Interleukin-6 (IL-6)/IL-6 receptor and persistence of inflammation in Giant Cell Arteritis. Effects of IL-6 receptor blockade with tocilizumab

Nekane Terrades Garcia

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Interleukin-6 (IL-6)/IL-6 receptor and persistence of inflammation in Giant Cell Arteritis. Effects of IL-6 receptor blockade with tocilizumab

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for the degree of Doctor by the University of Barcelona

Thesis directed by Maria Cinta Cid Xutglà

2018
## Index

Abbreviations ........................................................................................................................................ 11

Introduction .......................................................................................................................................... 17

Giant Cell Arteritis ................................................................................................................................. 19

  History and epidemiologic characteristics .......................................................................................... 19

  Clinical manifestations and diagnosis ................................................................................................. 19

  Pathophysiologic basis of GCA ........................................................................................................ 20

  Functional models .............................................................................................................................. 26

  Treatment ........................................................................................................................................... 27

  IL-6 ..................................................................................................................................................... 29

    Discovery and function ....................................................................................................................... 29

    Structure and expression .................................................................................................................. 30

    IL-6 receptors complex .................................................................................................................... 31

    IL-6 signaling pathway ..................................................................................................................... 33

    IL-6 and IL-6R in GCA ..................................................................................................................... 35

  TCZ .................................................................................................................................................... 35

  Biomarkers for TCZ treated patients ................................................................................................. 39

    Osteopontin ..................................................................................................................................... 39

    OPN as a biomarker in GCA patients ............................................................................................... 40

Citation

Hypothesis .............................................................................................................................................. 43

Aims of the study .................................................................................................................................. 43

Methods ................................................................................................................................................. 47

Patients serum ...................................................................................................................................... 49

IL6 and sIL-6R ...................................................................................................................................... 49
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPN</td>
<td>49</td>
</tr>
<tr>
<td>Temporal artery biopsy (TAB) processing</td>
<td>49</td>
</tr>
<tr>
<td>Culture of TABs</td>
<td>50</td>
</tr>
<tr>
<td>Cell culture</td>
<td>50</td>
</tr>
<tr>
<td>Primary cultures of VSMC</td>
<td>50</td>
</tr>
<tr>
<td>Peripheral blood mononuclear cells (PBMC)</td>
<td>51</td>
</tr>
<tr>
<td>Co-cultures of VSMC and PBMC</td>
<td>51</td>
</tr>
<tr>
<td>HUVEC culture</td>
<td>52</td>
</tr>
<tr>
<td>Cell culture with IL-6, sIL-6R and TCZ</td>
<td>52</td>
</tr>
<tr>
<td>RNA extraction</td>
<td>53</td>
</tr>
<tr>
<td>Reverse transcription and quantitative real-time PCR</td>
<td>53</td>
</tr>
<tr>
<td>Protein cell lysates</td>
<td>54</td>
</tr>
<tr>
<td>Protein quantification</td>
<td>55</td>
</tr>
<tr>
<td>Immunoblot</td>
<td>55</td>
</tr>
<tr>
<td>Enzyme-Linked Immunosorbent Assay (ELISA)</td>
<td>56</td>
</tr>
<tr>
<td>Immunofluorescence of TABs</td>
<td>56</td>
</tr>
<tr>
<td>Cell migration assay in Boyden chambers</td>
<td>57</td>
</tr>
<tr>
<td>Adhesion assay</td>
<td>58</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>58</td>
</tr>
<tr>
<td>Results</td>
<td>59</td>
</tr>
<tr>
<td>Distribution of IL-6 and IL-6R in GCA lesions</td>
<td>61</td>
</tr>
<tr>
<td>IL-6 and IL-6R expression in GCA lesions and patients’ serum</td>
<td>64</td>
</tr>
<tr>
<td>Under inflammatory conditions VSMCs contribute to IL-6 production whereas PBMCs increase expression of IL-6R</td>
<td>65</td>
</tr>
<tr>
<td>Effect of IL-6R blockade with TCZ on STAT3 phosphorylation in cultured GCA arteries and in co-cultured VSMC and PBMC</td>
<td>67</td>
</tr>
<tr>
<td>IL-6R blockade with TCZ selectively decreases STAT3 and SOCS3 mRNA expression in cultured GCA arteries</td>
<td>69</td>
</tr>
</tbody>
</table>
TCZ treatment modify chemokine expression in cultured GCA arteries by decreasing CCL2 and increasing CXCL9 and CXCL10 ................................................................. 71
CXCL9 and CXCL10 upregulation could be explained by a modulation of STAT1 induced by TCZ blockade ........................................................................................................ 73
Functional impact of TCZ treatment on PBMCs: reduction in adhesion and migration ..... 74
OPN as an activity biomarker for patients with GCA treated with TCZ ......................... 75
OPN expression and secretion in cultured GCA arteries is not suppressed by short-term IL-6 receptor blockade ................................................................................................. 75
Serum OPN is reduced but not abrogated in GCA patients in remission treated with tocilizumab ...................................................................................................................... 76
Discussion .......................................................................................................................... 79
Conclusions ....................................................................................................................... 89
References ......................................................................................................................... 93
Additional data .................................................................................................................. 105
Annex ............................................................................................................................... 109
Abbreviations
A

ADAM: A Disintegrin And Metalloproteinase
ANCA: Antineutrophil Cytoplasmic Antibody

B

BCL: B-cell lymphoma
BSA: Bovine Serum Albumin

C

CAR: Chimeric Antigen Receptor
CCL: Chemokine C-C motif ligand
CCR7: C-C chemokine receptor type 7
CD: Cluster of differentiation
cDNA: Complementary deoxyribonucleic acid
CLC: Cardiotropin-Like Cytokine
CNTF: Ciliary Neurotrophic Factor
COL1A1: Collagen type I alpha 1 chain
COL3A1: Collagen type III alpha 1 chain
CRP: C-reactive protein
CRS: Cytokine Release Syndrome
CT-1: Cardiotropin-1
CTLA-4: Cytotoxic T-Lymphocyte Antigen-4
CXCL: Chemokine C-X-C motif ligand
CXCR: Chemokine C-X-C motif receptor

D

DAPI: 4′,6-diamidino-2-phenylindole
DC: Dendritic cells
DMEM: Dulbecco's Modified Eagle Medium
DNA: Deoxyribonucleic acid

E

EDTA: Ethylene-diamine-tetra acetic acid
EGF: Epidermal growth factor
ELISA: Enzyme-Linked Immunosorbent Assay
ESR: Erythrocyte sedimentation rate
ET-1: Endothelin-1
<table>
<thead>
<tr>
<th>F</th>
<th>FCS: Fetal calf serum</th>
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<tbody>
<tr>
<td></td>
<td>FBS: Fetal bovine serum</td>
</tr>
<tr>
<td></td>
<td>FDA: Food and Drug Administration</td>
</tr>
<tr>
<td></td>
<td>FGF-2: Fibroblast growth factor-2</td>
</tr>
<tr>
<td></td>
<td>FLS: Fibroblast-like synovial cells</td>
</tr>
<tr>
<td></td>
<td>FOXP3: Forkhead box P3</td>
</tr>
<tr>
<td>G</td>
<td>GATA3: GATA binding protein 3</td>
</tr>
<tr>
<td></td>
<td>GCA: Giant Cell Arteritis</td>
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<tr>
<td></td>
<td>GUSB: Glucuronidase beta</td>
</tr>
<tr>
<td>H</td>
<td>HLA: Human leucocyte antigen</td>
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<tr>
<td></td>
<td>HUVEC: Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>I</td>
<td>ICAM-1: Intracellular adhesion molecule 1</td>
</tr>
<tr>
<td></td>
<td>IFN-γ: Interferon gamma</td>
</tr>
<tr>
<td></td>
<td>IgG: Immunoglobulin G</td>
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<tr>
<td></td>
<td>IL-: Interleukin-</td>
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<tr>
<td></td>
<td>IL-6R: IL-6 receptor</td>
</tr>
<tr>
<td>J</td>
<td>JAK: Janus kinase</td>
</tr>
<tr>
<td>L</td>
<td>LIF: Leukemia inhibitory factor</td>
</tr>
<tr>
<td>M</td>
<td>MAPK: Mitogen-activated protein kinase</td>
</tr>
<tr>
<td></td>
<td>MHC: Major histocompatibility complex</td>
</tr>
<tr>
<td></td>
<td>MMP: Matrix metalloproteinase</td>
</tr>
<tr>
<td></td>
<td>miR: microRNAs</td>
</tr>
<tr>
<td></td>
<td>mRNA: Messenger ribonucleic acid</td>
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<tr>
<td></td>
<td>MTX: Methotrexate</td>
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</table>
NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NOS2: Nitric oxide synthase 2
NPN: Neuropoietin

OPN: osteopontin
OSM: osteoclastin M

p: Phospho
P4HA2: Prolyl 4-hydroxylase subunit alpha 2
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate buffer saline
PD-1: Programmed death-1
PDGF: Platelet-derived growth factor
PD-L1: Programmed death ligand-1
PDN: Prednisone
PFA: Paraformaldehyde
PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIAS: Protein inhibitor of activated STATs
PMR: Polymyalgia Rheumatica
PMSF: Phenylmethylsulfonyl fluoride
PPR: Pattern recognition receptors

RA: Rheumatoid Arthritis
RIPA: Radio-Immunoprecipitation Assay
RNA: Ribonucleic acid
RORC: RAR related orphan receptor C
ROS: Reactive oxygen species
RPMI-1640: Roswell Park Memorial Institute-1640
RU: Relative units

s: Soluble
SAA: Serum amyloid A
**SDS**: Sodium dodecyl sulfate  
**SMA**: Smooth Muscle Actin  
**SOCS**: Suppressor of cytokine signaling  
**STAT**: Signal transducer and activator of transcription  

**TAB**: Temporal artery biopsy  
**TBST**: Tris Buffer Saline 1X with 0.1% Tween-20  
**TBX21**: T-box 21  
**TCZ**: Tocilizumab  
**Tfh**: T follicular helper cells  
**TGF-β**: Transforming growth factor beta  
**Th**: T-helper-  
**TLR**: Toll-like receptor  
**TNF-α**: Tumor necrosis factor alpha  
**Treg**: Regulatory T cells  

**VCAM-1**: Vascular cell adhesion molecule-1  
**VEGF**: Vascular endothelial growth factor  
**VSMC**: Vascular smooth muscle cells
Introduction
Giant Cell Arteritis

History and epidemiologic characteristics

Giant cell arteritis (GCA) is a chronic granulomatous vasculitis affecting large- and medium-sized vessels, particularly the carotid and its extracranial branches \(^1\to2\). However, as a systemic disease, there are other extracranial territories that may be affected, especially the aorta and its proximal branches \(^3\).

Epidemiological studies report differences in the incidence of GCA among ethnic groups, that ranges from 1.1–32.8 cases per 100,000 individuals aged ≥50 years depending on geographic location, with Scandinavians and North Americans having the highest incidence rates \(^4\to5\).

GCA affects patients older than 50 years showing a female predominance with women being two to three times more frequently affected than men. Its incidence markedly rises with increasing age, with a peak taking place between 70 and 80 years \(^6\to7\).

Clinical manifestations and diagnosis

GCA can lead to different symptoms related to vascular or systemic inflammation \(^1,3,8,9,10\). Common ischemic complications, due to persistent inflammation, include jaw claudication and visual loss. Visual manifestations represent the most severe complication and they usually range from transient diplopia and amaurosis fugax to sudden unilateral or bilateral partial or complete permanent visual loss. These visual manifestations may occur as a consequence of acute anterior ischemic optic neuritis, central retinal artery occlusion or retro-bulbar optic neuritis. Permanent visual loss affects ~15% of patients and once it is established, it is almost always permanent, but it can be prevented by early intervention \(^3\).

Systemic inflammation, derived from the acute-phase response associated to chronic inflammation, results in a wide range of symptoms such as fever, headache, anorexia, scalp tenderness, progressive weight loss, asthenia, general malaise or night sweats. GCA patients may also develop polymyalgia rheumatica (PMR) symptoms due to proximal bursitis and tenosynovitis, characterized by severe aching pain and morning stiffness in the neck, shoulder and pelvic girdles \(^1,3\).

Due to the acute-phase response GCA patients often present a significant elevation of the erythrocyte sedimentation rate (ESR) and an increase in acute-phase proteins such as C-reactive protein (CRP), haptoglobin or fibrinogen as well as proinflammatory cytokines like interleukin- (IL-) 6 (IL-6) or tumor necrosis factor alpha (TNF-α). These laboratory analytical findings can be very useful to monitor disease activity \(^9,11\).
In terms of diagnosis, 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. Imaging methods are also used for the diagnosis of GCA but despite they have proved to be important tools abnormal TAB findings still provide the best diagnostics specificity. Beyond their diagnostic value, TAB samples are an important source of tissue with great utility for investigating the pathogenesis of GCA.

The classical histological alterations of GCA consist of an inflammatory infiltrate in all three layers of the arterial wall with disruption of the internal elastic lamina and intimal thickening. Intimal hyperplasia may result in a partial or complete lumen occlusion leading to the ischemic complications typically observed in GCA. The infiltrating cells mainly comprise 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. Considerable histological variations can be found in GCA samples, not only among different patients, but also within the same sample, partially due to the so-called “skip lesions” referring to the segmental distribution of the inflammatory infiltrate.

![Figure 1](image.png)

**Figure 1.** Histopathological changes induced by GCA in temporal arteries. A. Normal temporal artery biopsy with clearly defined layers (I: intima; M: media; Ad: adventitia) and a preserved internal elastic lamina (arrowheads). B. Temporal artery biopsy from a patient with GCA highlighting the presence of typical transmural mononuclear infiltration (arrows) with disappearance of the organized medial layer and internal elastic lamina, along with prominent intimal hyperplasia (IH). Arrowheads point to some of the numerous giant-cells. Image from Terrades-Garcia and Cid, 2017 (in press).

**Pathophysiologic basis of GCA**

GCA seems to be the result of a complex interplay between three different factors: the individual genetic background, the environment and the specific immune system response. However, the exact etiology of the disease remains to be elucidated.
Genetic background

A genetic component on the development of GCA is supported by the high incidence in northern European countries and in northern European descendents along with observations of sporadic family clustering of affected members. Certainly, in candidate gene studies, an increase in GCA risk has been associated with polymorphisms in a variety of genes encoding molecules participating in immune, inflammatory, and vascular responses, such as IL-10, vascular endothelial growth factor (VEGF), nitric oxide synthase 2 (NOS2), IL-6, TNF, or intracellular adhesion molecule-1 (ICAM1), among others. However, it must be taking into account that there has been some contradictory results as it was the case for ICAM1 and also the fact that some of the results were not confirmed in other studies with independent cohorts.

A strong association has been found between GCA and genetic variants in the major histocompatibility complex (MHC) region, particularly with class II HLA-DRB1*04 alleles (usually DRB1*0401, but also DRB1*0404). Importantly, a large-scale fine mapping of genes related to immune responses has recently 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. This finding reinforces the role of the adaptive immune system in 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 recently related to increased GCA risk at genome-wide significance.

The environment

The environment can also be involved in the pathogenesis of GCA. Because of the cyclic patterns, geographical variations and seasonal fluctuations in the incidence of GCA the role of infectious agents in the etiology of the disease has been suspected. Different studies apparently found an association between GCA and the presence of cytomegalovirus, parvovirus B19, herpes simplex virus, human parainfluenzae 1, varicella zoster virus and Chlamydia pneumonia. However, until present, no clear causal relationship with a particular microorganism or viral agent has been demonstrated. A variety of pathogen sequences have been detected in temporal arteries but none of them have been consistently associated with GCA.

The reason why GCA affects people over 50 years and its increased incidence with age is not completely understood yet, but these facts point out that aging may play an important role in the pathogenesis of the disease. Multiple cells participating in the immune response and
vascular remodeling such as T cells, dendritic cells (DC), endothelial cells and vascular smooth muscle cells (VSMC) seem to be modified by the aging process. In addition, arterial vessels suffer structural and tissue modifications with age that, together with cellular alterations, might partially explain the impact of age in GCA development.

Finally, several proinflammatory genes have been reported to have an altered DNA methylation levels in temporal arteries from GCA patients. Epigenetic modifications promoted by environmental factors may modify gene expression altering the predisposition to GCA.

The immune system. Pathogenesis

The inflammatory response in GCA is probably initiated by DC, located at the media-adventitial junction, after their maturation from a non-stimulatory to a T-cell activating state. In normal arteries, these cells are suggested to act as sentinels conforming the first line immune defense of the vessel wall. Immature DCs are tolerogenic, thus supporting T-cell unresponsiveness. However, through the expression of pattern recognition receptors (PPR) such as toll-like receptor (TLR), these cells are able to sensing danger signals and initiate the immune response. After their activation via TLR, DCs start to produce chemokines, such as C-C motif ligand (CCL) 9 and 21 (CCL9 and CCL21, respectively), that induce the recruitment of CD4+ T cells as well as additional DCs that will be retained in the vessel wall due to the expression of the receptor of this chemokines (C-C chemokine receptor type 7; CCR7). In addition, DC express activation and co-stimulatory molecules (CD83 and CD86) making them able to activate CD4+ T cells (figure 2.A).

Recruited CD4+ T cells infiltrate the adventitia via vasa vasorum, where endothelial cells express adhesion molecules such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1). Programmed death-1 (PD-1) receptor is a surface protein expressed by activated T cells that binds to its ligand named programmed death ligand-1 (PD-L1) which is expressed in antigen presenting cells. Interaction between these two proteins induces T-cell apoptosis, T-cell anergy and the production of IL-10 by T cells or their polarization towards regulatory T (Treg) cells. A defect in this immune checkpoint has been recently observed in GCA-affected temporal arteries and it is thought to contribute to the excessive infiltration of activated T cells into medium- and large-size vessels. Once CD4+ T cells are activated they polarize toward T-helper-1 (Th1) and T-helper-17 (Th17) cells depending on the proinflammatory cytokines in the microenvironment (figure 2.A). Th1 cells are generated in the presence of IL-12 and IL-18 and produce interferon gamma (IFN-γ), a pro-inflammatory cytokine that has been shown in GCA-involved arteries (figure 2.B). IFN-γ is a potent activator of macrophages, the predominant cell population in GCA lesions, and is thought to drive the granulomatous
reaction and transformation of macrophages to giant cells in these lesions. Furthermore, recently published results showed that IFN-γ induces in vitro production of chemokines and adhesion molecules by VSMC which, in turn, can trigger the recruitment of other immune cells initiating a positive forward loop supporting the progression of inflammatory infiltrates to the artery wall.

Pro-inflammatory cytokines promoting the Th17 differentiation such as IL-1β, IL-21, transforming growth factor beta (TGF-β), IL-6 and IL-23 have been also observed in GCA patients. As a result, the generated Th17 cells produce IL-17A which expression has been seen increased in GCA lesions (figure 2.B). This cytokine has pleiotropic effects on a variety of cells including macrophages, neutrophils, endothelial cells and fibroblasts, and actively contributes to inflammatory cascades. It seems possible that T cell polarization may depend on the microenvironment, showing some plasticity between these cells under different conditions such as GCA treatment and/or the natural history of the disease that can modulate the milieu. In addition to Th1 and Th17 cells, CD8+ T cells also infiltrate the artery wall in response to chemokines and can produce cytokines and cytotoxic molecules such as granzymes and perforin (figure 2.A).

IFN-γ induces the differentiation of recruited monocytes into macrophages. Macrophages produce IL-6, IL-1β and TNF-α, which amplify the local inflammatory response. In addition, these cells produce wall damage through several mechanisms including reactive oxygen species generation, that cause lipid peroxidation of phospholipids, or matrix metalloproteinase-(MMP) 9 production, that may contribute to the destruction of the media and digestion of the internal elastic lamina (figure 2.C and 2.D). MMP-9 and MMP-2, mainly produced by macrophages and VSMC respectively, are up-regulated in GCA lesions. Their elastinolytic activity observed in GCA lesions may contribute to the disruption of elastic fibers and abnormal vascular remodeling (figure 2.D).

Macrophages, giant cells or injured VSMC also produce angiogenic factors, such as VEGF, fibroblast growth factor-2 (FGF-2) and platelet-derived growth factors (PDGFs) (figure 2.D). These growth factors can promote the formation of new vessels which in turn facilitates the recruitment of additional immune cells in the arterial wall. Because of this neoangiogenesis process, vasa vasorum can be observed in the media and the intima of GCA arteries (figure 3).
Figure 3. Neovessel formation in GCA lesions. Immunofluorescence staining of endothelial cells in yellow using an antibody against CD31. Nuclei are show in blue and were stained with DAPI. A: adventitia; I: intima; L: lumen; M: media. Image from Terrades-Garcia and Cid, 2017 (in press).

Growth factors as PDGFs, as well as other molecules including TGF-β1 and endothelin-1 (ET-1) are expressed in GCA lesions and may contribute to vascular remodeling inducing myofibroblast differentiation of VSMC, migration towards the intimal layer and deposition of extra-cellular matrix proteins thus resulting in intimal hyperplasia \textsuperscript{49, 63, 64, 65}. Indeed, blockade of PDGF receptor by imatinib mesylate or blocking ET-1 receptors (A and/or B) result in reduced myointimal cell outgrowth from cultured temporal arteries of patients with GCA \textsuperscript{60, 65}.

