clinical data were collected subject to legal, contractual or consent provisions that prohibit transfer to third parties. All those receiving access to data will be required to enter into a Data Use Agreement, which shall contain terms and conditions that are customary for similar agreements and similar companies in the industry. For requests, please email JFP, Kiniksa Pharmaceuticals's Chief Medical Officer, at jpaolini@kiniksa.com.

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