Additional mechanisms contributing to GCA pathogenesis

Although GCA has been primarily considered a T-cell mediated disease, B-lymphocytes are crucial to T cell activation. B cell lymphocytes are not abundant in GCA, however, their presence has been observed in vascular inflammatory lesions \textsuperscript{66, 67, 68}, and more recently forming tertiary lymphoid structures \textsuperscript{69}. Circulating levels of B cells are decreased in patients with active GCA but recovered with glucocorticoid treatment and are thought to be recruited into inflamed vessels \textsuperscript{68}. B-cell depletion in relapsing patients using rituximab, a monoclonal antibody against CD20, has reported therapeutic benefit. However, the efficacy of rituximab has only been described in two isolated case reports and has not been further investigated \textsuperscript{70, 71}. Different auto-antibodies have been detected in sera from patients with GCA including anti-ferritin antibodies, anti-endothelial or anti-vascular smooth muscle cell antibodies recognizing
different antigens (i.e., vinculin, annexin V, among others) \(^{72, 73}\). However, since there is no specific association with GCA it is likely that many of these antibodies are generated because of inflammation and tissue injury rather than having a primary pathogenic role \(^{74}\).

**Functional models**

To date, most of the conclusions related to GCA pathogenesis are based on the previously known functions of the molecules identified in observational studies and their correlation with clinical or histological abnormalities. In addition, two functional models based in the use of TAB have also help to better understand the pathogenic process.

Subcutaneous engraftment of GCA-involved TA fragments into mice with severe combined immunodeficiency have been used for functional studies. Using this model, it was possible to postulate an important role of dendritic cells in GCA pathogenesis since their depletion reduce inflammation in the explants \(^{37}\). 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 \(^{75}\). This model has also been useful to detect changes in cytokine expression in TA after pharmacological treatment of engrafted mice with corticosteroids \(^{76, 77}\).

More recently a TA culture in 3-D matrix has been introduced to investigate pathogenic pathways. As an advantage, this model substitutes the use of mouse with severe combined immunodeficiency by Matrigel\(^{\text{TM}}\) as a biological support reducing the complexity and expense \(^{47}\). In this model, it has been shown that glucocorticoids decrease production of inflammatory cytokines but do not influence factors involved in vascular remodeling \(^{47}\).

Using this *ex vivo* system it was observed that IFN-\(\gamma\) may play an important role in the recruitment of macrophages in GCA by inducing production of specific chemokines and adhesion molecules \(^{48}\). Blocking PDGF receptor signaling with imatinib or ET-1 signaling with receptor antagonists has been shown to reduce myointimal cell outgrowth \(^{60, 65}\).

Using target isolated tissue represents one of the main drawback since the interaction with a functional immune system cannot be assessed. However, functional models can be useful to study the impact of therapeutic drugs on vascular inflammation and remodeling. These data, together with the ones derived from clinical trials, will help to better understand the effects of some specific therapies. In addition, functional models will provide unique insight into pathogenic mechanisms of vascular inflammation and repair.
Treatment

Currently, GCA treatment is based on high dose glucocorticosteroid administration, which should be tapered usually after 2-4 weeks \(^3\). Most patients are able to discontinue treatment after 1-2 years, but the duration is highly variable. Importantly, 40-60% of patients relapse when glucocorticosteroids are tapered and/or experience recurrences after treatment removal \(^3, 78\). In addition, there are multiple adverse effects associated with corticosteroid therapy, including: infections, hypertension, gastrointestinal bleeding, diabetes mellitus and osteoporosis \(^3\). Altogether, the disadvantages associated with corticosteroids highlight the importance of looking for alternative or steroid-sparing therapies.

Methotrexate

Three clinical trials have investigated the use of methotrexate (MTX) as an adjuvant therapy to corticosteroids for GCA \(^79, 80, 81\). MTX is an analog of folic acid with potent anti-inflammatory effects. In a study of 42 patients with GCA, treatment with prednisone and MTX reduced the proportion of patients who experienced at least one relapse as well as the proportion of patients who experienced multiple relapses, compared to treatment with combined prednisone and placebo \(^79\). However, a second study carried out by Spiera et al. found that no corticosteroid-sparing benefit could be attributed to the combination of MTX with corticosteroid therapy for the treatment of patients with GCA \(^80\). In concordance, the results of a randomized, multicenter trial in 98 patients over four years found no significant difference in treatment failure rates, corticosteroid dose, inflammatory markers, or treatment toxicity in patients treated with adjuvant MTX therapy compared to placebo \(^81\). The discrepancies between these trials may be explained by the differing trial designs. In 2007, Mahr et al. performed a meta-analysis of individual patients from these 3 randomized trials and found that adjunctive treatment with MTX was useful to reduce corticosteroids exposure and to decrease the risk of relapses \(^82\).

TNF-α

TNF-α is a potent pro-inflammatory cytokine that promotes infiltration of leucocytes. TNF blockade has shown important benefits in other chronic inflammatory or granulomatous diseases. In GCA, TNF-α expression has been associated with persistent disease activity \(^51\). This evidence provided the rationale for conducting clinical trials to investigate TNF inhibition with infliximab or adalimumab in GCA. Unfortunately, TNF-α blockade did not provide an advantage over placebo in maintaining remission in newly diagnosed patients \(^83, 84, 85\).
**Tocilizumab**

IL-6 pathway inhibition represents a promising therapeutic approach in GCA. IL-6 is a potent pro-inflammatory cytokine with pleiotropic effects on a variety of cell types. This cytokine is highly expressed in GCA patients lesions, especially in those with a strong systemic inflammatory reaction and it is hypothesized that IL-6 might contribute to disease pathogenesis. Recently, tocilizumab (TCZ), a humanized monoclonal antibody against IL-6 receptor (IL-6R), has been shown to be more effective than placebo in maintaining remission and sparing glucocorticoids in phase 2 and phase 3 clinical trials. Short-term clinical outcomes seem to be clearly improved by TCZ. IL-6R blockade strongly inhibits the systemic inflammatory response which is an important burden in patients with GCA, as well as cranial and polymyalgic clinical symptoms. However, longer follow-up studies are necessary to determine the durability of remission and safety of this treatment. In addition, the impact of TCZ on vascular inflammation and vascular remodeling, needs to be evaluated; this will provide unique insights into pathogenic mechanisms of vascular inflammation and repair.

**Other alternatives**

The blockade of IL-23 or IL-17 have been postulated as interesting alternative due to their role in GCA pathogenesis. Use of ustekinumab, a monoclonal antibody against subunit p40 of IL-12 and IL-23, can be useful to reduce glucocorticoid dose in patients with refractory GCA. However, the efficacy and safety of this therapeutic alternative must be evaluated in future randomized controlled trials.

Targeting T cell responses may be also a therapeutic strategy. Blocking CD28-mediated T cell co-stimulation with abatacept, a recombinant Ig-cytotoxic T-lymphocyte antigen-4 (CTLA-4) molecule, has demonstrated efficacy in maintaining remission in a recent randomized controlled trial. Alternatively, as previously mentioned, B cell depletion with rituximab has provided benefit in a few case reports.

IL-1β is also highly expressed in inflamed arterial walls of patients with giant cell arteritis and may contribute to the pathogenesis of this disease. Three cases of refractory GCA were successfully treated with anakinra, an IL-1 blockade therapy which competitively inhibits the union of IL-1 to its receptor. There is also an ongoing clinical trial aimed to analyze the beneficial effects of gevokizumab, a monoclonal antibody against IL-1β, for the treatment of relapsing GCA patients (www.clinicaltrials.gov).
IL-6

The disadvantages associated with the current treatment of GCA patients, based on glucocorticoids administration, have led to the search for therapeutic alternatives. Blockade of IL-6 signaling with TCZ represents a newly promising alternative supported by the results of two recently published clinical trials 86,87. However, beyond its implication in the acute phase response, the role of IL-6 in the pathogenesis of GCA and vascular inflammation is still unknown.

Discovery and function

IL-6 was first identified as a B-cell differentiation factor 93, 94. Various molecules were also described by other groups and termed different depending on the research area in which they were identified. Thanks to the molecular cloning of one of these molecule in 1986 it was possible to observe that all of them were in fact the same molecule which is currently known as IL-6 95.

IL-6 is a multifunctional cytokine that plays an important role in the immune response, hematopoiesis and inflammation, driving the acute phase response 96. IL-6 is quickly synthesized in response to infections or tissue damage and activates the acute phase response. Upon IL-6 stimulation, liver hepatocytes produce acute-phase proteins such as CRP, serum amyloid A (SAA) and fibrinogen 97. These proteins act as an emergency stress signal promoting host defense. Moreover, CRP is often used as a biomarker of inflammation or infection 11.

IL-6 induces the differentiation of activated B cells into immunoglobulin-producing plasma cells and promotes its survival and maintenance. Beyond B cells, IL-6 also acts as a polarizing cytokine guiding the differentiation of naïve CD4+ T cells into effector T-cell subsets 98. IL-6 promotes the differentiation of naïve CD4+ T cells into Th17 98 that are important for immunity to certain microorganisms infections and produce IL-17. In addition, IL-6 inhibits the TGF-β induction of Treg and prevents Th17 cells from converting to Treg cells 99, 100, 101. Treg are a subset of T cells that prevent other immune cells from attacking the body’s own tissues and other harmless environmental materials, such as food and commensal organisms. The imbalance of Th17 cells over Treg may lead to the alteration of immune tolerance promoting the development of some autoimmune and inflammatory disease 102. Furthermore, IL-6 promotes T follicular helper cell (Tfh cells) differentiation by induction of the transcriptional repressor B-cell lymphoma (BCL)-6 and controlling IL-21 production 103. Tfh cells are a specialized subset of CD4+ T that promote B cell proliferation and immunoglobulin class switching in B cell follicles contributing to humoral response 103.
Upon IL-6 stimulation, endothelial cells and smooth-muscle cells increase the expression of adhesion molecules, such as ICAM-1, and release chemokines, like CCL2, that contribute to the recruitment of more immune cells at the inflammation site\textsuperscript{104, 105}.

Regarding hematopoiesis, IL-6 promote the formation of multilineage blast cell colonies and also induces megakaryocyte and macrophage differentiation \textsuperscript{96}. IL-6 seems to participate in bone homeostasis and lipid metabolism and it has been associated with poor prognosis in many cancers such as lymphoma, ovarian cancer or melanoma \textsuperscript{96, 106}. Furthermore, this cytokine is involved in angiogenesis and it has been related with vascular wall remodeling and neointima formation in a rat model of carotid artery balloon injury \textsuperscript{107} (the multifunctional role of IL-6 is summarized in figure 4).

**Figure 4.** Scheme of the pleiotropic role of IL-6. IL-6 is a multifunctional cytokine that plays an important role in inflammation driving the acute phase response as well as participating in the differentiation and activation of B and T cells. This cytokine is also implicated in hematopoiesis, bone homeostasis and lipid metabolism, as well as in angiogenesis.

**Structure and expression**

IL-6 is expressed as a secreted protein of 184 amino acids with two potential N-glycosylation sites and four cysteine residues \textsuperscript{102}. The core protein is about 21kDa but several isoforms of 21-
28kDa are found due to different glycosylation status. IL-6 isoforms might modify protein stability or half-life, but glycosylation does not seem to be essential to its function.

Many immune and non-immune cells can produce and secrete IL-6 such as T and B cells, macrophages, monocytes, fibroblasts, endothelial cells, adipocytes and tumor cells. Normal physiological concentration of IL-6 in human serum of healthy individual are relatively low, about 1-5 pg/mL. However, its concentration rises dramatically during inflammatory conditions and can reach up to a range of µg/mL in cases of sepsis.

There are several pathways that can promote IL-6 synthesis including Toll-like receptors, prostaglandins, adipokines, stress response and cytokines, among which IL-1β and TNF are major activators of IL-6 expression. IL-6 mRNA levels in turn are regulated by several factors that modulates its production and fate both at transcriptional or post-transcriptional level.

This includes diverse transcription factors, such as nuclear factor kappa B (NFκB), several miRNAs that suppress IL-6 mRNA acting on its 3’ UTR, as well as RNA binding proteins, like regnase-1, which can promote mRNA decay via its ribonuclease activity.

**IL-6 receptors complex**

The IL-6 receptor system comprises a specific IL-6 binding molecule called IL-6R (also known as IL-6Rα, gp80 or CD126) and a signal transducer molecule termed gp130 (also referred to as IL-6Rβ or CD130). An initial binding of IL-6 to IL-6R is essential to trigger the association of this complex to gp130 which will then transduce the signal inside the cell. IL-6 binding to its receptor leads to the formation of a hexameric complex consisting of two IL-6, IL-6R and gp130 molecules (figure 5).

While gp130 is expressed by most if not all cells of the body, the expression of IL-6R is limited to few cell types such as hepatocytes, neutrophils, monocytes/macrophages and some lymphocytes. Thus, due to lack of the IL-6R, the majority of cells in the human body are not responsive to IL-6. Nevertheless, there are two forms of the IL-6R: an 80kDa transmembrane IL-6R (mIL-6R) and a 50 to 55kDa soluble IL-6R (sIL-6R), lacking the cytoplasmic and transmembrane domains. sIL-6R can bind to its ligand IL-6 with the same affinity as the mIL-6R, and the complex IL-6/sIL-6R can interact with gp130 in cells lacking the mIL-6R, thereby initiating signaling. This signaling mode is called IL-6 trans-signaling, whereas IL-6 signaling via mIL-6R is referred to as classic signaling (figure 5.A).
Figure 5. Schematic representation of IL-6 receptors complex. Classical IL-6 receptor signaling occurs in cells that express IL-6R and gp130. A fully functioning IL-6 receptor complex consists of a hexameric structure in which IL-6, IL-6R and gp130 exist in a 2:2:2 stoichiometry. A. A soluble form of IL-6R is released from the cell surface by proteolysis and splicing of IL6R mRNA and can bind IL-6 to form an agonistic complex that signals through gp130. This mechanism of trans-signaling allows IL-6 to act on cells that lack IL-6R. B. The soluble form of gp130 can agonistically bind IL-6/sIL-6R complex. Image adapted from Hunter and Jones, 2015 with the hexameric structure proposed by Boulanger et al., 2003.

The soluble form of IL-6R can be found in body fluids such as urine and blood. Normal levels of sIL-6R in human plasma of healthy individual are found at about 25-35 ng/mL and can rise during inflammatory conditions. Although the cytoplasmic tail of the IL-6R does not seem to participate in signal transduction, it may play a role in its basolateral membrane location in epithelial cells. sIL-6R is mainly generated by proteolytic cleavage of the membrane bound form. However, a reduced amount (10%) can be also produced via alternative splicing. The enzymes responsible of IL-6R shedding belong to the a disintegrin and metalloproteinase (ADAM) gene family of metallopeptases and are ADAM10 and ADAM17. These proteases cleave the receptor close to the transmembrane region and they may play an important role in some inflammatory diseases like rheumatoid arthritis (RA).

Beside IL-6, gp130 is also a receptor subunit of other members of the IL-6 family of cytokines, including IL-11, leukemia inhibitory factor (LIF), cardiotoxin-like cytokine (CLC), ocostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotropin-1 (CT-1), neuropoietin (NPN) and IL-27 with the exception of IL-31. The sharing of the gp130 receptor subunit by several cytokines may partially explain their functional redundancy. Soluble forms of gp130 (sgp130) can also be found in human serum of healthy individual at high concentration (200-400 ng/mL) which remain mostly unaltered during inflammation. These soluble isoforms are generated by...
alternative splicing and inhibit IL-6 trans-signaling by blocking IL-6/sIL-6R complex, thus acting as a physiological buffer. Furthermore, sgp130 does not bind IL-6 or IL-6R alone and has no effect on classical signaling (figure 5.B).

**IL-6 signaling pathway**

Binding of IL-6/IL6R complex to gp130 receptors allows their dimerization that in turn leads the activation of constitutively bound Janus kinases (JAKs), specifically JAK1, JAK2 and TYK2. The activated JAKs phosphorylate tyrosine residues in the cytoplasmic domain of gp130. This domain possesses six tyrosine residues, among which the four most membrane distal represent recruitment sites for signal transducer and activator of transcription (STAT) factors, mainly STAT3, but also STAT1 and to a lesser extent, STAT5. STAT factors are then phosphorylated by JAKs, dimerize forming either homo- or heterodimers and travel to the nucleus where they can stimulate transcription of target genes. In addition, tyrosine Y759 residue of gp130 cytoplasmic tail is a docking site for the tyrosine phosphatase SHP2, which stimulates the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway (figure 6).

IL-6 signaling is regulated by negative feedback by the suppressor of cytokine signaling (SOCS), and the protein inhibitor of activated STATs (PIAS). Between SOCS proteins, SOCS1 and SOCS3 are the ones more functionally related with IL-6 signaling. These two proteins are target genes of the JAK/STAT pathway and act as classical feedback inhibitors. PIAS family proteins are important transcriptional co-regulators of the JAK/STAT pathway that interact with tyrosine phosphorylated STAT factors. PIAS3 was found to bind specifically to STAT3, blocking the DNA-binding activity of this factor and inhibiting gene transcription activation.
Figure 6. Schematic representation of IL-6 signaling. The receptor complex formation leads the activation of Janus kinases: JAK1, JAK2 and TYK2. These kinases phosphorylate the tyrosine residues of the cytoplasmic domain of gp130 that are recruitment sites for STAT3 and to a lesser extent STAT1. Once activated, STATs form dimers and travel to the nucleus to act as transcription factors. One of the target genes encodes for SOCS3, an IL-6 pathway inhibitor that binds the phosphorylated tyrosine 759 motif in gp130 and counteracts JAKs activation, generating a negative feedback loop. PIAS3 inhibits gene transcription activation by binding to phosphorylated STAT3 and blocking its DNA-binding activity. Additionally, IL-6 signaling can also activate the MAPK pathway as well as the PI3K pathway through the tyrosine phosphatase SHP2, which can bind to the tyrosine 759 of gp130 cytoplasmic tail. Image adapted from Costa-Pereira, 2014. 

126
IL-6 and IL-6R in GCA

IL-6 is highly expressed in GCA lesions at mRNA level, but their transcripts are also present in normal temporal arteries. IL-6 protein expression has been also observed by immunohistochemistry in GCA lesions, being significantly higher in patients with GCA with a strong systemic inflammatory response.

Serum IL-6 is elevated in patients with GCA and positively correlates with disease activity. Patients with ischemic complications have lower levels of circulating IL-6 and the expression of this cytokine in GCA lesions is decreased compared with non-ischemic patients. Moreover, persistently increased serum IL-6 is found in patients with relapsing disease. Regardless IL-6 has been widely identified by several authors as a biomarker of disease activity in GCA, its functional role in the pathogenesis of this disease has not been investigated.

As it has been mentioned before there is an increasing interest in blocking the IL-6 pathway with TCZ in GCA, an antibody addressed to IL-6R. Specially, after the promising results recently obtained in two clinical trials. However, little is known about IL-6 receptor expression in GCA. In a recent study, serum levels of soluble IL-6R were found significantly higher in GCA patients but there was no correlation with disease activity.

TCZ

As it has been mentioned before, TCZ is a humanized monoclonal antibody that blocks IL-6 signaling by binding to both mIL-6R and sIL-6R. The clinical development of TCZ for the treatment of RA starts in 1997 by the Japanese company Chugai Pharmaceuticals. The European Medicines Agency approved the use of this drug as RoActemra for the treatment of RA in January 2009. In 2010, the US Food and Drug Administration (FDA) approved it for the same purpose as Actemra and, later, also allowed its use for the treatment of Systemic Juvenile Idiopathic Arthritis and Polyarticular Juvenile Idiopathic Arthritis. Recently, in 2017, FDA has approved the use of this monoclonal antibody for the treatment of adult patients with GCA and adult and pediatric patients with chimeric antigen receptor (CAR) T cell-induced severe or life-threatening cytokine release syndrome (CRS).

Beyond TCZ, several companies are currently evaluating other IL-6 signaling blockers which either bind to IL-6 or IL-6R. Including soluble forms of gp130 that specifically inhibits the response mediated by sIL-6R without altering the signaling associated to the membrane-bound IL-6R.

Despite the increasing interest in using TCZ for the treatment of different immune related disease, little is known about the biological impact of this antibody. A better understanding of
how this drug modulates cellular response and signaling may allow preventing side effects associated with its use, as well as improving the treatment conditions of patients and extending its use to other diseases.

Results of two clinical trials with TCZ in GCA have been recently published with positive results. In a phase 2, double-blind, clinical trial 20 patients with GCA were randomized to receive TCZ and prednisolone and 10 to receive placebo and glucocorticoids therapy. Oral prednisolone started at 1mg/Kg, in both group, and was tapered and discontinued according to a standardized protocol defined in the study. 85% of patients in the TCZ group, compared with 40% in the placebo reached the primary outcome of complete remission at a prednisolone dose of 0.1mg/Kg/day at week 12 (p=0.03). At week 52, relapse-free survival was observed in 85% of patients in the TCZ group compared with 20% in the placebo group (p=0.001). The cumulative prednisolone dose after 52 weeks was significantly lower in the TCZ group (43mg/kg) compared with the placebo group (110mg/kg) (p=0.0005). Adverse events were observed in 75% of patients in the TCZ group (15 patients) and 70% in the placebo group (7 patients). Of them 35% in the TCZ group had serious adverse events versus 50% in the placebo group.

More recently, results from a phase 3 clinical trial with 251 newly diagnosed and relapsing GCA patients with active disease have been published. Patients were randomized in four groups in a 1:1:2:1 ratio to receive 26 week prednisone taper and placebo, 52 week prednisone taper and placebo, 26 week prednisone taper and TCZ (at a dose of 162mg subcutaneous) weekly or 26 prednisone taper and TCZ every other week. The primary outcome was the proportion of patients in sustained glucocorticoid-free remission at week 52 in each TCZ group compared with the 26-week prednisone and placebo group. The key secondary endpoint was the rate of remission in both TCZ groups versus the 52-week prednisone taper group. At week 52 sustained remission was observed in 56.0% of patients treated weekly with TCZ and in 53.1% of those treated with TCZ every other week compared with 14.0% in the placebo group that underwent the 26-week prednisone taper and 17.6% in the placebo 52-week prednisone taper group (p < 0.001 for comparisons of either active treatment vs placebo at each time point). The cumulative median prednisone dose over 52 weeks was 1862 mg in each TCZ group compared with 3296 mg in the placebo 26-week group (p < 0.0001) and 3818 mg in the placebo group that underwent the 52-week taper (p < 0.0003). Adverse effects were observed in most of patients from all groups: 98.8% in the group treated weekly with TCZ, 95.9% in every other week TCZ group, 96% in the 26-week prednisone arm and in 92.2% of patients in the 52 week prednisone group. Serious adverse events occurred in 15% of
patients treated with TCZ weekly, 14.3% of TCZ every other week, 22% of those in the placebo group underwent the 26-week taper and 25.5% of placebo 52-week taper patients. In both clinical trials, TCZ was superior to placebo in the induction of sustained glucocorticoid-free remission in GCA patients. However, safety of TCZ and the durability of induced remission needs to be assessed with long-term follow-up studies since TCZ withdrawal might result in disease recurrence.

Since the IL-6-dependent systemic inflammatory response has clinical implications, there is some concern about whether TCZ acts on disease activity or provides symptomatic release only. Currently, there is no available information regarding the functional impact of TCZ treatment on signaling pathways and cell responses in GCA lesions. A recent study with peripheral blood cells has pointed out that one of the efficiency of TCZ could be partially due to its role in Treg population regulation in GCA patients. Authors analyze the frequency, phenotype and function of peripheral blood Treg in four different groups: patients with active GCA, patients in remission with corticosteroids or TCZ and healthy controls that were included for comparison. Even though no differences were found in Treg population, defined as CD4+ T cells expressing forkhead box P3 (FOXP3) among groups, they observed that treatment with TCZ, compared with corticosteroids, increased the numbers of activated Treg cells (defined as CD45RA-FOXP3hi) in addition, TCZ in contrast to corticosteroids, was able to restore the replicative potential of Treg cells without affecting the proliferation of non-regulatory CD4+T cells. Patients with GCA seem to possess more Treg cells with an increased expression of FOXP3Δ2, a spliced variant of FOXP3 lacking exon 2 that is associated with less suppressive capacity. In this sense, treatment with TCZ, in contrast to corticosteroid therapy, was able to correct the Treg abnormalities observed in active GCA patients. Summarizing, this first report showed that TCZ was able to increase the proliferation and activation of Treg, and also reverted the pathogenic phenotype observed in Treg cells in active GCA patients.

A functional impact of IL-6 blockade with TCZ on Treg population has also been previously reported in patients with RA. In this study Samson et al., found that the imbalance between Th17 (CD4+IL-17+) cells and Treg (CD4+CD25hiFoxP3+) cells was corrected after TCZ treatment. Thereby, TCZ induced a significant decrease in the percentage of Th17 cells and in turn, an increase in the percentage of Treg cells in the peripheral blood of patients. TCZ may also play a role on B cell population. It has been observed that treatment with TCZ modified, at least partially, the cytokine production profile of B cells from RA patients, thus resembling the one observed in healthy donors.
Decrease in circulating neutrophils have been reported in RA patients treated with TCZ. However, neutropenia may be due to neutrophil trafficking to the bone marrow since treatment with TCZ does not seem to alter neutrophil apoptosis or functions. In an in vitro study using human DC, obtained from stimulated CD14+ monocytes, it was observed that treatment with TCZ enhanced mIL-6R expression and increase sIL-6R secretion, turning DC into an important source of IL-6 trans-signaling activation.

Given the multifunctional role associated with IL-6, its blockade with TCZ can also affect other cell populations different from the inflammatory cells. Treatment with TCZ may alter angiogenesis in inflamed tissues. In inflammatory lesions there is production of angiogenic molecules regulating endothelial cell proliferation and survival, as well as their migration and activation. These molecules include VEGF, epidermal growth factor (EGF), IL-6, IL-1 and TNF-α, among others, and regulate angiogenesis by modulating endothelial cell responses. It has been shown that the use of an anti-IL6R monoclonal antibody normalized serum levels of VEGF in RA patients. IL-6, in combination with sIL-6R, induced tube formation in a co-culture system of human umbilical vein endothelial cells (HUVEC) and fibroblast-like synovial cells (FLS) from RA patients, and this angiogenic response was completely inhibited by the addition of an anti-VEGF antibody or TCZ. In this study, TCZ was also able to suppress VEGF production triggered by IL-6/sIL-6R complex but had no effects on other angiogenic factors, indicating that VEGF may play a crucial role in IL-6 induced angiogenesis. The anti-angiogenic role of TCZ may be also useful in the treatment of oral squamous cell carcinoma since in vitro culture of SAS human tongue carcinoma cell line with TCZ showed a decreased expression of VEGF. In the same study, tumors growth in TCZ-treated mice exhibited a reduction in microvessel density and vessel diameter.

Additionally, endothelial cells can play an important role in leukocytes adhesion and transmigration. IL-6 in combination with sIL-6R induced production of CCL2 chemokine and expression of adhesion molecule ICAM-1 in the cell surface of HUVEC endothelial cells. IL-6/sIL-6R cocktail also promoted the adhesion of monocyte cell line U937 to HUVEC. All these effects were suppressed when TCZ was used to block IL-6R.

Use of TCZ for IL-6R blockade could be also useful for the treatment of some type of tumors. It has been seen, for instance, that TCZ suppressed growth of human lung squamous cell carcinoma-derived cell and it could be useful to inhibit metastasis of this tumor cells to the brain. In another study, it was found that TCZ significantly reduce in vivo growth of an oral squamous cell carcinoma cell line. Growth reduction was accompanied with a decrease in STAT3 phosphorylation levels in tumor cells in mice.
Biomarkers for TCZ treated patients

One important concern for the management of inflammatory diseases is the rapid decrease of CRP levels after TCZ treatment, since IL-6 is the main inducer of this protein. As previously mentioned, inflammatory markers such as ESR and CRP are elevated in GCA patients because of the acute-phase response triggered by IL-6. Consistently, these markers are widely used in clinical practice to monitor disease activity in those patients. However, treatment with TCZ inhibits the hepatic synthesis of acute phase reactants, razing levels of CRP and ESR. TCZ may suppress GCA manifestations without eliminating the arteritis, therefore, TCZ treatment turns these two inflammatory markers unreliable to monitor disease activity. For these reason, there is an urgent need of novel biomarkers, not directly related with IL-6 signaling, that allow to monitor disease activity in the era of TCZ treatment for GCA. In the present thesis we propose osteopontin (OPN) as potential alternative biomarker.

Osteopontin

OPN is a multifunctional protein composed of about 300 amino acids. Its molecular weight can range from 40 to 80kDa, due to alternative splicing, as well as post-translational modifications, such as glycosylation and phosphorylation. OPN can be found as an intracellular or a secreted glycoprotein. Intracellular OPN exerts its function by binding to the adapter protein MyD88, which is involved in the Toll-like receptor signaling pathway. On the other hand, extracellular OPN needs to bind to some cell surface receptors expressed in target cells to trigger its effect. OPN can interact with different types of integrins, depending on the binding site exposed due to different proteolytic cleavage. It also possesses a binding site for certain variant forms of CD44. This glycoprotein is expressed by a variety of cell type related with the immune and inflammatory response such as T and B lymphocytes, DC, neutrophils, macrophages and natural killer cells. In addition, high expression of OPN can be found in liver, brain, joints, lung, bone, adipose tissue and body fluids such as urine and blood.

OPN participates in innate and adaptive immune responses. It promotes Th1 and Th17 polarization and inhibits Th2-mediated responses. OPN appears to play a crucial role in immune cell recruitments since it supports lymphocyte and monocyte/macrophage migration, adhesion and their survival. In addition, OPN can also act on endothelial and VSMC promoting their migration, thus may contribute to angiogenesis and vascular remodeling.
OPN expression was investigated by immunohistochemistry in different granulomatous diseases, based on its production by activated macrophages. In this descriptive study, increased tissue expression of OPN was observed in TA biopsies from two patients with GCA. Due to the functions associated with OPN and its elevated expression at sites of inflammation and tissue injury it could play also an important role in the pathogenesis of GCA. Furthermore, elevated levels of soluble circulating OPN have been observed in a variety of inflammatory diseases of blood vessels including Antineutrophil Cytoplasmic Antibody (ANCA)-associated vasculitis and Behcet’s disease. Patients with other vascular conditions, such as non-vasculitic thoracic or abdominal aortic aneurysms, have also showed elevated tissue and serum concentrations of OPN. Serum levels of OPN in GCA patients has not been investigated, however, recently work carried out in this lab has pointed out its potential as a biomarker in this disease.

**OPN as a biomarker in GCA patients.**

Serum level of sOPN was analyzed in 76 biopsy-proven GCA patients, selected from two reported cohorts. Cohort 1 included 42 patients and cohort 2 included 34. Serum samples were included based on their availability at the time of diagnosis, before the initiation of glucocorticoid treatment. Concentration of sOPN in serum (ng/ml; mean ± SD) was found to be significantly higher in patients with active GCA compared with controls in both the cohort 1 (GCA 119.59 ± 70.36 vs controls 42.82 ± 24.58; p<0.001) and cohort 2 (GCA 113.21 ± 69.56 vs controls 39.24 ± 21.28; p<0.001) (figure 7.A and 7.B respectively), as well as in the pooled cohorts of active GCA patients (116.75 ± 69.61) compared to pooled healthy controls (figure 7.C) (41.10 ± 22.65; p<0.001). In the 36 patients from the cohort 1 who underwent a second sample collection at the time of disease remission, a significant decline in sOPN concentrations was observed (active disease at baseline 102.45±57.72 vs remission 46.47±23.49; p<0.001) (figure 7.D).
Figure 7. Serum osteopontin (sOPN) concentration in patients with GCA and healthy controls. A. sOPN concentration in active patients with GCA from cohort 1 and in controls (subgroup A). B. sOPN concentration in active patients with GCA from cohort 2 and controls (subgroup B). C. sOPN concentration in the pooled cohorts of active GCA patients and controls. Box-plot in A, B and C represents median, 25-75% percentil and range. **p<0.005. D. sOPN concentration in GCA patients at diagnosis and when in remission by paired comparison.

Moreover, patients with the highest sOPN concentrations at diagnosis experienced more relapses and cumulated higher glucocorticoid doses. Specifically, within the group of relapsers, patients with more than one disease relapse demonstrated significantly higher sOPN levels than those with only one relapse (figure 8.A). The proportion of patients requiring a prednisone maintenance dose ≥ 10 mg/day over time was significantly higher among patients with sOPN levels above the mean sOPN concentration at the time of diagnosis (p=0.036) (figure 8.B).
Figure 8. Baseline serum osteopontin in patients with GCA as predictor of relapses and duration of glucocorticosteroid treatment. A. Baseline sOPN concentrations in patients with no subsequent relapses, with one relapse and with ≥ two relapses. *p<0.05. B. Percentage of patients requiring a daily maintenance prednisone dose ≥ 10mg over time according to baseline sOPN (≥ mean sOPN v s < mean sOPN); p<0.05.

Previous studies have indicated that an intense systemic inflammatory response is associated with recurrent disease. Accordingly, sOPN was significantly higher in patients with strong systemic inflammatory response than in patients with weak acute phase reaction. On the contrary, sOPN concentrations were significantly lower in patients with cranial ischaemic complications compared to patients without these complications. This observation has also previously described for serum IL-6. Interestingly, sOPN showed a positive correlation with serum IL-6 at the time of GCA diagnosis and with the IL-6-dependent acute phase reactants ESR and CRR and negatively correlated with haemoglobin concentration. Moreover, sOPN performed better than individual detection of ESR, CRP, haemoglobin or IL-6 as predictor of relapsing course. The correlation between sOPN and IL-6 and their association with an intense systemic inflammatory response may suggest a coordinated regulation of these two molecules.

All together, these results pointed out the potential value of sOPN as a biomarker of disease activity and predictor of relapsing disease and glucocorticoid requirements in GCA patients. Although OPN synthesis can be induced by IL-6 it is not exclusively IL-6 dependent since it can also be induced by IL-1β TNF-α and IFN-γ among others. The fact that OPN is not totally dependent of IL-6 and its potential as a biomarker in GCA, are two interesting characteristics to consider this protein as a suitable option for TCZ treated patients. However, its possible use to monitor patients treated with TCZ, as well as, the effect of IL-6R blockade on its expression needs to be widely investigated.
Hypothesis

Aims of the study
IL-6 is a pleiotropic cytokine which plays a relevant role as a proinflammatory molecule and major driver of the acute phase response in various diseases and has been widely studied in different conditions. It is, in fact, an important biomarker in GCA, a disease characterized by a strong systemic inflammatory response. Results from two new clinical trials have pointed out the potential benefits of IL-6 signaling blockade with TCZ in the management of GCA patients. In fact, the FDA has recently approved the use of this humanized monoclonal antibody against IL-6R for the treatment of patients with GCA. Thereby, the incorporation of this new therapeutic drug brings out the need to expand our knowledge about the role of IL-6 signaling in GCA. However, beyond its association with disease activity and acute phase response, little is known about its functional role in GCA. In addition, there is scarce information about IL-6R in this context and despite accumulating evidence for the therapeutic efficacy of TCZ, the functional effect of this monoclonal antibody at the cellular level remains largely unexplored. Finally, the use of TCZ in GCA patients also remarks the urgency to find alternative biomarkers not being totally abrogated by this treatment, as it happens with the related to the acute phase response.

In this context, we hypothesize that, in addition to inducing the acute phase response, IL-6 has a role in promoting and sustaining vascular inflammation in GCA. Consequently, blocking IL-6 receptor would reverse activation of signaling pathways and modulation of gene expression induced by IL-6 in inflamed arteries from patients with GCA. Furthermore, since blocking IL-6R abrogates the acute phase response, OPN could be a suitable alternative biomarker of disease activity in TCZ treated patients.

To test these hypothesis, the aims of the present study are the following:

- To investigate the expression of IL-6 and IL-6R in GCA lesions.
- To identify which cells are contributing to the expression of IL-6 and IL-6R in GCA.
- To analyze the ex-vivo effects of TCZ treatment on IL-6 activated signaling pathways in GCA-involved arteries and in peripheral blood mononuclear cells.
- To explore changes induced by IL-6R blockade with TCZ on the expression of candidate genes relevant to disease pathogenesis in cultured temporal arteries from patients with GCA.
- To determine the functional impact of IL-6R blockade with TCZ on specific cell subsets present in GCA lesions.
- To explore the potential of sOPN as a serum biomarker of disease activity in GCA patients treated with TCZ.
Methods
**Patients serum**

**IL6 and sIL-6R**

Patient inclusion in the present study was based on the availability of serum samples collected at the time of diagnosis, before the initiation of glucocorticoid treatment. IL-6 and sIL-6R were measured in the serum of 26 patients from a previously reported cohort \(^{157}\). Patients were followed for at least 2 years (mean 187 weeks, range 114–360), and time to first disease relapse, number of disease relapses, time to reach a stable (at least for 3 months) prednisone maintenance dosage <10 mg/day or <5 mg/day, as well as time to prednisone discontinuation and cumulative prednisone dose were recorded. A second sample was obtained after approximately one year (402 ± 48 days) of glucocorticoid treatment following a uniform, previously reported tapering protocol \(^{160, 164}\). At the time of the second sample collection, patients were receiving a median daily prednisone dose of 5 mg/day (range 2.5 - 10 mg/day) and all patients were in clinical remission defined by the absence of disease-related manifestations and the presence of ESR and CRP levels within the normal range. The control group consisted of 13 healthy individuals with no chronic inflammatory diseases and matched for age and gender. The study was approved by the ethics committee of Hospital Clinic (Barcelona, Spain) and patients signed informed consent.

**OPN**

Soluble OPN (sOPN) was measured in 17 additional GCA patients in prednisone-maintained remission. Seven patients were receiving ≥20 mg/day (high dose) and 10 were receiving ≤10 mg/day (low-dose). sOPN was subjected to cross-sectional comparison with that from 15 patients in remission treated with TCZ (4-8 mg/Kg/month) (as monotherapy or together with low-dose prednisone) to explore the effect of tocilizumab on sOPN concentration. CRP was simultaneously measured in the same samples for comparison purposes. The clinical characteristics of these additional 32 patients have been previously described \(^{136}\). Study of these patients was approved by the IRB from the Massachusetts General Hospital (Boston, MA) and patients signed informed consent.

**Temporal artery biopsy (TAB) processing**

TABs were performed to 45 consecutive patients with suspected GCA for diagnostic purposes. Twenty-nine biopsies disclosed histopathological features of GCA and the remaining 16 showed no inflammatory infiltrates and were used as controls. Patients with negative biopsies
were eventually diagnosed with other conditions. The study was approved by the local Ethics Committee (Hospital Clinic of Barcelona) and patients signed informed consent.

After the histopathological examination and confirmation of GCA, the remaining TAB tissue was processed depending on the requirements. These TABs were treated for the further extraction of mRNA, using the method explained in section: RNA extraction, or homogenized in a lysis buffer to obtain protein lysates, as it is described in section: Protein cell lysates. Moreover, in some cases, biopsies were preserved to investigate protein expression by immunofluorescence (see section: Immunofluorescence of TABs).

Beyond the use of fresh TABs for the mentioned purposes, it is also feasible to culture them under different conditions. The possibility to culture the TABs allows to work with an ex vivo model, as well as to obtain a primary culture of VSMC as it is further described in the following sections.

**Culture of TABs**

As previously described, 1mm thick sections from TABs were placed onto reconstituted basement membrane Matrigel (BD Biosciences), which preserves the viability of the artery in culture conditions. Each section was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Waltham, Massachusetts, USA), 2mM of L-glutamine (Gibco), 50µg/mL of gentamicin (Braun, Melsungen, Germany) and 2.5 µg/mL of amphotericin B (Invitrogen). TABs sections were cultured at 37°C and 5% CO₂.

In order to analyze the impact of TCZ treatment using this artery culture model, sections from a GCA positive biopsy were cultured in medium alone or in medium supplemented either with 10µg/mL of TCZ (Roche), 10µg/mL of IgG isotype control (Sigma) or 0.5µg/mL of dexamethasone (Sigma). After 5 days in culture, sections were recovered and processed for RNA or protein extraction. Supernatants were also collected and preserved at -80°C for detection of secreted molecules.

**Cell culture**

**Primary cultures of VSMC**

To obtain primary cultures of VSMC, TAB sections were cultured with Dulbecco's Modified Eagle Medium (DMEM) medium (Lonza, Verviers, Belgium) supplemented with 10% FBS (Gibco, Life Technologies, Waltham, Massachusetts, USA), 2mM of L-glutamine (Gibco),
50µg/mL of gentamicin (Braun, Melsungen, Germany) and 2.5 µg/mL of amphotericin B (Invitrogen); at 37°C and 5% CO₂. Under these conditions VSMC, from the tunica media, are able to expand and colonize the well. VSMCs usually start growing out from the arteries between day 3 and 6 of culture, and they reach confluence after approximately 1 month. Subcultures were realized using 0.05%-Trypsin EDTA (Gibco) when cells reached confluence, at 1:2 ratio. Cell count was performed with a Neubauer chamber and trypan blue solution 0.4% (Sigma) was used to assess cellular viability. VSMCs were used between passage 3 and 8 for in vitro experiments.

Peripheral blood mononuclear cells (PBMC)

PBMC were obtained by density gradient centrifugation using Ficoll-Lymphoprep™ (Histopaque®-1077, Sigma Diagnostics, St. Louis, MO) in sterile conditions. Fresh blood was collected from healthy volunteers in tubes with EDTA as an anticoagulant agent (Vacutainer EDTA tubes) and diluted 1:1 with phosphate buffer saline (PBS) 1x. 30mL of diluted blood were deposited over 15mL of Ficoll® with caution, avoiding mixing, in a 50mL conical centrifuge tube. Tubes were centrifuged for 30 min at 660 G at room temperature, without break. Using this procedure, PBMC, because of their density, stay in the interface within the Ficoll® solution and blood plasma. PBMC were recovered and washed three times with 20mL of PBS 1x and centrifuged for 10min at 360 G. Prior to the last wash, PBMC were stained with trypan blue solution 0.4% (Sigma) to assess viability and counted with a Neubauer chamber. PBMC were cultured with RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% FBS (FBS; Gibco, Life Technologies, Waltham, Massachusets, USA), 2mM of L-glutamine (Gibco), 50µg/mL of gentamicin (Braun, Melsungen, Germany) and 2.5 µg/mL of amphotericin B (Invitrogen), at 37°C and 5% CO₂.

Co-cultures of VSMC and PBMC

VSMC were seeded at 0.15·10⁶ cells/well, onto 6 well-plates and cultured overnight with DMEM medium, supplemented as previously described, to allow cells to attach to the plate. The day after, media from the VSMC was replaced by PBMC in RMPI medium supplemented as described. 1 million of PBMC was seeded per well. The co-culture conditions were compared with situations containing only isolated VSMC or PBMC. After, 24 or 48 hours supernatants were collected, and cells were processed to obtain RNA or protein lysates as explained bellow. Different cell populations of the co-culture were obtained and processed separately. Supernatants from wells containing PBMC were centrifuged at 1000G for 10min to also
recover non-attached cells. In co-culture conditions, before collecting VSMC, cells were rinsed with cold Versene® (Invitrogen) to remove attached PBMC. Co-cultures were also conducted using trans-well inserts of 0.4 µm of pore size (Nunc™) that prevent cell contact but allow exchange of soluble factors.

To analyze the effect of TCZ in these conditions, PBMC where pre-incubated with medium alone or medium supplemented with 10µg/mL of TCZ (Roche) or 10µg/mL of IgG control (Sigma) during 1h at 37C and 5% CO₂; prior to co-culture with VSMC. The co-culture conditions were also compared with situations containing only isolated VSMC or PBMC treated with TCZ, the IgG control or medium alone.

**HUVEC culture**

HUVEC, obtained from human umbilical embryo cordon, were cultured in RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 20% fetal calf serum (FCS; Gibco, Life Technologies, Waltham, Massachusetts, USA), 100mg of endothelial cell growth supplement (Collaborative Biomedical Products), 2500 units of sodium heparin (Rovi), 2mM of L-glutamine (Gibco), 50µg/mL of gentamicin (Braun, Melsungen, Germany) and 2.5 µg/mL of amphotericin B (Invitrogen), at 37°C and 5% CO₂. HUVEC were split 1:4 when reached confluence and were used between passage 3 and 5 for in vitro experiments.

**Cell culture with IL-6, sIL-6R and TCZ**

VSMC, HUVEC and PBMC were cultured in vitro to analyze the effect of TCZ treatment on chemokine expression. VSMC (90,000 cells/well) and HUVEC (200,000 cells/well) were seeded in a 6-well plate and let to adhere overnight at 37°C and 5% CO₂. The day after, the different stimuli or blockers, diluted in the corresponding mediums, were added to the PBS 1x pre-washed VSMC or HUVEC. PBMC were obtained as previously described and directly seeded (1·10⁶ cells/well) with the corresponding molecules. Specifically, the following molecules alone or in combination were used:

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Company</th>
<th>Working concentration</th>
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</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>R&amp;D</td>
<td>10ng/mL</td>
</tr>
<tr>
<td>sIL-6R</td>
<td>R&amp;D</td>
<td>100ng/mL</td>
</tr>
<tr>
<td>TCZ</td>
<td>Roche</td>
<td>10µg/mL</td>
</tr>
<tr>
<td>IgG Control</td>
<td>Sigma</td>
<td>10µg/mL</td>
</tr>
</tbody>
</table>
VSMC and HUVEC were cultured with both IL-6 and its soluble receptor. Whereas, PBMC were treated only with IL-6. This experiment was set up based on previously observed capacity of these cells to respond to either IL-6 alone or in combination with sIL-6R. (See the Additional data section for more information).

After 24h, supernatants were recovered, and cells were collected to RNA extraction as further explained. For PBMC non-adherent cells were also recovered after supernatant centrifugation at 1000G for 10 min.

**RNA extraction**

RNA extraction, both from the artery sections as well as from cellular lysates, was carried out using the chloroform-isopropanol method. Samples were recovered in 1mL of TRIzol reagent (Life Technologies). Addition of TRIzol promotes cellular lysis and contributes to the preservation of samples, avoiding RNA degradation. To the RNA extraction samples in TRIzol were mixed with chloroform and then centrifuged to obtain different phases. For artery cultured sections, 2 to 3 sections per condition were homogenized with a Polytron® homogenizer (IKA, Staufen, Germany), prior to RNA extraction. RNA, that remains in the aqueous phase, was recovered in a new eppendorf 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 with 20µL of DEPC water (Ambion, Life Technologies). Finally, RNA concentration was quantified using a 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, considering the results obtained after quantifications. For the reverse transcription the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used. Specifically, the reactions, per each sample, were achieved with 10µL of RT buffer, 4µL of dNTP mix, 10µL of random primers, 5µL of MultiScribe® Reverse Transcriptase, 21µL of DEPC water (Ambion) and 50µL of the RNA diluted in DEPEC water. The reaction was performed in a programed thermocycler.

Gene expression was measured by quantitative real-time PCR. For the reaction, 1 µL of sample cDNA was mixed with 6µL of TaqMan Universal PCR Master Mix (Applied Biosystem) which contains the necessary elements for the reaction, 0.6µL of the appropriate probe (Applied
Biosystem) and 9.25µL of DEPC water (Ambion, Life Technologies). Fluorescence was detected using ViiA™ 7 Real-Time PCR System and results were analyzed with the QuantStudio Real-Time PCR software v1.1 (both from Applied Biosystems). Gene expression was normalized to the expression of the endogenous control gene GUSB using comparative ΔCt method. GUSB encodes for glucuronidase beta, a hydrolase that degrades glycosaminoglycans. mRNA concentration was expressed in relative units with respect to GUSB.

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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Probe reference</th>
<th>Gene name</th>
<th>Probe reference</th>
</tr>
</thead>
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<td>GUSB</td>
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<td>CXCL8</td>
<td>Hs99999034_m1</td>
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<td>TBX21</td>
<td>Hs00894392_m1</td>
<td>CXCR4</td>
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<td>Hs00171022_m1</td>
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<td>Hs01076112_m1</td>
<td>ICAM1</td>
<td>Hs99999152_m1</td>
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<tr>
<td>STAT3</td>
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<td>VCAM1</td>
<td>Hs01003372_m1</td>
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<td>SOCS3</td>
<td>Hs02330328_s1</td>
<td>VEGFA</td>
<td>Hs00900055_m1</td>
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<tr>
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<td>TGFβ</td>
<td>Hs00171257_m1</td>
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<tr>
<td>IL6R</td>
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<td>COL1A1</td>
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<td>Hs01013996_m1</td>
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<tr>
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<td>SPP1 (OPN)</td>
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<tr>
<td>CCL5</td>
<td>Hs00174575_m1</td>
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</tr>
</tbody>
</table>

**Protein cell lysates**

Cultured cells or temporal artery sections were treated with RIPA (Radio-Immunoprecipitation Assay, Sigma-Aldrich, Ayrshire, UK) lysis 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), aprotinin (Thermo Scientific) and 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. Specifically, 2 to 3 artery sections were homogenized with Polytron® homogenizer (IKA, Staufen, Germany) in 150µL of buffer. For cell lysates 100µL of buffer was used per each well of a 6 well plate.
Protein quantification

Protein lysates were quantified prior to analyze protein expression by immunoblot using the Pierce BCA protein Assay kit (Pierce, Thermo Scientific), to load the same concentration in all the different conditions. Quantifications where done in a 96 well plate and the standard curve was prepared with bovine serum albumin (BSA). Both the samples as well as BSA from the standard curve were diluted with RIPA and prepared in duplicate. Once the diluted samples were loaded to the plate, 200µL of a mixed solution from the kit was added to each well. The mixed solution, composed by two reagents included in the detection kit, reacts with the proteins present in the samples allowing their quantification by absorbance. The plate was incubated for 30 min at 37°C before determining the absorbance at 580nm using a plate reader (Multiskan Ascent, Thermo Scientific).

Immunoblot

Twenty µg of protein per condition were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide (BioRad) electrophoresis gels at reducing conditions. The percentage of acrylamide used to prepare the resolving gel was determined depending on the molecular weight of the analyzed protein. For the present work gels were prepared at 10% or 15% of acrylamide. Samples were diluted with load buffer NuPAGE® LDS Sample Buffer (Invitrogen) and β-mercaptoethanol (Sigma), used as a reducing agent, and they were boiled for 5 min at 95°C to completely denature the proteins. Electrophoresis was carried out at 125V with the running buffer 1X prepared from the pre-made Tris Base (25mM)-Glycine (192mM)-SDS (0.1% w/v) 10X buffer (BioRad) during 2h. After electrophoresis, the gels were blotted onto nitrocellulose membranes (Invitrogen) using iBlot Blotting System (Thermo Fisher). Blocking was performed by incubating the membranes one hour at room temperature with Tris Buffer Saline 1X with 0.1% Tween-20 (TBST 1X) and a 5% of nonfat milk. Immunodetection was performed by incubating primary antibodies diluted following the manufactures recommendations, in TBST 1X with 5% BSA at 4°C, overnight. The day after, membranes were washed 3 times with TBST 1X and incubated with the corresponding secondary antibodies diluted in TBST1X and 5% BSA for 1h at room temperature. Chemiluminescence signal was measured with the ImageQuant LAS-4000 imaging system (GE HealthCare Life Science) after incubating the membranes with Supersignal West Dura or Pico Substrate kit (Thermo scientific) for 5 min. In some cases, membranes were treated for 30 min at room temperature with a stripping buffer with 100mM of glycine (Sigma) and 100mM of NaCl (Sigma) in water at pH 2.5 with HCl, in order to reincubate the membrane with different
antibodies. Image analysis and quantification was done using the ImageQuant TL 8.1 software.

The antibodies and working dilutions used were as follow:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution</th>
<th>Host animal</th>
<th>Clonality</th>
</tr>
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<tbody>
<tr>
<td>Anti-IL-6</td>
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<td>Polyclonal</td>
</tr>
<tr>
<td>Anti-IL6R</td>
<td>Genetex</td>
<td>1:2000</td>
<td>Rabbit</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Anti-pSTAT3 (Y705)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>Monoclonal</td>
</tr>
<tr>
<td>Anti-STAT3</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Mouse</td>
<td>Monoclonal</td>
</tr>
<tr>
<td>Anti-pSTAT1 (Y701)</td>
<td>Cell Signaling</td>
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<td>Rabbit</td>
<td>Monoclonal</td>
</tr>
<tr>
<td>Anti-STAT1</td>
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<td>Anti-Goat-HRP</td>
<td>Santa Cruz</td>
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<td>Mouse</td>
<td>Monoclonal</td>
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**Enzyme-Linked Immunosorbent Assay (ELISA)**

The ELISA is a technique that allows to analyze 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 ELISAs 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 in a plate reader (Multiskan Ascent, Thermo Scientific) and the concentration was extrapolated from a standard curve.

ELISA kits, all from R&D, were used to detect the following molecules: IL-6, sIL-6R in serum of GCA patients and controls; CCL2, CXCL9 and CXCL10 in supernatants of cultured arteries with TCZ; sOPN in supernatants of cultured arteries with TCZ; and CRP and sOPN in serum from patients treated with TCZ.

**Immunofluorescence of TABs**

Fresh biopsies were fixed with paraformaldehyde 4X (PFA) in PBS 1X, pretreated with increasing concentrations of sucrose, 15% and 30%, before being embedded in Tissue-Tek OCT Compound (Sakura) and preserved at -80°C until use. Sections of 7µm were obtained with a
cryotome cryostat (Leica Microsystems) and re-fixed with PFA 4X in PBS 1X for 20min at room temperature. After washing the samples three times with PBS1X for 5min, sections were permeabilized with 0.1% Triton solution in PBS1x for 15min. Blockade of non-specific interactions was performed with a blocking solution with 10% donkey serum (Sigma) in 0.1% Triton PBS1X for 1h at room temperature. Next, samples were incubated overnight at 4°C with the primary antibodies against the target molecules diluted in blocking solution, following the manufacture recommendations. Negative controls were carried out using blocking solution without primary antibodies. The day after, samples were washed 5 times with 0.1% Triton in PBS1X, and they were incubated with the corresponding secondary antibodies conjugated with different fluorochromes for 1h at room temperature. Finally, after 5 more washes, mounting medium with 4′,6-diamidino-2-phenylindole (DAPI Fluoromount-G, Southern Biotech) was used to stain the nuclei and to preserve the preparations. Immunofluorescence samples were observed with a SP5 Leica confocal microscopy (Leica Microsystems) at the Unitat de Microscòpia Confocal de la Universitat de Barcelona and the images were analyzed using the ImageJ software (National Institutes of Health). The antibodies and working dilutions used were as follows:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution</th>
<th>Host animal</th>
<th>Clonality</th>
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<tbody>
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<td>Donkey</td>
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</tr>
</tbody>
</table>

Cell migration assay in Boyden chambers

PBMC cell migration was measured in 48-well Boyden chamber using 5µm pore polyester membranes (Poretics, Osmonics Inc). PBMC were pre-incubated with RPMI 1%FBS medium alone or supplemented with 10µg/mL of TCZ (Roche) or 10µg/mL of IgG control (Sigma) during 1h at 37°C and 5% CO₂. Next, 125,000 PBMC were added to each upper well and allowed to migrate for 5h at 37°C and 5% CO₂. Membrane was then recovered and fixed with methanol (Sigma) for 10min at room temperature. After the staining with hematoxylin Gil III (Leica) for 30min mounting medium (Merck) was applied. Each condition was tested in four different
wells and four randomly selected fields per well were counted under an optic microscope at 40x magnification.

**Adhesion assay**

VSMC were seeded in a 96-well plate the day before at 5,000 cells per well. The day after, PBMC were pre-incubated with medium alone or supplemented with 10µg/mL of TCZ (Roche) or 10µg/mL of IgG control (Sigma) during 1h at 37°C and 5% CO₂. 100,000 untreated or treated PBMC were added to each well over pre-washed VSMC monolayer and allowed to adhere during 30 min at 37°C and 5% CO₂. After removal of non-adherent cells and washing the plate with PBS 1X, cells were stained with 100µL/well of 0.2% crystal violet (Sigma) solutions in 20% methanol and PBS 1X, during 10min. The plate was then washed three times with water and let dry overnight at room temperature. The day after, 100µL/well of SDS 1% was used to lyse the cells and the absorbance was measured with a plate reader (Multiskan Ascent, Thermo Scientific) at 580 nm. Each condition was tested in eight different wells and possible effects of TCZ and IgG isotype control over VSMC was also considered, subtracting the absorbance of VSMC cultured with the corresponding supplemented mediums without PBMC.

**Statistical analysis**

Mann-Whitney U test and Student’s t-test, when applicable, were used for quantitative independent or paired data. Calculations were performed with the IBM SPSS Statistics (Version 20.0, Armonk, NY).
Results
Distribution of IL-6 and IL-6R in GCA lesions

As it has been previously described, IL-6 was intensively expressed in GCA-involved arteries (figure 9.A). IL-6R expression was found to be especially intense in areas with clusters of infiltrating inflammatory cells (figure 9.A arrows) as well as in the neointima and the luminal endothelium (figure 9.A). In control arteries, compared with GCA arteries, a very slight expression of both IL-6 and its receptor was observed in organized VSMC in the media and in the luminal endothelium (figure 9.B. arrows).

Specific cell markers were used to assess which cells in GCA lesions were able to express IL-6 and/or IL-6R (figure 10). Both IL-6 and IL-6R were intensely detected in macrophages, identified as CD68\(^+\) positive cells (figure 10.A). Some CD20\(^+\) positive B cells localized in the adventitia, also expressed IL-6 and IL-6R (figure 10.B). However, their expression was found to be weaker compared with the expression by CD68\(^+\) cells. VSMC, identified as a Smooth Muscle Actin (SMA) positive cells, also showed positive staining for both IL-6 and its receptor (figure 10.C). IL-6 and IL-6R positive staining was also observed in endothelial cells (CD31 positive cells), both the ones forming adventitial vessels as well as the ones that conforms the luminal endothelium (figure 10.D).
Figure 9. IL-6 and IL-6R expression in GCA temporal arteries compared with controls. A. Immunostaining of IL-6 (red), IL-6R (green) and nuclei (blue) in GCA temporal artery. White arrows point out an area with infiltrating inflammatory cells intensively stained for IL-6 and IL-6R. B. Immunostaining of IL-6 (red), IL-6R (green) and nuclei (blue) in control arteries. White arrows highlight the slight expression of IL-6 and IL-6R in the media and the luminal endothelium. (I: intima; M: media; Ad: adventitia). The scale bar represents 200µm in all images.
Figure 10. IL-6 (red) and IL-6R (green) expression by different cellular subsets in GCA lesions using different cell markers (white). A. Macrophages identified as CD68+ cells. B. Expression in B cells marked as CD20+. C. VSMC positive for SMA (Smooth Muscle Actin). D. Endothelial cells identified as CD31 positive cells. Upper image shows a CD31 positive vessel from the adventitia. Bottom image represents the luminal endothelium. The scale bar represents 50µm in all images. Nuclei are represented in blue.
IL-6 and IL-6R expression in GCA lesions and patients’ serum

IL-6 mRNA levels were higher in temporal arteries from GCA patients compared with control arteries, but the difference did not reach statistical significance. Unexpectedly, IL-6R mRNA was significantly less abundant in GCA lesions than in control arteries (figure 11.A). By contrast, IL-6R protein expression was found increased in GCA lesions compared with controls (figure 11.B). This result was in concordance with the previous observations made in immunofluorescence stained biopsies.

**Figure 11.** IL-6 and IL-6R in GCA lesions. A. mRNA expression of IL-6 and IL-6R in fresh GCA biopsies (n=28) compared with control arteries (n=24). Results are expressed in relative units (RU). **p<0.005. B.** Representative image of IL-6R protein expression measured by immunoblot in two GCA biopsies compared with two control arteries. IL-6R protein expression was assessed in a total of 4 GCA biopsies and 4 control arteries with similar results.

In concordance with previous publications, IL-6 concentrations (pg/mL; mean ± SD) were significantly elevated in patients serum with active GCA compared with controls (active disease versus controls; 45.19 ± 36.99 vs. 18.67 ± 12.08, p=0.006). In addition, a significant decline in IL-6 concentrations was observed in patients in remission after one year of treatment with glucocorticoids (remission; 14.27 ± 5.53, p=0.000) (figure 12.A). No differences were found in serum levels of sIL-6R in patients with active GCA compared with controls (active disease versus controls; 76534.88 ± 16693.99 vs. 81829.59 ± 15965.49, p=0.192), nor after one year of treatment with glucocorticoids (remission; 77995.52 ± 20431.99, p=0.192) (figure 12.B).
Under inflammatory conditions VSMCs contribute to IL-6 production whereas PBMCs increase expression of IL-6R

Co-cultures of PBMCs from healthy donors with human temporal artery-derived VSMC, the main component of the arterial wall, where used to mimic the vascular inflammatory microenvironment. The aim was to better characterize the cell types responsible of IL-6 and IL-6R expression in GCA, as well as their possible regulation under inflammatory conditions.

In concordance with the previous results, IL-6 expression was significantly increased in VSMCs and PBMCs when they were in co-culture conditions, both at mRNA as well as protein level (figure 13.A and 13.C). When compared with PBMCs, the expression of IL-6 by VSMC in co-culture was 50 times higher than the expression by PBMCs at 24h (figure 13.A). IL-6R mRNA expression was significantly increased in co-cultured VSMC. However, PBMCs in co-culture significantly decreased the expression of IL-6R at 24h (figure 13.B). For IL-6R the mRNA expression levels were much higher in PBMCs than in VSMC, in all tested conditions. Contrary to the mRNA expression, IL-6R is downregulated at protein level in co-cultured VSMC whereas its protein levels increased in PBMCs when in co-culture (figure 13.C).
Figure 13. IL-6 and IL-6R expression in co-cultured VSMC with PBMC. A. mRNA expression, in relative units (RU), of IL-6 in co-cultured VSMC (left) or co-cultured PBMCs (right) at 24 and 48h. B. mRNA expression (RU) of IL-6R in co-cultured VSMC (left) or co-cultured PBMCs (right) at 24 and 48h. Bars represents the mean ± SEM. **p<0.005. The IL-6 and IL-6R expression was assessed in 5 independent experiments. C. IL-6 and IL-6R protein expression measured by immunoblot in isolated or co-cultured VSMC (left) and PBMC (right) at 24h. Protein expression was analyzed in three independent experiments with similar results.

IL-6R expression by PBMCs in co-culture agreed with the previously observations made in GCA lesions, in which there was a downregulation at mRNA level (figure 11.A) but protein levels were increased (figure 11.B). In addition, IL-6R expression was associated with clusters of inflammatory cells by immunofluorescence (figure 9 and 10). Nevertheless, the results obtained with VSMC in co-culture were apparently contradictory. Co-cultures between VSMCs
and PBMCs were assessed at 24 and 48h to avoid changes induced by prolonged culture. This fact represents a limitation to analyze the effects of long term inflammatory conditions. For this reason, IL-6 and IL-6R mRNA expression was investigated in VSMC, at passage zero, derived from GCA temporal arteries compared with VSMC obtained from control temporal arteries. As shown in figure 14, patient-derived VSMC conserved the mRNA expression pattern of IL-6 and its receptor observed in temporal artery biopsies (figure 11). IL-6 expression was increased in VSMC derived from GCA patients with no significative differences (figure 14.A). On the other hand, IL-6R expression was significantly decreased in these VSMC compared with the ones obtained from control temporal arteries (figure 14.B).

**Figure 14.** mRNA expression, in relative units (RU), of IL-6 (A) and IL-6R (B) in VSMC at passage zero derived from GCA (n=8) or control (n=11) temporal arteries. **p<0.005.

**Effect of IL-6R blockade with TCZ on STAT3 phosphorylation in cultured GCA arteries and in co-cultured VSMC and PBMC**

The impact of IL-6R blockade with TCZ was investigated using the previously described artery cultured model. In this case, GCA positive biopsies were treated with TCZ for 5 days (see materials and methods: Culture of TAB sections for more details) and after that, the phosphorylation state of STAT3, a target of IL-6 signaling pathway, was analyzed to check the blockade effect on these conditions. No differences were found regarding to the activation state of this transcription factor (figure 15.A).
Figure 15. Impact of TCZ blockade on phosphorylation levels of STAT3. A. Representative protein expression of pSTAT3 and STAT3 measured by immunoblot in GCA cultured temporal arteries treated with TCZ. Protein was analyzed in four different cultured arteries with similar results. B. Representative protein expression of pSTAT3 and STAT3 measured by immunoblot in isolated and co-cultured VSMC or PBMC treated with TCZ at 24h. C. Same experiment as in B, but in this case, the co-cultures were carried out in trans-well supports. Protein expression in co-cultures was measured in three independent experiments with same results.

One possibility is that the microenvironment as well as other factors may be conditioning the effects of TCZ. To test this hypothesis, the phosphorylation levels of STAT3 were analyzed in VSMCs and PBMCs in co-culture treated with TCZ. Co-culture conditions induced an increase in phospho-STAT3 (pSTAT3) levels in VSMC, but no effect of treatment with TCZ was observed on these cells at 24h (figure 15.B, left). In PBMCs, TCZ blockade completely abrogated the phosphorylation of STAT3 when these cells were cultured alone and a slightly increase in total STAT3 was observed in this condition. Nevertheless, phosphorylation levels of STAT3 in TCZ treated PBMCs were unmodified by the IL-6R blockade when these cells were co-cultured with VSMC (figure 15.B, right). The same effect was observed when PBMCs were co-cultured with VSMC in a trans-well to avoid cell-cell contact (figure 15.C).
IL-6R blockade with TCZ selectively decreases STAT3 and SOCS3 mRNA expression in cultured GCA arteries

The mRNA expression of a variety of candidate molecules relevant to the pathogenesis of GCA was also analyzed and compared with the expression in the same untreated biopsies or treated with an IgG control. The analyzed molecules included transcription factors involved in T-cell functional differentiation, proinflammatory cytokines, chemokines and chemokine receptors, adhesion molecules, growth factors and extracellular matrix proteins (table 1).

After 5 days, GCA positive biopsies showed a significant increase in mRNA expression of TBX21, GATA3, RORC, FOXP3, IL6, IL1B, IFNG, CCL5 and VEGFA when compared with controls. Moreover, mRNA expression of CXCL12 and TGFB was found to be significantly lower in biopsies from GCA patients than in control biopsies (table 1).

Table 1 also shows the effect of blocking IL-6R with TCZ on analyzed molecules. TCZ treatment appeared not to have a specific effect on the expression of those molecules. However, the relatively low number of specimens analyzed (n=16) and the wide individual variability in expression of inflammatory products could also partially explain these results. Treatment with dexamethasone was able to markedly decrease additional relevant molecules not influenced by TCZ (table 1).
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Number of specimens analyzed: negative biopsies: 8; untreated GCA: 15; GCA treated with TCZ: 16; GCA treated with IgG isotype control: 13; GCA treated with dexamethasone: 5. *p<0.05 and **p<0.005. **TBX21**: T-box 21; **GATA3**: GATA binding protein 3; **RORC**: RAR related orphan receptor C; **FOXP3**: Forkhead box P3; **IL-6**: Interleukin-6; **IL-6R**: Interleukin-6 receptor; **IL-1β**: Interleukin-1 beta; **TNF-α**: Tumor necrosis factor alpha; **IFN-γ**: Interferon gamma; **IL-17A**: Interleukin-17A; **CCL5**: Chemokine C-C motif ligand 5; **IL-8**: Interleukin-8; **CXCR4**: Chemokine C-X-C motif receptor 4; **CXCL12**: Chemokine C-X-C motif ligand 12; **ICAM-1**: Intracellular adhesion molecule 1; **VCAM-1**: Vascular cell adhesion molecule-1; **VEGFA**: Vascular endothelial growth factor; **TGF-β**: Transforming growth factor beta; **COL1A1**: Collagen type I alpha 1 chain; **COL3A1**: Collagen type III alpha 1 chain.
Despite other factors may be contributing to the phosphorylation of STAT3, partially masking TCZ effects, the blockade of IL-6R induced a significant decrease in mRNA expression of STAT3 and SOCS3 in cultured biopsies (figure 16).

*Figure 16. Effect of TCZ blockade on mRNA expression of STAT3 (left) and SOCS3 (right). *p<0.05. A total of 8 control biopsies and 15 GCA cultured temporal arteries were analyzed.*

**TCZ treatment modify chemokine expression in cultured GCA arteries by decreasing CCL2 and increasing CXCL9 and CXCL10**

In addition to its effect on STAT3 and SOCS3 mRNA expression, IL-6R blockade with TCZ also decreased CCL2 mRNA expression in cultured arteries. On the contrary, it significantly increased mRNA expression of CXCL9 and CXCL10 when compared with untreated sections of the same biopsy (figure 17.A). Consequently, CCL2 concentration in the supernatant fluid was also reduced upon TCZ blockade and CXCL9 and CXCL10 concentration were found to be significantly increased (figure 17.B).
To better understand the mechanisms of chemokine regulation induced by TCZ treatment, mRNA expression of CCL2, CXCL9 and CXCL10 was assessed in VSMC, HUVEC and PBMCs in vitro, upon stimulation with IL-6 and sIL-6R and TCZ blockade. The purpose was to study how TCZ may differentially modulate the expression of chemokines in the main cell types present in GCA lesions. Both in VSMC as well as in HUVEC mRNA expression of CCL2, CXCL9 and CXCL10 was increased upon stimulation with IL-6 and sIL-6R. TCZ addition was sufficient to abrogate this induction (figure 18.A and 18.B). In PBMCs, CCL2 expression was also modulated in a similar way, since the induction observed after IL-6 stimulation was inhibited with TCZ. However, interesting results were found for CXCL9 and CXCL10 expression in these cells. IL-6 showed no clear effect on the expression of these two chemokines but blockade of IL-6R with TCZ increased CXCL9 and CXCL10 mRNA expression in PBMCs (figure 18.C).
Figure 18. mRNA expression (relative units, RU) of CCL2 (left panels), CXCL9 (central panels) and CXCL10 (right panels) in VSMC (A), HUVEC (B) and PBMC (C) treated in vitro. Cells were stimulated with IL-6 (10ng/mL) alone (in case of PBMC) or in combination with sIL-6R (100ng/mL) (when working with VSMC and HUVEC) and/or treated with TCZ (10µg/mL) or IgG isotype control (10µg/mL). Graphs show the mRNA expression of a representative experiment. These results were confirmed in two independent experiments.

**CXCL9 and CXCL10 upregulation could be explained by a modulation of STAT1 induced by TCZ blockade**

CXCL9 and CXCL10 are two chemokines regulated by STAT1\(^{165,166}\) which activation has been showed to be highly dependent on IFN-γ in GCA context \(^{48}\). Considering this, the upregulation of these two chemokines could be the consequence of a dysregulation between STAT3 and STAT1 pathways induced after IL-6R blockade with TCZ. To check this hypothesis STAT1 expression was analyzed in GCA temporal arteries cultured with TCZ. Although differences were no statistically significant, mRNA expression of STAT1 showed a tendency to increase upon TCZ treatment when compared with the untreated sections of the same biopsy (\(p=0.053\)) (figure 19.A). Provisional results to be confirmed showed an increase in pSTAT1 levels in TCZ treated sections from cultured GCA biopsies and a slightly increment in total STAT1 expression (figure 19.B).
Figure 19. Effect of TCZ blockade on STAT1 expression in cultured GCA temporal arteries treated with TCZ. A. mRNA expression of STAT1 in GCA cultured temporal arteries. A total of 8 control biopsies and 15 GCA cultured temporal arteries were analyzed. #p=0.053. B. Immunoblot showing the expression of pSTAT1 and STAT1 in GCA cultured temporal arteries.

Functional impact of TCZ treatment on PBMCs: reduction in adhesion and migration

The previous results suggest that TCZ may have a direct effect on membrane receptor-bearing cells, such as PBMCs; especially when they are not in a tissue context or in contact with other cells. For this reason and considering the importance of inflammatory cell adhesion and migration during the pathogenesis of GCA, these functional capacities of PBMCs were evaluated after treatment with TCZ.

Blockade of IL-6R with TCZ significantly decreased PBMC adhesion to VSMC (figure 20.A). Incubation of PBMCs with TCZ also reduced their chemotaxis in Boyden chambers compared with untreated PBMCs (figure 20.B).

Figure 20. Effect of TCZ blockade on PBMC adhesion and migration capacity. A. Lower adhesion capacity of PBMC to VSMC after IL-6R blockade with TCZ. B. Reduced migration capacity of PBMC after treatment with TCZ. Each functional effect was analyzed in three independent experiments. **p<0.005
OPN as an activity biomarker for patients with GCA treated with TCZ

As it has been mentioned before in the introduction section, previous results showed that sOPN was significative elevated in serum of patients with active GCA compared with controls but returned to normal when patients achieved remission with glucocorticoid treatment (see figure 7 in introduction section). Interestingly, sOPN showed a positive correlation with serum IL-6 at the time of GCA diagnosis and both molecules presented a similar pattern since patients with a strong systemic inflammatory response possessed higher levels of sOPN than patients with weak acute phase reaction. On the contrary, sOPN concentrations were significantly lower in patients with cranial ischaemic complications compared to patients without these complications.

All together, these results pointed out the potential value of sOPN as a biomarker of disease activity to monitor GCA patients. The fact that OPN is not totally dependent of IL-6 and its potential as a biomarker in GCA, point out that this protein may be a suitable option for TCZ treated patients. The following experiments were carried out to study the effect of IL-6R blockade on OPN expression and to assess the possible utility of this molecule to monitor patients treated with TCZ.

OPN expression and secretion in cultured GCA arteries is not suppressed by short-term IL-6 receptor blockade

The effect of IL-6R blockade with TCZ was firstly studied using the artery culture model. Short-term treatment with TCZ did not modify OPN mRNA expression or protein secretion in cultured GCA arteries (figure 21). These findings support that, despite the correlation between serum IL-6 and sOPN, OPN production in GCA arteries is not exclusively dependent on IL-6 signaling.
Figure 21. Effect of tocilizumab on OPN expression in cultured temporal arteries from GCA patients and controls. A. OPN mRNA expression by cultured temporal arteries from negative controls and from patients with GCA untreated, treated with TCZ at 10 μg/ml, or treated with non-immune human control IgG at 10 μg/ml. B. OPN concentrations in the supernatants of cultured temporal arteries from negative controls (n=15) and from patients with GCA untreated (n=14), treated with TCZ at 10 μg/ml (n=15), or treated with non-immune human control IgG at 10 μg/ml (n=16). Sensitivity of the immunoassay (minimal detectable concentration) was 0.011 ng/mL.

Serum OPN is reduced but not abrogated in GCA patients in remission treated with tocilizumab

Based on the results on cultured arteries, serum concentrations of sOPN and CRP were measured in a subset of GCA patients that were in remission maintained with glucocorticoids or tocilizumab (alone or in combination with low-dose prednisone) to see whether sOPN could be a useful biomarker in patients receiving IL-6 blockade therapy. As shown in figure 22.B, serum CRP was significantly lower and sometimes undetectable in tocilizumab-treated patients compared with prednisone only -treated patients at high dose or low-dose. In contrast, sOPN was detected in all cases without significant differences between patients under tocilizumab or glucocorticoid only treatment either at high dose or low-dose figure 22.A. These results suggest that, unlike CRP, sOPN might be an interesting disease activity biomarker to be explored in tocilizumab-treated patients.
Figure 22. Serum osteopontin (sOPN) and C-reactive protein (CRP) in patients with giant-cell arteritis in remission according to treatment. **A.** sOPN in patients with GCA in remission with high-dose prednisone (PDN) (≥ 20 mg/day), low-dose prednisone (≤ 10 mg/day) or with tocilizumab (TCZ). **B.** Serum CRP concentration in patients with GCA in remission with high-dose prednisone (≥ 20 mg/day), low-dose prednisone (≤ 10 mg/day) or with tocilizumab (TCZ). **p = 0.017 vs high-dose prednisone group and p< 0.001 vs low-dose prednisone group. Sensitivity (minimal detectable concentration) of OPN and CRP immunoassay are 0.011 ng/mL and 0.010 ng/mL respectively.
Discussion
The results from the present study show that IL-6 and IL-6R are remarkably increased in temporal artery lesions from patients with GCA compared with normal arteries. In accordance with previously reported results 43, 46, 47, 51, 68, 127, 128, 129, 130, IL-6 was found to be upregulated both at tissue level as well as in the serum of patients with GCA.

The expression pattern of IL-6R appears to be more complex and some discrepancies were found between tissue and the peripheral compartment. The results from the present work show, for the first time, that IL-6R is upregulated in GCA lesions, particularly at the granulomatous areas. This distribution agrees with the known expression of IL-6R by leukocytes 116. In addition, co-culture experiments supported this result since, in an inflammatory microenvironment, IL-6R protein production was increased in mononuclear cells. The intense staining of IL-6R observed in CD68+ cells seems to indicate that macrophages may play an important role as a source of IL-6R in GCA lesions. Co-cultures with isolated mononuclear cells, as previously reported 65, may help to deeper understand their function in IL-6 signaling and to confirm its main role as a source of IL-6R.

Unexpectedly, IL-6R mRNA was decreased in GCA lesions. Other molecules have also shown dissociation between protein and mRNA expression in GCA temporal arteries 64, and it may be due to the complexity of regulatory mechanisms. This discrepancy could be explained by counterregulatory mechanisms that may influence mRNA expression or stability. In some systems, accumulation of the final products exerts a negative feedback loop on de novo expression of molecules participating in the corresponding signal pathway. In fact, it has been shown that IL-6 exerts a negative feedback loop decreasing IL-6R expression in a murine B-cell hybridoma and a natural killer cell line 167, 168.

In addition, mRNA expression may be regulated by controlling its stability. Multiple mechanisms have been described to regulate IL-6 expression including some microRNAs (miR), RNA-binding proteins, as well as RNAse 98. It would not be surprising that, some of these mechanisms also participate in the regulation of IL-6R expression. In this sense, several microRNAs, such as miR-451 or miR-449a 169, 170, 171, have been demonstrated to decrease IL-6R mRNA expression.

When analyzing IL-6R mRNA expression by VSMC co-cultured with PBMC, some differences were found compared to the expression observed in whole GCA temporal arteries and with the expression in PBMCs co-cultured with VSMC. However, in unexpanded VSMC directly derived from GCA lesions, IL-6R was decreased when compared with VSMC obtained from control temporal arteries. Thus, IL-6R expression by VSMC may be modulated by prolonged inflammatory conditions that would produce a decrease in IL-6R mRNA in these cells. In addition, it should be noticed that the expression levels of IL-6R in PBMCs were notably higher.
than the ones observed in VSMC, indicating that VSMC could have a minor role in terms of IL-6R expression compared with mononuclear cells in inflamed arteries.

At the protein level, an interesting result was also obtained in co-cultured VSMC, since a decrease in IL-6R was observed. VSMC may be the main target cells to respond to the IL-6R produced by mononuclear cells in this culture model. IL-6 and sIL-6R complex present in co-culture supernatant may interact with membrane gp130, expressed on VSMC, triggering the activation of IL-6 trans-signaling pathway on these cells. As a consequence, the IL-6 receptor complex assembly could promote the internalization of IL-6R and its degradation in lysosomal vesicles, leading to a decrease in IL-6R protein content in co-cultured VSMC cells lysates.

Contrary to what was observed in tissue, serum levels of sIL-6R showed no differences between GCA patients and controls. This result agrees with observations made in a related inflammatory disease such as PMR. Currently, there is only one publication referring to sIL-6R levels in serum of GCA patients. In this recently published work, Pulsatelli et al. reported a significant increase of sIL-6R serum levels in GCA patients. However, this study was carried out with a small cohort of patients.

Regarding to the expression of IL-6, it was shown to be increased under co-culture conditions. IL-6 was especially enhanced in co-cultured VSMC, showing that these cells may be an important source of this cytokine in GCA. This result agrees with previous observations where VSMC increase IL-6 production when in co-culture with isolated monocytes. Positive IL-6 staining in endothelial cells of GCA lesions suggest that these cells may also contribute to the increased levels of IL-6 observed in GCA. Co-cultures with PBMCs and endothelial cells may help to assess this possibility.

The artery culture model, where various sections of the same biopsy are cultured under different conditions and compared, was used to better understand the impact of TCZ blockade. No variations in pSTAT3 levels were observed after treatment with TCZ for 5 days. In this case, limitations of established culture conditions may partially explain the absence of effect on STAT3 phosphorylation state. STAT3 is a direct target of IL-6 signaling pathway and its activation and transient translocation to the nucleus occur rapidly after IL-6 treatment. Consequently, the possible impact of TCZ on STAT3 phosphorylation may disappear after 5 days in culture.

In addition, there are other molecules that may lead to an increase in the phosphorylation of STAT3 beyond IL-6, such as other cytokines from the IL-6 family, many growth factor receptors as well as other proinflammatory molecules present in GCA, like IFN-γ or ET-1. These molecules may be upregulated under inflammatory conditions and may contribute to counteract the effect of IL-6R blockade with TCZ on STAT3 phosphorylation. In accordance with
this hypothesis, the results of the present study show that while TCZ completely abrogated
STAT3 phosphorylation in isolated PBMC, when these cells where co-culture with VSMC
simulating inflammatory conditions, no effect of TCZ on pSTAT3 levels was observed,
regardless of whether PBMC where in contact with VSMC or separated using trans-wells. In
isolated PBMCs, a slight increase in total STAT3 levels was also observed that may reflect a
compensatory mechanism. Among the different molecules that can contribute to STAT3
phosphorylation in co-culture conditions, at least two, IFN-γ and ET-1, have been reported to
be present in the supernatants. Nevertheless, other molecules may be increased under
these conditions and future studies using this model may be useful to identify other possible
STAT3 phosphorylation triggers.
Considering the discrepancy observed between isolated or co-cultured PBMCs treated with
TCZ, the effect of IL-6R blockade could depend on stimuli present in the micro-environment.
Thus, in tissues where mononuclear cells are surrounded by other cells, such as VSMC, the
effect of TCZ blockade may be at least partially compensated by interactions between
inflammatory cells and the consequently generated environment. This could be the scenario in
GCA lesions. On the contrary, peripheral mononuclear cells could be highly sensitive to TCZ
blockade, showing an important decrease in STAT3 phosphorylation levels after treatment. To
test this hypothesis, an interesting point would be analyzing the activation state of STAT3 and
other directly regulated molecules, in mononuclear cells obtained from GCA patients treated
with TCZ.
Even though no evident effect of TCZ was observed on STAT3 phosphorylation after 5 days in
the artery culture model and in spite of the possible protective effect of the microenvironment
suggested by the co-cultured system, TCZ treatment resulted in a significant decrease in the
expression of some IL-6-induced genes, specifically STAT3 and SOCS3. These results
demonstrate a specific impact of TCZ in regulating the expression of molecules implicated in
the IL-6 signaling pathway, which are known to be modulated through this via.
The changes in expression of the molecules explored in untreated artery sections from GCA
patients and those treated in non-immune IgG not always were both significant with respect to
TCZ treated sections. Two important things need to be considered in this regard. First, the
heterogeneous distribution of the inflammation throughout the temporal artery and the
variability introduced during the technical procedure may contribute to this variability. Second,
the IgG control was used to control for non-specific changes induced by IgG or changes
achieved through Fc receptors. Unlike TCZ, which has been approved as a therapeutic drug and
consequently it is a pure reagent with low non-specific interactions, the control IgG used may
have induced some uncontrolled effects increasing the variability between the treated sections.

The number of cultured arteries included in the present work is too small to study possible correlations between the results after TCZ treatment and patient characteristics. Around 40% of patients did not respond to TCZ in the reported phase 3 clinical trial pointing out the necessity of predictive markers of response. When the expression of selected molecules was analyzed independently in each artery culture experiment, response to TCZ was variable and not always present. Therefore, expanding the number of culture temporal arteries, as well as, analyzing gene expression profiles between responders and non-responders, may help to better identify specific predictors of response to be analyzed on patient cohorts.

It has been found that GCA patients with a strong systemic inflammatory response have elevated circulating levels of IL-6 and an increased tissue expression; and are more resistant to corticosteroid therapy. Because of this, one interesting point could be to study whether patients with strong systemic inflammation respond better to TCZ.

After analyzing the expression of different candidate molecules relevant to GCA pathogenesis in the artery culture model, results agreed with previously observed, demonstrating the reliability of this model. As already described, the expression of TBX21, GATA3, RORC, IL1-β, IFN-γ and CCL5 was significantly increased in GCA positive biopsies when compared with control arteries. In addition, the decrease in TGF-β expression has been also previously observed. Nonetheless, some differences in terms of statistical significance were found for STAT3, IL-6 and TNF-α, probably due to the wide individual variability in expression of inflammatory products. It is important to remark that, despite some difference in statistical significance, the tendencies observed in mRNA expression in the present study were consistent with previously published results.

In the artery culture model, glucocorticoids, the cornerstone of remission-induction treatment in GCA, induced a decrease of additional relevant molecules in GCA not influenced by TCZ. Based on the findings obtained for STAT3 phosphorylation levels, compensatory mechanisms may partially mask the impact of TCZ treatment. Moreover, the specific blockade of IL-6 pathway with TCZ may not be sufficient to abrogate inflammatory activity in full-blown GCA lesions, which may require blockade of multiple pathways. Glucocorticoid treatment also promoted a decrease in SOCS3 expression, probably as a consequence of its effect on IL-6 expression. In addition, as previously shown, glucocorticoid treatment received by some patients prior to the temporal artery biopsy may also have influenced results obtained in the ex vivo model, contributing to dispersion of results.
In addition to the effect on STAT3 and SOCS3 mRNA expression, IL-6R blockade with TCZ also decreased CCL2 and increased the expression of CXCL9 and CXCL10 in cultured temporal arteries. This effect on chemokine expression may be due to a specific and particular response induced by TCZ treatment in each of the different cell types present in the artery vessel. Based on the in vitro results presented here, IL-6R blockade may promote an upregulation of CXCL9 and CXCL10 expression in mononuclear cells, that may explain the increased expression observed in cultured arteries. An increase in CCL2 expression has been previously reported in human aortic VSMC as well as HUVEC upon IL-6 pathway activation \(^{105, 145, 178}\). Therefore, blockade of IL-6R with TCZ may result in a decrease in CCL2 expression by vascular wall components VSMC and endothelial cells. The different response observed depending on which cell type was used may be due to the specific cellular response to cytokines \(^{179}\). The response specificity may depend of three different factors that are not mutually exclusive: the combination of downstream molecules expressed in each cell type; the balance of opposite stimuli that will determine the resulting signal; and the variations in downstream pathways that may lead to different output signals \(^{179}\). For instance, this complexity may also explain why in human lung fibroblasts, obtained from patients with idiopathic pulmonary fibrosis, IL-6 shows a mitogenic role, whereas, in normal fibroblasts it inhibits proliferation \(^{180}\).

It has been shown that STAT1 regulates the expression of CXCL9 and CXCL10 in other settings \(^{165, 166}\). In GCA context, IFN-γ induces expression of CXCL9 and CXCL10 most likely through the activation of STAT1 that may interact with interferon-stimulated responsive elements presents in the promoter of these two chemokines \(^{48}\). Thereby, the upregulation of CXCL9 and CXCL10 in mononuclear cells could be the consequence of a dysregulation between STAT3 and STAT1 pathways induced by TCZ blockade. Although, some stimuli have been shown to activate both STAT1 and STAT3, opposite roles have been associated to these two transcription factors in terms of cell proliferation, apoptosis or inflammation \(^{177}\). STAT3 may down-regulate STAT1 activity, and vice versa, through different mechanisms depending on the cellular context, including induction of SOCS3 or SOCS1 respectively, competition for common receptor docking sites and heterodimer formation \(^{177, 181}\). It has been shown that STAT1-induced expression of CXCL9 and CXCL10 is decreased after STAT3 activation, by preventing STAT1 to form homodimers in myeloid cells \(^{181}\). Therefore, changes in the expression of STAT3 caused by TCZ blockade could be altering the expression and/or activation of STAT1 and its induced responses in this context.

When STAT1 mRNA expression was analyzed in cultured arteries treated with TCZ, a trend towards an increased expression was observed when compared with controls (p=0.053). It should be considered that if this effect is only affecting mononuclear cells present in the artery
vessel, the analysis of the whole artery may be partially masking this result. Preliminary experiments also shown an increase in pSTAT1 in TCZ treated biopsies and a slightly increment in total STAT1 expression. Nevertheless, this result must be confirmed in additional samples before drawing a conclusion. Other interesting approach, to better develop this hypothesis, would be analyzing the expression of STAT1 in VSMC and PBMCs cells treated with TCZ in vitro, both at mRNA as well as protein level. In accordance with this hypothesis, STAT1 should be somehow upregulated in PBMCs when they are treated with TCZ, whereas the expression in VSMC should not be altered after TCZ treatment.

The present results showed that PBMCs were the more sensitive cells to TCZ treatment, especially when they are not in a tissue context or in contact with other cells. For this reason, the functional impact of IL-6 blockade in the migration and adhesion capacity of these cells was investigated. Both processes mediate recruitment of inflammatory cells into the vessel wall and are crucial to the development of inflammatory infiltrates in GCA. IL-6 signaling inhibition with TCZ treatment was able to reduce the adhesion and migratory capacity of mononuclear cells. Further research is needed to better understand the molecules involved in TCZ modulation of these two processes.

An increase in ICAM-1 expression has been reported after treatment of HUVEC cells with IL-6 in combination with sIL-6R. In the same work, authors observed an increased adhesion capacity of a monocyte cell line after HUVEC stimulation with IL-6 and sIL-6R. This adhesion was suppressed when cultured endothelial cells were also treated with TCZ. The induction of ICAM-1 after IL-6 stimulation have been also reported in other cell types such as oral squamous cell carcinoma cells. However, in the present work, the short time under which the adhesion assay was conducted (30min) make unlikely that the effect of TCZ on PBMC adhesion will be mediated through regulation of ICAM1 expression in VSMC. In addition, despite PBMC remained more time with TCZ during the experiment, it is also scarcely possible that a modulation in ICAM-1 expression in PBMC would explain this result.

One possible explanation could be that IL-6 blockade with TCZ prevents integrin activation on mononuclear cells, downregulating cell adhesion. Integrins are transmembrane receptors that poses two directions of signaling: inside-out and outside-in. In addition, these adhesion molecules present different conformation states related with the affinity to their ligands, that are modulated through the bidirectional signaling. Regulation of integrin activity is a complex process in which different intracellular proteins may interact with the integrin cytoplasmic domain modifying it ligand binding affinity. IL-6R blockade with TCZ could induce a dysregulation of intracellular kinase activity leading to a downregulation of integrins activation and a subsequent decrease in mononuclear cell adhesion.
Future experiments will also help to better understand the role of TCZ in mononuclear cell migration. It has been shown that IL-6 induced rat VSMC migration in a dose-dependent manner through activation of gp130/STAT-3 signaling\(^{107}\). IL-6 also induced cell motility by increasing phosphorylation of focal adhesion kinase (FAK) and paxillin and by promoting actin polymerization in cultured VSMC from rat aortas\(^{185}\). IL-6 role in inducing cell migration have been considerably investigated in cancer research field because its importance in tumor progression\(^{186,182}\). It has been seen, for instance, that IL-6, produced by cancer associated fibroblasts, enhances gastric cancer cell migration by activating JAK2/STAT3 pathway\(^{186}\). Despite there are some evidence indicating that IL-6 may play an important role in cell motility, specific mechanisms by which activation of this pathway regulates this process are less investigated. Since TCZ modified the chemokine pattern expression in mononuclear cells it would be interesting to investigate whether this treatment also regulates the expression of chemokine receptors. In addition, the upregulation of CXCL9 and CXCL10 observed in PBMCs after IL-6R blockade might be acting in an autocrine way, preventing cells from migration.

Results from the present study suggest an important role of TCZ treatment on lymphocyte and monocyte recruitment into the involved arteries. IL-6R blockade may contribute to decrease tissue inflammation by limiting the advent of new inflammatory cells. However, our study has some limitations that need to be considered. The ex vivo artery culture system is isolated from a functional immune system. Therefore, overall benefits of TCZ therapy could be wider and more complex. Ex vivo cultured arteries are not exposed to recruitment of inflammatory cells into the inflamed artery. Whereas, in vivo, it is likely that inhibition of inflammatory cell recruitment induced by IL-6R blockade, would result in a greater impact on the downregulation of inflammatory products in the target tissue. In addition, the consequence of the potentially counter-acting induction of CXCL9 and CXCL10 chemokines needs to be better delimited as might reduce efficacy in some patients. However, the direct impact of TCZ at adhesion and migration of PBMC would hamper the effect of increased chemokine expression.

Future studies are necessary to identify predictive factors of response, contributing to a more tailored administration of TCZ to GCA patients.

TCZ represents a powerful new tool for the treatment of GCA patients, allowing reduction of glucocorticoid administration and, in consequence, some of its associated adverse effects. As shown in the present study, the inhibition of inflammatory cell recruitment may partially explain the therapeutic benefit associated to IL-6R blockade. Future research will help to determine which type of GCA patients will take more advantage from this treatment.
The last aim of the present work was to analyze the potential of sOPN as a biomarker of disease activity in TCZ treated GCA patients. Previous results indicated that sOPN may be a useful biomarker of disease activity and predictor of relapsing disease and glucocorticoid requirements. The assessment of disease activity in individuals treated with TCZ is difficult because neutralizing IL-6R blocks the hepatic synthesis of acute phase proteins usually used to monitor disease activity in GCA patients. Beyond IL-6, different proinflammatory molecules such as IL-1-β, TNF-α or IFN-γ, can promote OPN induction. Thus, its synthesis is not exclusively IL-6 dependent. Preliminary results from the present study indicate that short-term TCZ treatment of cultured arteries does not have a selective impact in OPN expression in GCA lesions. Consistently, while levels of CRP were virtually undetectable after IL-6R blockade, serum concentration of OPN was similar in patients on glucocorticoid or TCZ maintained remission.

All together, these data suggest that sOPN could be a useful biomarker of disease activity for TCZ treated patients. However, the role of sOPN as a marker of disease activity in patients in tocilizumab versus glucocorticoid induced remission could not be specifically assessed, since no longitudinal samples or samples during relapses were available. Baseline samples from TCZ treated patients would also allow to assess the role of sOPN as a predictor of response to TCZ, as demonstrated in rheumatoid arthritis. For all these reasons, the role of sOPN needs to be further explored in larger studies with longitudinal cohorts. Based in these results, sOPN will be analyzed in serum of patients enrolled in the phase III clinical trial (GiACTA).
Conclusions
The conclusions from the present work are as follows:

1. In agreement with previous publications, IL-6 is increased in giant-cell arteritis (GCA) lesions as well as in serum from patients with GCA.
2. IL-6 receptor expression is increased in GCA arteries. However, there are no differences in sIL-6R levels in serum between GCA patients and controls.
3. In GCA lesions, infiltrating mononuclear cells represent the main source of IL-6R. Whereas VSMC would be the main producers of IL-6 under inflammatory conditions.
4. IL-6R blockade with TCZ abrogates STAT3 phosphorylation in isolated peripheral blood mononuclear cells but does not substantially affect the phosphorylation state of STAT3 in PBMC co-cultured with VSMC or STAT3 phosphorylation in cultured GCA arteries, suggesting a compensatory effect by other STAT3 activating stimuli present in the inflammatory microenvironment.
5. However, TCZ treatment of GCA cultured arteries has functional effects and selectively decreases STAT3 and SOCS3 expression.
6. Among STAT-3 regulated molecules, IL-6R blockade with TCZ significantly decreases CCL2 production. Surprisingly, TCZ treatment increases expression of chemokines CXCL9 and CXCL10.
7. In vitro treatment of VSMC, HUVEC and PBMCs with TCZ differentially modulates the expression of CCL2, CXCL9 and CXCL10 in these cells. These results suggest that mononuclear cells may be the source of CXCL9 and CXCL10 in response to TCZ treatment in GCA cultured arteries.
8. TCZ treatment has a direct and rapid functional impact on PBMCs decreasing their adhesion and migration capacity which may prevent recruitment of circulating leukocytes into the arteries.
9. Contrary to CRP, OPN expression and secretion is not exclusively dependent on IL-6 and consequently it is not suppressed by short-term exposure of cultured GCA arteries to TCZ.
10. Serum OPN is reduced but not abrogated in GCA patients in TCZ-maintained remission whereas CRP levels are virtually undetectable, pointing out the potential of OPN detection to monitor disease activity in TCZ-treated GCA patients.
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Additional data
Induction of CCL2 mRNA expression in VSMC and HUVEC was only observed when cells were cultured with IL-6 and sIL-6R (Supplementary figure 1. A and B). While PBMC were able to respond to either IL-6 alone or in combination with its soluble receptor (Supplementary figure 1. C). This is provably due to the expression of membrane IL-6R in PBMC cells. On the contrary, VSMC and HUVEC do not express the membrane receptor or its expression is not enough to make this cells responsiveness.

Supplementary figure 1. mRNA expression (relative units, RU) of CCL2 in VSMC (A), HUVEC (B) and PBMC (C) treated in vitro. Cells were stimulated with IL-6 (10ng/mL) alone or in combination with sIL-6R (100ng/mL) and/or treated with TCZ or an IgG isotype control (both at 10µg/mL).
Annex
Original articles


Reviews

- Terrades-Garcia N and Cid MC. Pathogenesis of giant-cell arteritis: how targeted therapies are influencing our understanding of the mechanisms involved Rheumatology. In press.

ORIGINAL ARTICLE

Serum osteopontin: a biomarker of disease activity and predictor of relapsing course in patients with giant cell arteritis. Potential clinical usefulness in tocilizumab-treated patients

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ABSTRACT

Background Osteopontin (OPN) is a glycoprotein involved in Th1 and Th17 differentiation, tissue inflammation and remodelling. We explored the role of serum OPN (sOPN) as a biomarker in patients with giant cell arteritis (GCA).

Methods sOPN was measured by immunoassay in 76 treatment-naïve patients with GCA and 25 age-matched and sex-matched controls. In 36 patients, a second measurement was performed after 1 year of glucocorticoid treatment. Baseline clinical and laboratory findings, as well as relapses and glucocorticoid requirements during follow-up, were prospectively recorded. sOPN and C reactive protein (CRP) were measured in 32 additional patients in remission treated with glucocorticoids or tocilizumab (interleukin 6 (IL-6) receptor antagonist). In cultured temporal arteries with glucocorticoids or tocilizumab, OPN mRNA expression and protein production were measured by reverse transcription polymerase chain reaction (RT-PCR) and immunoassay, respectively.

Results sOPN concentration (ng/mL; mean±SD) was significantly elevated in patients with active disease (116.75±65.61) compared with controls (41.10±22.65; p<0.001). A significant decline in sOPN was observed in paired samples as patients entered disease remission (active disease 102.45±57.72, remission 46.47±23.49; p<0.001). sOPN correlated with serum IL-6 (r=0.55; p<0.001). Baseline sOPN concentrations were significantly higher in relapsing versus non-relapsing patients (relapsers 129.08±74.24, non-relapsers 90.63±41.02; p=0.03). OPN mRNA expression and protein production in cultured arteries were not significantly modified by tocilizumab. In tocilizumab-treated patients, CRP became undetectable, whereas sOPN was similar in patients in tocilizumab-maintained (51.91±36.25) or glucocorticoid-maintained remission (50.65±23.59; p=0.49).

Conclusions sOPN is a marker of disease activity and a predictor of relapse in GCA. Since sOPN is not exclusively IL-6-dependent, sOPN might be a suitable disease activity biomarker in tocilizumab-treated patients.

INTRODUCTION

Giant cell arteritis (GCA) is an inflammatory disease of large-sized and medium-sized arteries with a chronic and relapsing course.1 2 About 43%–64% of patients experience recurrences3 4 5 and require long-term glucocorticoid treatment with substantial toxicity.4 6 7 For years, attempts to identify glucocorticoid-sparing agents have not been
clearly successful, but a new treatment paradigm based on the inhibition of interleukin 6 (IL-6) signalling is emerging in the field of GCA, supported by two recent randomised controlled trials with the IL-6 receptor neutralising antibody tocilizumab.

The inflammatory markers erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) are widely used in clinical practice to monitor disease activity in patients with GCA. However, IL-6 receptor blockade with tocilizumab abrogates the hepatic synthesis of acute-phase reactants and renders CRP and ESR measurement unreliable for the purpose of monitoring disease activity. For these reasons, there is an urgent need for novel biomarkers that reflect overall disease activity in the era of tocilizumab treatment for GCA.

Osteopontin (OPN) is a multifunctional intracellular and secreted glycoprotein that functions as a matrix protein or as a soluble mediator. It is expressed by a variety of cells involved in immune and inflammatory responses, including dendritic cells, T and B lymphocytes, macrophages, neutrophils and eosinophils. OPN participates in innate and adaptive immune responses. It is highly induced after T lymphocyte activation, stimulates Th1 and Th17 differentiation and inhibits Th2-mediated responses. It also promotes B cell differentiation and immunoglobulin production. OPN is not expressed by circulating monocytes, but it is highly upregulated during macrophage differentiation. OPN has integrin and CD44 binding sequences and supports lymphocyte and monocyte migration and survival. In addition, OPN enhances endothelial and vascular smooth muscle cell (VSMC) migration, contributing to angiogenesis and vascular remodelling. According to these functions, OPN is highly expressed at the sites of inflammation and tissue injury and reflects concomitant activation of different pathways relevant to immune and inflammatory responses that participate in the pathogenesis of GCA.

Based on its production by activated macrophages, OPN expression was investigated by immunohistochemistry in a variety of granulomatous diseases. In this survey, increased tissue expression of OPN was observed in two temporal artery biopsies from patients with GCA. Moreover, increased circulating soluble OPN has been shown in several inflammatory diseases of blood vessels, including Behçet’s disease and anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis.

Elevated tissue and serum concentrations of OPN have been demonstrated in patients with other vascular conditions, such as non-vasculitic thoracic or abdominal aortic aneurysms. To date, serum OPN (sOPN) concentrations have not been explored in patients with GCA.

In this study, we investigated the value of sOPN as biomarker of disease activity and risk of disease relapse in patients with GCA. In addition, we explored the temporal artery expression of OPN in the context of IL-6 receptor blockade. Finally, we compared the serum concentrations of OPN and CRP in patients in remission receiving tocilizumab (with or without low-dose glucocorticoids) or glucocorticoids alone.

MATERIALS AND METHODS

Patients
The study group consisted of 76 biopsy-proven patients with GCA, selected from two reported cohorts. The first cohort consisted of patients with GCA prospectively imaged in order to detect large vessel involvement at diagnosis (cohort 1). The second cohort incorporated patients who were cross-sectionally evaluated to detect aortic dilatation during follow-up (cohort 2). Patient inclusion in the present study was based on the availability of serum samples collected at the time of diagnosis, before the initiation of glucocorticoid treatment. Cohort 1 included 42 patients and cohort 2 included 34.

Cranial symptoms (headache, jaw claudication, scalp tenderness), systemic manifestations (weight loss, fever), polymyalgia rheumatica and disease-related cranial ischemic complications were recorded at the time of diagnosis. CRP, haemoglobin concentration and ESR were determined by laboratory standardised systems.

Patients were followed for at least 2 years (mean 187 weeks, range 114–360), and time to first disease relapse, number of disease relapses, time to reach a stable (at least for 3 months) prednisone maintenance dosage (<10mg/day or <5mg /day, as well as time to prednisone discontinuation and cumulative prednisone dose were recorded. We used a consensus definition of relapse established in the context of international multicentre clinical trials. Relapses were defined as reappearance of GCA manifestations, usually accompanied by elevation of acute-phase reactants, that required treatment adjustment. Disease-related symptoms considered were polymyalgia rheumatica, cranial symptoms (headache, scalp tenderness, jaw claudication, cranial ischemic complications), systemic manifestations (anaemia, fever and/or weight loss) or symptomatic large vessel involvement (extremity claudication). Cranial ischemic manifestations included amaurosis fugax, GCA-related visual loss, diplopia, transient ischaemic attacks or stroke. Isolated increases in ESR or CRP were not considered relapses unless the above symptoms occurred after close follow-up.

In 36 patients from the discovery cohort, a second sample was obtained after approximately 1 year (402±48 days) of glucocorticoid treatment following a uniform, previously reported tapering protocol. At the time of the second sample collection, patients were receiving a median daily prednisone dose of 5mg/day (range 2.5–10mg/day), and all patients were in clinical remission defined by the absence of disease-related manifestations and the presence of ESR and CRP levels within the normal range.

The control group consisted of two sets of 13 (A) and 12 (B) healthy individuals with no chronic inflammatory diseases and matched for age and gender. sOPN was measured in 17 additional patients with GCA in
prednisone-maintained remission. Seven patients were receiving ≥20 mg/day (high dose) and 10 were receiving ≤10 mg/day (low-dose). sOPN was subjected to cross-sectional comparison with that from 15 patients in remission treated with tocilizumab (4–8 mg/kg/month) (as monotherapy or together with low-dose prednisone) to explore the effect of tocilizumab on sOPN concentration. CRP was simultaneously measured in the same samples for comparison purposes. The clinical characteristics of these additional 32 patients have been previously described. 29

sOPN, CRP and IL-6 measurement
Serum samples were stored at −80°C until use. sOPN, IL-6 and CRP in sera were determined by immunoassay (Quantikine R&D Systems, Minneapolis, Minnesota, USA). OPN was also detected in the supernatant fluid from cultured temporal arteries.

Culture of temporal arteries from patients with GCA and controls
Temporal artery fragments from 16 patients with GCA and normal temporal arteries from 15 controls (patients with suspected GCA who eventually were diagnosed with other conditions) were cut into 0.8–1 mm sections and were embedded in Matrigel (Corning Matrigel Basement Membrane Matrix, Life Sciences, Tewksbury, Massachusetts, USA) to ensure prolonged survival as described 30 31 with or without tocilizumab (RoActemra, purchased from Hoffman-La Roche, Basel, Switzerland) or an IgG isotype control (Sigma, Ayrshire, UK) both at 10µg/mL. Each condition was tested in two to three replicate wells. Temporal artery biopsies were obtained for diagnostic purposes and, since appropriate diagnosis was the first priority, all conditions could not be tested in all samples due to the small size of tissue spared for research. After 5-day culture, sections were homogenised and frozen in TRizol reagent (Life Technologies, Carlsbad, California, USA) for RNA extraction.

RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR)
Total RNA was obtained from 11 cultured arteries from six patients with GCA and five controls, following the instructions of the manufacturer; and reverse-transcribed to cDNA using Archive Kit (Applied Biosystems, Life Technologies) in a final volume of 100µL, employing random hexamer priming. OPN and signal transducer and activator of transcription 3 (STAT3) expression, as well as expression of the housekeeping gene GUSb, was investigated using specific predeveloped TaqMan probes (Hs00959010_m1, Hs00374280_m1 and Hs9999908_m1, respectively) from Applied Biosystems (TaqMan Gene Expression Assays). Fluorescence was detected using Viia 7 Real-Time PCR System and results were analysed with the QuantStudio Real-Time PCR V.1.1 software (both from Applied Biosystems). Gene expression was normalised to the expression of the endogenous control GUSb using comparative threshold cicles (ΔCt) method. mRNA concentration was expressed in relative units with respect to GUSb.

RESULTS
sOPN concentrations are increased in patients with active GCA
Table 1 summarises clinical and laboratory data from cohort 1 and cohort 2 of patients with GCA. sOPN concentrations (ng/mL; mean±SD) were significantly elevated in patients with active GCA compared with controls in both cohorts: GCA 119.59±70.36 vs controls 42.82±24.58 (p<0.001) in cohort 1, and GCA 113.21±69.56 vs controls 39.24±21.28 (p<0.001) in cohort 2 (figure 1A,B); as well as in the pooled cohorts of patients with active GCA (116.75±69.61) compared with pooled healthy controls (41.10±22.65; p<0.001) (figure 1C). In the 36 patients who underwent a second sample collection at the time of disease remission, a significant decline in sOPN concentrations was observed (active disease at baseline 102.45±57.72 vs remission 46.47±23.49; p<0.001) (figure 1D). Using ROC analysis, an sOPN cut-off of 59.79 ng/dL resulted in a sensitivity and specificity of 80% and 84%, respectively, for patients with active GCA compared with healthy controls (area under the curve (AUC) 0.862, 95% CI 0.788 to 0.937; p<0.001) (figure 1E). Moreover, a sOPN cut-off of 67.28 ng/dL resulted in a sensitivity and specificity of 77% and 78%, respectively, to detect disease activity when analysing active patients and those in remission (AUC 0.836, 95% CI 0.764 to 0.907; p<0.001) (figure 1F). No significant differences in sOPN concentrations were found between patients in remission (48.78±23.97) and healthy controls (41.10±22.65; p=0.213).

Patients with GCA with active disease and systemic symptoms demonstrated significantly higher sOPN concentrations than those with no systemic symptoms (118.45±61.70 vs 82.70±57.50; p=0.028) (table 2). Contrarily, and as previously described for serum IL-6, 32 sOPN concentrations were significantly lower in patients with cranial ischaemic complications compared with patients without these complications (79.91±57.90 vs 117.29±61.32; p=0.028) (table 2). There were no differences in baseline sOPN levels between patients with or
Table 1  Baseline clinical, laboratory and follow-up data of patients with giant cell arteritis

<table>
<thead>
<tr>
<th>Cohort 1</th>
<th>Cohort 2</th>
<th>Pooled cohorts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range) years</td>
<td>80 (57–92)</td>
<td>79 (63–89)</td>
</tr>
<tr>
<td>Gender, male/female, n</td>
<td>10/32</td>
<td>8/26</td>
</tr>
<tr>
<td>Duration of symptoms, median (range) days</td>
<td>35 (8–365)</td>
<td>56 (10–728)</td>
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<td>Cranial symptoms (%)</td>
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<tr>
<td>Headache</td>
<td>64</td>
<td>79.5</td>
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<tr>
<td>Scalp tenderness</td>
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<td>38</td>
</tr>
<tr>
<td>Jaw claudication</td>
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<td>38</td>
</tr>
<tr>
<td>Stroke/visual events (%)*</td>
<td>9/24</td>
<td>3/23.5</td>
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<td>Systemic symptoms (%)</td>
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<td>Fever</td>
<td>18</td>
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<tr>
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<td>Polymyalgia rheumatica (%)</td>
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<td>ESR, mm/1 hour</td>
<td>90±34</td>
<td>90±29</td>
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<td>Haemoglobin, mg/dL</td>
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<td>114±13</td>
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<td>IL-6, pg/mL</td>
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</tr>
<tr>
<td>Glucocorticoid treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to &lt;10 mg daily, median (range) weeks</td>
<td>29 (12–51)</td>
<td>27 (12–172)</td>
</tr>
<tr>
<td>Time to &lt;5 mg/day, median (range) weeks</td>
<td>79 (48–154)</td>
<td>105 (23–423)</td>
</tr>
<tr>
<td>Cumulated dose at treatment withdrawal, mean±SD (mg)</td>
<td>5875±674</td>
<td>5370±962</td>
</tr>
</tbody>
</table>

Strong SIR was defined as the presence of ≥3 of the following: ESR ≥85 mm/hour, haemoglobin <110 g/L, fever >37°C and weight loss >3 kg as described.25

*Visual events include diplopia and visual loss due to anterior ischaemic optic neuropathy.

†Relapses were clinically defined as depicted in the Materials and methods section and, except for three patients, were always accompanied by a rebound in ESR and CRP.

CRP, C reactive protein; ESR, erythrocyte sedimentation rate; IL-6, interleukin 6; SIR, systemic inflammatory response.

without vascular imaging compatible with large vessel inflammation (table 2). In addition, there was no correlation between baseline sOPN concentrations and subsequent aortic dilatation (table 2).

sOPN correlates with acute-phase reactants and circulating IL-6

At the time of GCA diagnosis, sOPN showed a positive correlation with serum IL-6 (r=0.55; p<0.001) and with the IL-6-dependent acute-phase reactants ESR (r=0.32; p=0.009) and CRP (r=0.42; p<0.001). In addition, baseline sOPN negatively correlated with haemoglobin concentrations (r=0.34; p=0.005).

Baseline sOPN concentration predicts disease relapse and glucocorticoid use

Baseline sOPN was significantly higher in patients who developed disease relapses during follow-up (relapers 129.08±74.24 vs non-relapers 90.63±41.02 ng/mL; p=0.03). Within the group of relapers, patients with more than one disease relapse demonstrated significantly higher sOPN levels (194.00±77.02) than those with only one relapse (98.52±50.72; p=0.007) (figure 2A).

Furthermore, the percentage of patients in remission over time was significantly lower in patients with baseline sOPN concentrations superior to the mean value (p=0.03) (figure 2B).

The proportion of patients requiring a prednisone maintenance dose ≥10 mg/day over time was significantly higher among patients with sOPN levels above the mean sOPN concentration at the time of diagnosis (p=0.036) (figure 2C).

Moreover, there was a positive correlation between the baseline sOPN and the time to achieve a stable glucocorticoid dose <10 mg/day (r=0.43, p=0.02) or <5 mg/day.
Figure 1  Serum osteopontin (sOPN) concentrations in patients with giant cell arteritis and healthy controls. (A) sOPN concentrations in active patients with giant cell arteritis (GCA) from the cohort 1 and in controls (subgroup A). (B) sOPN concentrations in active patients with GCA from the cohort 2 and controls (subgroup B). (C) sOPN concentrations in the pooled cohorts of patients with active GCA and controls. Box-plot in (A), (B) and (C) represent median, 25%-75% percentile and range. (D) sOPN concentrations in patients with GCA at diagnosis and when in remission by paired comparison. (E) Receiver-operator characteristic (ROC) analysis of sOPN concentrations in patients with active GCA versus controls. (F) ROC curve of patients with active GCA versus patients in remission.

Table 2  Serum OPN concentrations according to clinical/imaging data in the pooled cohort of patients with giant cell arteritis

<table>
<thead>
<tr>
<th></th>
<th>Presence</th>
<th>Absence</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial symptoms</td>
<td>104.78±59.60</td>
<td>118.56±75.16</td>
<td>0.491</td>
</tr>
<tr>
<td>Systemic symptoms</td>
<td>118.45±61.70</td>
<td>82.70±57.50</td>
<td>0.028</td>
</tr>
<tr>
<td>Ischaemic symptoms</td>
<td>79.91±57.90</td>
<td>117.29±61.32</td>
<td>0.028</td>
</tr>
<tr>
<td>Strong SIR</td>
<td>132.56±77.56</td>
<td>97.46±53.40</td>
<td>0.039</td>
</tr>
<tr>
<td>PMR</td>
<td>118.17±74.27</td>
<td>101.15±54.45</td>
<td>0.287</td>
</tr>
<tr>
<td>LVV*</td>
<td>109.44±61.88</td>
<td>115.55±69.45</td>
<td>0.799</td>
</tr>
<tr>
<td>Aortic dilation†</td>
<td>111.58±46.74</td>
<td>105.56±70.96</td>
<td>0.754</td>
</tr>
</tbody>
</table>

*Evaluated in 42 patients.
†Evaluated in 34 patients.

LVV, large vessel vasculitis; OPN, osteopontin; PMR, polymyalgia rheumatica; SIR, systemic inflammatory response.
In addition, patients with the highest sOPN concentrations at diagnosis experienced more relapses and cumulated higher glucocorticoid doses. Previous studies have indicated that an intense systemic inflammatory response assessed by a composite combination of clinical and biological data is associated

between serum IL-6 and sOPN, OPN production in GCA arteries is not exclusively dependent on IL-6 signalling.

sOPN is reduced but not abrogated in patients with GCA in remission treated with tocilizumab

Based on our results on cultured arteries, we measured sOPN and serum CRP concentrations in a subset of patients with GCA who were in remission maintained with glucocorticoids or tocilizumab (alone or in combination with low-dose prednisone) to see whether sOPN could be a useful biomarker in patients receiving IL-6 blockade therapy. As shown in figure 4, serum CRP was significantly lower and sometimes undetectable in tocilizumab-treated patients (0.06±0.16 mg/dL) compared with prednisone only-treated patients at high dose (0.25±0.24 mg/dL; p=0.017) or low dose (0.28±0.19 mg/dL; p<0.001). In contrast, sOPN was detected in all cases without significant differences between patients under tocilizumab (51.91±36.25) or glucocorticoid only treatment either at high dose (43.55±21.36; p=0.861) or low dose (55.62±24.87; p=0.24). These results suggest that, unlike CRP, sOPN might be an interesting disease activity biomarker to be explored in tocilizumab-treated patients.

DISCUSSION

In this study, we demonstrated that sOPN was significantly elevated in patients with active GCA compared with age-matched and sex-matched healthy individuals but returned to normal when patients achieved remission with glucocorticoid treatment. In addition, patients with the highest sOPN concentrations at diagnosis experienced more relapses and cumulated higher glucocorticoid doses. Previous studies have indicated that an intense systemic inflammatory response assessed by a composite combination of clinical and biological data is associated

**Figure 2** Baseline serum osteopontin in patients with giant cell arteritis as predictor of relapses and duration of glucocorticosteroid treatment. (A) Baseline sOPN concentrations in patients with no subsequent relapses, with 1 relapse and with ≥2 relapses. (B) Percentage of patients in remission over time according to baseline sOPN (≥mean sOPN vs <mean sOPN). (C) Percentage of patients requiring a daily maintenance prednisone dose ≥ 10 mg over time according to baseline sOPN (≥ mean sOPN vs < mean sOPN).

(r=0.48, p=0.03), and between baseline sOPN and the cumulated prednisone dose at the time of prednisone discontinuation (r=0.37; p=0.01).

In contrast to the sOPN findings, no significant differences were found in baseline IL-6 (49.76±41.52 vs 28.00±30.86 pg/mL; p=0.237), ESR (83.88±31.10 vs 95.28±20.38 mm/1 hour; p=0.188), CRP (9.77±8.37 vs 6.70±3.91 mg/dL; p=0.117) or haemoglobin (116.56±14.33 vs 113.11±12.15 mg/dL; p=0.412) concentrations between relapsing patients and patients who achieved sustained clinical remission (online supplementary table S1). As with sOPN, the proportion of patients who relapsed over time was significantly higher in patients with CRP concentrations above the mean level than in patients with lower concentrations (p=0.022), but no differences were found according to IL-6, ESR or haemoglobin values (online supplementary figure S1).

Together, these data underline the predictive value of baseline sOPN in terms of disease relapse and long-term glucocorticoid requirements in patients receiving glucocorticoid monotherapy.  

**OPN expression and secretion in cultured GCA arteries are not suppressed by short-term IL-6 receptor blockade**

IL-6 receptor signalling results in activation and expression of transcription factor STAT-3. We have previously shown that cultured temporal arteries have constitutive IL-6 and STAT3 expression and activation. As previously shown, STAT3 expression was higher in cultured control arteries than in cultured GCA involved arteries, possibly reflecting loss of STAT3-producing VSMCs (figure 3A). Short-term treatment with the anti-IL-6 receptor monoclonal antibody tocilizumab for 5 days, at concentrations able to reduce STAT3 expression (figure 3A), did not modify OPN mRNA expression or protein secretion in cultured GCA arteries (figure 3B,C). These findings support that, in spite of the correlation
Effect of tocilizumab (TCZ) on osteopontin (OPN) and STAT3 expression in cultured temporal arteries from patients with giant cell arteritis (GCA) and controls. (A) STAT3 mRNA expression in relative units (RU) by cultured temporal arteries from negative controls (n=5) and from patients with GCA untreated (n=6), treated with TCZ at 10 µg/mL (n=6) or treated with non-immune human control IgG at 10 µg/mL (n=4). *p=0.030 vs negative biopsies, #p=0.046 vs untreated GCA biopsies (paired comparison). (B) OPN mRNA expression in RU by cultured temporal arteries from negative controls and from patients with GCA untreated, treated with TCZ at 10 µg/mL or treated with non-immune human control IgG at 10 µg/mL as in (A). (C) OPN concentrations in the supernatants of cultured temporal arteries from negative controls (n=15) and from patients with GCA untreated (n=14), treated with TCZ at 10 µg/mL (n=15) or treated with non-immune human control IgG at 10 µg/mL (n=16). **p<0.001 vs negative control samples. Sensitivity of the immunoassay (minimal detectable concentration) was 0.011 ng/mL.

Figure 4 Serum osteopontin (sOPN) and C reactive protein (CRP) in patients with giant cell arteritis (GCA) in remission according to treatment. (A) sOPN in patients with GCA in remission with high-dose prednisone (PDN) (≥20 mg/day), low-dose prednisone (≤10 mg/day) or with tocilizumab (TCZ). (B) Serum CRP concentration in patients with GCA in remission with high-dose prednisone (≥20 mg/day), low-dose prednisone (≤10 mg/day) or with TCZ. **p=0.017 vs high-dose prednisone group and p<0.001 vs low-dose prednisone group. Sensitivities (minimal detectable concentration) of OPN and CRP immunoassay are 0.011 ng/mL and 0.010 ng/mL, respectively.

with recurrent disease. Accordingly, in the present study, sOPN was significantly higher in patients with strong systemic inflammatory response than in patients with weak acute-phase reaction. Interestingly, although sOPN at baseline correlated with ESR, CRP and IL-6, and negatively with haemoglobin concentration, sOPN performed better than individual detection of ESR, CRP, haemoglobin or IL-6 as predictor of relapsing course. sOPN is increased in a variety of conditions conveying tissue injury and remodelling, including malignancies, infection and other systemic vasculitis. Therefore, sOPN may not be a suitable diagnostic
biomarker. However, if confirmed in larger studies, our data indicate that sOPN may be a useful biomarker of disease activity and predictor of relapsing disease and glucocorticoid requirements.

Regarding additional clinical data, patients with cranial ischaemic complications had significantly lower sOPN concentrations. A similar pattern has been previously shown for serum IL-6,\(^3\) and, in fact, in the present study sOPN and sIL-6 concentrations significantly correlated, suggesting coordinated regulation. As hypothesised for IL-6, angiogenic activity of OPN might compensate for ischaemia at distal sites.\(^3\)\(^5\)\(^6\)

Although glucocorticoids are very efficient in inducing remission in the majority of patients, glucocorticoid tapering results in relapse in a substantial number of individuals. No clear alternatives to prolonged use of glucocorticoids were available to treat patients with GCA until very recently.\(^8\)\(^-\)\(^1\)\(^2\)\(^3\)\(^7\)\(^3\) A phase II and a phase III trial have demonstrated the efficacy and safety of blocking IL-6 receptor with tocilizumab in maintaining remission and sparing glucocorticoid in GCA.\(^1\)\(^1\)\(^2\) However, neutralising IL-6 receptor blocks the synthesis of acute-phase response and leads to a transient rebound in serum IL-6, making difficult the assessment of disease activity in individuals treated with tocilizumab.\(^3\)\(^8\) OPN synthesis is activated through different pathways. Its promoter region has binding sites for transcription factors relevant to immune and inflammatory responses such as nuclear factor κB (NFκB), activator protein 1 (AP-1) and STAT3, which also regulate IL-6 expression.\(^3\)\(^9\)\(^1\)\(^0\) Although OPN can be induced by IL-6, it is not exclusively IL-6-dependent. It can be also induced by IL-1β, tumour necrosis factor-α and interferon-γ, among others.\(^1\)\(^6\) Our preliminary results indicate that short-term treatment exposure of ex vivo cultured GCA arteries to tocilizumab does not have a selective impact on OPN expression in GCA lesions. Consistently, while tocilizumab virtually abolished serum CRP, sOPN was similar in patients in glucocorticoid-maintained or tocilizumab-maintained remission. All together, these data suggest that sOPN could be a useful biomarker of disease activity for tocilizumab-treated patients.

Our data on baseline sOPN were obtained from two patient cohorts subjected to imaging.\(^2\)\(^5\)\(^-\)\(^2\)\(^7\) As with serum IL-6, sOPN did not seem to be associated with anatomical disease extension in these patients since there was no relationship between sOPN and computed tomography angiography (CTA) detection or extension of large-vessel inflammation at diagnosis. Moreover, as shown for serum IL-6,\(^2\)\(^6\)\(^-\)\(^2\)\(^7\) sOPN at diagnosis did not predict subsequent development of aortic dilatation in our patients.

Our study has important strengths: it is performed in a sizeable and unique cohort of patients with treatment-naïve, biopsy-proven GCA, subjected to standardised clinical evaluation, imaging and treatment. However, detection of sOPN in patients in tocilizumab-induced vs glucocorticoid only-induced remission was cross-sectional, and the performance of sOPN as a marker of disease activity in these patients could not be specifically assessed, since no baseline samples or samples during relapse were available. The absence of baseline samples from patients treated with tocilizumab also prevents assessment of sOPN as predictor of response to tocilizumab as demonstrated in rheumatoid arthritis.\(^4\) This would be an interesting point to be investigated in future studies.

In summary, our data suggest that OPN might be a biomarker of disease activity in patients with GCA, and our preliminary data suggest that it might be particularly useful for patients treated with tocilizumab. Its performance in this setting deserves to be explored in larger prospective, longitudinal cohorts or in sera collected during clinical trials.

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Competing interests GE-F, SP-G, JR-H, SHU and MCC have participated in the GACTA trial sponsored by Hoffmann-La Roche. MCC has received consultation fees from Hoffman-La Roche.

Ethics approval The study was approved by the ethics committee of Hospital Clinic (Barcelona, Spain) and the Massachusetts General Hospital (Boston, Massachusetts, USA).

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement No additional data are available.

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Serum osteopontin: a biomarker of disease activity and predictor of relapsing course in patients with giant cell arteritis. Potential clinical usefulness in tocilizumab-treated patients

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EXTENDED REPORT

Endothelin-1 promotes vascular smooth muscle cell migration across the artery wall: a mechanism contributing to vascular remodelling and intimal hyperplasia in giant-cell arteritis

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ABSTRACT

Background Giant-cell arteritis (GCA) is an inflammatory disease of large/medium-sized arteries, frequently involving the temporal arteries (TA). Inflammation-induced vascular remodelling leads to vaso-occlusive events. Circulating endothelin-1 (ET-1) is increased in patients with GCA with ischaemic complications suggesting a role for ET-1 in vascular occlusion beyond its vasoactive function. Objective To investigate whether ET-1 induces a migratory myofibroblastic phenotype in human TA-derived vascular smooth muscle cells (VSMC) leading to intimal hyperplasia and vascular occlusion in GCA. Methods and results Immunofluorescence/confocal microscopy showed increased ET-1 expression in GCA lesions compared with control arteries. In inflamed arteries, ET-1 was predominantly expressed by infiltrating mononuclear cells whereas ET receptors, particularly ET-1 receptor B (ETBR), were expressed by both mononuclear cells and VSMC. ET-1 increased TA-derived VSMC migration in vitro and α-smooth muscle actin (αSMA) expression and migration from the media to the intima in cultured TA explants. ET-1 promoted VSMC motility by increasing activation of focal adhesion kinase (FAK), a crucial molecule in the turnover of focal adhesions during cell migration. FAK activation resulted in Y397 autophosphorylation creating binding sites for Src kinases and the p85 subunit of PI3Kinases which, upon ET-1 exposure, colocalised with FAK at the focal adhesions of migrating VSMC. Accordingly, FAK or PI3K inhibition abrogated ET-1-induced migration in vitro. Consistently, ET-1 receptor A and ETBR antagonists reduced αSMA expression and delayed VSMC outgrowth from cultured GCA-involved artery explants. Conclusions ET-1 is upregulated in GCA lesions and, by promoting VSMC migration towards the intimal layer, may contribute to intimal hyperplasia and vascular occlusion in GCA.

INTRODUCTION

Giant-cell arteritis (GCA) is a granulomatous vasculitis targeting large and medium-sized arteries in aged individuals.1 2 Inflammation-induced vascular remodelling results in intimal hyperplasia leading to symptoms of vascular insufficiency or irreversible ischaemic complications in 20%–30% of patients.3 4 It is generally assumed that vascular smooth muscle cells (VSMC) migrate through disrupted elastic fibres towards the intima where they produce abundant matrix proteins.5 However, underlying mechanisms remain virtually unexplored. Several growth factors, including PDGF, TGFβ, EGF, NGF or BDNF, are expressed in GCA lesions and may participate in this process based on their ability to stimulate proliferation and/or migration of VSMC in vitro.6 7 8

Visual loss, the most frequent ischaemic complication in GCA, is frequently preceded by transient episodes of blindness (amaurosis fugax) suggesting that reversible vasospasm may initially contribute to flow reduction in small arteries supplying the optic nerve.1 2 9 Endothelin-1 (ET-1) is a potent vasoactive peptide that might potentially participate in this process.9 10 ET-1 is mainly synthesised by endothelial cells although VSMC and macrophages may also produce it.11 ET-1 signals through two G-protein coupled receptors (GPCR): E-1 receptors A and B (ETAR and ETBR). Both ETAR and ETBR mediate VSMC contraction. Signalling through ETAR on endothelial cells may also produce vasodilatation by stimulating nitric oxide and prostacyclin production.9 10 12

Although the majority of previous studies on ET-1 functions have focused on VSMC regulation of the vascular tone, in recent years, skin, liver and lung fibroblasts have been identified as important targets of ET-1.13 14 ET-1 promotes myofibroblast differentiation of fibroblasts, a crucial step in the development of fibrogenic diseases such as systemic sclerosis and cardiac, pulmonary or hepatic fibrosis.13–15

The inflammatory milieu of GCA is enriched in cytokines and growth factors able to enhance ET-1 expression such as TGFβ among others.9 16 We and others have recently shown that ETAR, ETBR and ETβR are increased in GCA lesions, although the specific cells expressing the ET-1 system components have not been determined.17 18 In spite of the short half-life of circulating ET-1, plasma ET-1 concentrations are elevated in patients with GCA-related cranial ischaemic complications.18

Since arteries involved by GCA are usually larger than resistance arteries controlling vascular tone, we hypothesised that, in addition to its vasoactive function, ET-1 might contribute to the development of intimal hyperplasia by stimulating a myofibroblast

phenotype in VSMC and promoting their migration towards the intimal layer. Consequently, we investigated the effect of ET-1 on human temporal artery (TA)-derived VSMC migration in vitro and ex vivo as well as the signalling pathways involved.

**METHODS**

**Patient samples**

TA biopsies were performed to 10 patients with suspected GCA (see online supplementary table S1). Five biopsies disclosed typical GCA histopathological features and were used in the indicated experiments. The remaining five showed no inflammatory lesions and served as controls. Patients with negative biopsies were eventually diagnosed with other conditions (see online supplementary table S2). The study was approved by the local Ethics Committee (Hospital Clinic of Barcelona) and patients signed informed consent.

**Isolation and culture of VSMC derived from human TA**

Human TA-derived VSMCs were obtained from explanted TA sections from the above patients cultured on Matrigel and characterised by flow cytometry, as described. In specific experiments, VSMCs were cocultured with peripheral blood mononuclear cells (PBMC) or purified subsets (CD4+ T cells or CD14+ monocytes) (online supplementary methods).

**Reagents**

See online supplementary methods.

**Immunofluorescence**

Immunofluorescence staining was performed in cultured VSMC or in fresh-frozen or cultured TA sections. Antibodies used, dilutions and detailed steps are depicted in online supplementary methods.

**Quantitative real-time reverse transcription PCR**

RNA was extracted from cultured VSMC using TRIzol Reagent (Life Technologies, Paisley, UK). Prepro-ET-1 and α-smooth muscle actin (αSMA) mRNAs (1 μg) were measured by quantitative reverse transcription PCR with specific TaqMan gene expression assays from Applied Biosystems as reported.

**ET-1 immunoassay**

ET-1 in cell supernatants was measured using R&D Quantikine ELISA Kit.

**Migration assay**

VSMC migration was assessed using Boyden chambers with 10 μm pore polyester filters. Further details are exposed in the online supplementary methods.

**Scratch wound-healing assay**

VSMCs were seeded at 80% confluence onto 0.1% gelatin-precoated 12-well plates and cultured overnight. One scratch per well was done before adding fresh Dulbecco’s modified Eagle medium supplemented with 50 μmol/L of HEPES (Sigma-Aldrich) and BQ123, BQ788 (20 μmol/L) or combination of both inhibitors. ET-1 (10−9 mol/L) or fresh medium was added to each corresponding well. Time-lapse video microscopy was applied to record cell movement and results were analysed as depicted in online supplementary methods. A proliferation assay was also performed to assess the potential impact of ET-1 on cell growth (see online supplementary figure S1).

**Western blot and gelatin zymography**

See details in online supplementary methods.

**Transient transfection**

Focal adhesion kinase (FAK) wild-type cDNA and FAK point mutants Y397F and Y925F, cloned into the pCDNA3 expression vector, were kindly provided by Kazue Matsumoto and Kenneth M Yamada (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland) and generated as previously described. Lipoctactimine 2000 Reagent (Invitrogen) was used for transient transfection of VSMC. Transfection efficiency was about 30% (see online supplementary figure S2).

**Ex vivo-cultured TA sections from patients with GCA**

TA sections from four treatment-naive patients with GCA and four controls were cultured on Matrigel as described, with or without BQ123 or BQ788 (20 μmol/L). VSMC outgrowth was scored in three arteries at various time points by two investigators (EPR and MCB) blinded to the conditions tested.

**Statistical analysis**

Mann-Whitney U test for independent variables was applied using SPSS software, PASW V.22.0.

**RESULTS**

**Distribution of the ET-1 system in GCA lesions compared with controls**

In control arteries, slight ET-1 expression was observed in organised VSMC in the media and in the luminal endothelium (figure 1A). In GCA-involved arteries, ET-1 was intensively expressed by clusters of infiltrating inflammatory cells (figure 1B, b.1 and figure 1C, c.1) and by scattered remaining VSMC (figure 1B, b.2). In addition, ET-1 expression by the luminal endothelium was increased (figure 1B,C) compared with control arteries (figure 1A).

To further characterise the cell types responsible for ET-1 production in GCA, primary cultures of VSMC were obtained from normal TA and cocultured with purified CD4+ T lymphocytes or monocytes (CD14+) from healthy donors in order to mimic vascular inflammation. Interestingly, a slight but consistent increase in prepro-ET-1 mRNA expression was observed in CD4+ T lymphocytes and to a lesser extent in CD14+ monocytes, when cocultured with VSMC (figure 1D). VSMC remarkably expressed and secreted mature ET-1 (figure 1E). When in coculture, unprocessed big ET-1 increased in PBMC and decreased in VSMC lysates. Overall, secreted ET-1, mainly produced by VSMC, decreased in coculture supernatants (figure 1F). The increase in prepro-ET-1 mRNA in PBMC cocultured with VSMC was confirmed in three paired experiments performed with PBMC and VSMC from the same GCA donor (figure 1G).

Expression of ET-R and ETaR was explored in the same TA specimens. In control arteries, ET-R was expressed by VSMC in the media whereas ETaR was hardly detected (figure 1H,I). In GCA, both ET-R and ETaR receptors were expressed by αSMA-positive cells at the intima-media border (figure 1J,K). Endothelial cells and inflammatory cells also expressed both ET receptors (figure 1J,K).

**ET-1 promotes VSMC cytoskeleton reorganisation and migration through ET-R and ETaR**

To investigate whether ET-1 promoted a myofibroblast phenotype in VSMC, we explored changes in cytoskeleton organisation induced by ET-1 in cultured TA-derived VSMC. ET-1 elicited...
Figure 1 ET-1, ETAR and ETBR expression in GCA lesions compared with control TA. (A) Immunostaining of ET-1 (red), αSMA (green) and nuclei (blue) in a control TA. (B) Immunostaining of ET-1 (red), αSMA (green) and nuclei (blue) in a TA with typical GCA involvement. White arrow highlights ET-1 expression by the endothelium. (b.1 and b.2) Magnifications of 1B showing independent expression or coexpression of αSMA and ET-1, respectively. (C) Immunostaining of ET-1 (red), CD45 (green) and nuclei (blue) in a GCA-involved TA. (c.1) Magnification of 1C showing CD45+ cells expressing ET-1. ET-1 distribution was confirmed in three different GCA and control arteries. L, lumen; I, intima; M, media; Adv, adventitia. (D) Prepro-ET-1 mRNA expression by purified CD4+ T cells or CD14+ monocytes isolated or cocultured with VSMC for 24 hours. Bars represent mean and SEM of triplicates. *p<0.05 cocultured versus isolated. (E) Immunoassay of supernatants from isolated CD4+ T lymphocytes, CD14+ monocytes or VSMC, or supernatants from cocultures of CD4+ T cells or CD14+ monocytes with VSMC for 24 hours. Bars represent mean and SEM of triplicates. *p<0.05 cocultured versus isolated. (F) Big-ET-1 detection by western blot in lysates (20 µg/lane) of isolated PBMC or PBMC cocultured with VSMC, and in lysates of VSMC isolated or cocultured with PBMC for 24 hours. (G) Prepro-ET-1 mRNA expression by PBMC from three patients with GCA and their corresponding VSMC isolated or in coculture for 24 hours. (H, I) Immunofluorescence staining of ETAR (red) or ETBR (red) together with αSMA (green) and nuclei (blue) in a control TA and their corresponding magnifications (h.1, i.1). (J, K) Immunofluorescence staining of ETAR (red) or ETBR (red) together with αSMA (green) and nuclei (blue) in a GCA-involved TA and their corresponding magnifications of the endothelium (j.1, k.1), neointima (j.2, k.2) and media (j.3, k.3). αSMA, α-smooth muscle actin; ET-1, endothelin-1; ETAR, ET-1 receptor A; ETBR, ET-1 receptor B; GCA, giant-cell arteritis; RU, relative units; PBMC, peripheral blood mononuclear cells; TA, temporal arteries; VSMC, vascular smooth muscle cells.
spreading of VSMC with a striking formation of cytoplasm protrusions (figure 2A). Cell spreading was not induced in VSMC cultured on polylysine and was more remarkable when VSMCs were cultured on fibronectin (figure 2A), suggesting participation of integrin-mediated signalling pathways in this process. 25 Spreading was reverted by blocking ET-1 signalling with ET AR antagonist BQ123 and ETBR antagonist BQ788 (figure 2A). ET-1-induced VSMC morphology changes were associated with increased migratory activity (see online supplementary movie). ET-1 exposure resulted in significantly faster scratch-wound closure (figure 2B). ET R R and ET R R antagonists (BQ123 and BQ788, respectively) and combination of both inhibitors significantly abrogated ET-1-induced VSMC migration, indicating implication of both receptors in this process (figure 2B). ET-1 did not accelerate scratch-wound closure by stimulating VSMC growth, since no significant increase in VSMC proliferation was elicited by ET-1 (online supplementary figure S1). ET-1 induced VSMC migration in Boyden chambers when added to the upper compartment (figure 2C). In contrast, when ET-1 was added to the lower compartment, no differences in migration were observed, indicating that ET-1 has no chemo-attractant activity and primarily stimulates motility (figure 2C). ET-1-induced migration was abrogated by BQ788 and BQ123 (figure 2D).

Figure 2  Effect of ET-1 on TA-derived VSMC cytoskeleton reorganisation and migration. (A) Immunofluorescence of VSMC f-actin with phalloidin-rhodamine (red) and nuclei (blue). VSMCs were preincubated with ET R antagonist BQ123 (20 µmol/L), ET R antagonist BQ788 (20 µmol/L) or both in suspension for 45 min. ET-1 (10−9 mol/L) was added at the time of VSMC seeding on plastic, fibronectin (5 µg/cm2) or polylysine (10 µg/mL) and VSMCs were incubated for 3 hours before fixing and staining. (B) Scratch wound healing assay of VSMC untreated or exposed to ET-1 (10−9 mol/L), with or without the presence of BQ123 (20 µmol/L), BQ788 (20 µmol/L) or both. Graph represents percentage of scratch closure over time in three independent experiments. (C) Boyden chamber migration assay. ET-1 was added either to the lower or in the upper compartment at the indicated concentrations. Cells were counted at 4× magnification. **p<0.005 for untreated cells versus ET-1-treated cells. Bars represent number of cells (mean and SEM of quadruplicates). (D) Boyden chamber migration assay where ET-1 was added to the upper compartment with or without preincubation with antagonists BQ123, BQ788 or both. Cells were counted at 10× magnification. Bars represent number of cells (mean and SEM of quadruplicates). (B, D) *p<0.05/**p<0.005 for untreated cells versus ET-1-treated cells. #p<0.05/##p<0.005 comparing ET-1-treated cells versus cells incubated with ET-1 receptor antagonists BQ123 or BQ788. ET-1, endothelin-1; ET R R, ET-1 receptor A; ET R, ET-1 receptor B; TA, temporal arteries; VSMC, vascular smooth muscle cells.
FAK phosphorylation at Y397 is essential for ET-1 induction of VSMC migration

Based on the relevance of integrin engagement in ET-1-induced cytoskeleton reorganisation, and the seminal role of FAK in integrin-mediated cell motility, we explored the involvement of FAK in ET-induced VSMC migration. FAK is a docking and signalling tyrosine kinase with a seminal role in focal adhesion turnover required for cell migration in response to integrin binding or growth factor signalling.23–27

FAK activation results in autophosphorylation of crucial tyrosine residues.26–27 One of the best characterised is Y397 which provides a binding site for Src-type tyrosine kinases promoting their recruitment to focal adhesions and allowing their phosphorylation. This interaction is essential for cell migration in fibroblasts and malignant cells.26–28 Src, in turn, phosphorylates additional tyrosine residues, including Y925, located within the focal adhesion targeting sequence at the FAK C-terminal domain.29 Phosphorylated Y925 may recruit the adaptor protein Grb2, leading to activation of the GTP-binding protein Ras, and to ERK1/2 activation.30

ET-1 increased phosphorylation of Y397 and Y925 FAK residues (figure 3A), particularly when cells were cultured on plastic or fibronectin whereas this effect was absent in cells plated on polylysine (figure 3A,B). FAK phosphorylation was reduced by ET, R or ET, R antagonists BQ123 and BQ788 (figure 3A). As G-coupled receptors, ET-1 receptors may activate heterotrimetric G proteins which have important roles in integrin inside-out and outside-in signalling.31 Pertussis toxin induces ADP-ribosylation of several Go, subunits inhibiting their activity.25 As shown in figure 3C, ET-1-induced Y397 FAK phosphorylation was abrogated by pertussis toxin confirming the participation of heterotrimetric G proteins in ET-1-induced FAK activation.

To confirm the role of FAK in ET-1-induced VSMC migration, we investigated the effect of PF-573228, an inhibitor of FAK kinase activity. At concentrations able to inhibit FAK phosphorylation (see online supplementary figure S3), PF-573228 significantly decreased ET-1-induced VSMC cytoskeleton organisation and migration in a dose-dependent manner (figure 3D,E). Interestingly, at low concentrations, PF-573228 inhibited ET-1-induced migration whereas at higher concentrations it was also able to reduce baseline VSMC migration.

Consistent with a seminal role of FAK in mediating ET-1-induced migration, transient transfection of VSMC with FAK wild type significantly increased VSMC migration through Boyden chambers overcoming the effect of ET-1 which was not able to increase migration in FAK-overexpressing cells. However, transient transfection with an expression vector containing FAK Y397F point mutation abrogated ET-1-induced motility. In spite that ET-1 also increased Y925 phosphorylation, transfection of Y925F point mutant had no impact on ET-1-induced migration (figure 3F). These results indicate the crucial participation of FAK Y397 in ET-1-mediated migration in primary TA-derived VSMC.

It has been previously reported that integrin engagement and FAK signalling trigger rapid secretion of gelatinases MMP9 and MMP2 by lymphoid cells.29–31 Based on the important role of ET-1 in inducing FAK activation, we explored whether ET-1 modulated secretion of gelatinases by VSMC. ET-1 slightly increased secretion of pro-MMP2 (figure 3G) and this effect was reduced by BQ788 (ET, R antagonist) (figure 3H).

ET-1 induced FAK phosphorylation and recruitment of phosphorylated FAK at the focal adhesions in the leading and rear edges and colocalisation with αSMA (figure 4A,B). To confirm the relevance of the above results in GCA, cultured TA sections from patients with GCA were assessed for FAK phosphorylation. As shown in figure 4C,D, Y397-phosphorylated FAK was detected in GCA lesions, particularly at the intima and intima/media junction and FAK phosphorylation decreased upon exposure to ET, R and ET, R antagonists.

Src and PI3kinases mediate ET-1-induced VSMC migration

Considering the relevance of FAK Y397 in ET-1-induced VSMC migration, we next explored ET-1 downstream pathways involved in cell migration including ERK, Src and PI3K.26–30 ET-1 promoted Src activation revealed by increased phosphorylation of the Y416 Src residue and this was inhibited by both ET, R and ET, R antagonists (figure 5A).

ERK1/2 activation has a crucial role in cell motility, by phosphorylating myosin light chains and as scaffolding molecule.26 27 34 Although transfection with Y925F point mutant did not substantially reduce ET-1-induced migration, ET-1 increased ERK1/2 phosphorylation and this was reduced by ET-1 receptor antagonists (figure 5A), consistent with the existence of alternative ERK activating mechanisms dependent and independent of FAK.26 30 Although the effect of ET-1 on baseline-activated Src and ERK phosphorylation was modest, it was consistently observed.

In accordance with the crucial role of Src in cell migration, Src inhibitor PP2 reduced baseline and ET-1-induced VSMC migration. Interestingly, PI3kinase inhibition with LY294002 selectively reduced ET-1-induced migration (figure 5B). ERK inhibition of ET-1-induced migration could not be assessed with PD98059 due to the decreased viability observed after the 6-hour exposure required for migration experiments (see online supplementary figure S4). Short-term exposure to Src and ERK inhibitors, not reducing cell viability, virtually impeded cell spreading (figure 5C) whereas PI3kinase inhibition only reduced the increase in cell protrusions induced by ET-1 (figure 5C,D).

FAK Y397 interaction with p85, the regulatory subunit of PI3kinase, is crucial to cell migration in other experimental contexts.35 36 ET-1 promoted colocalisation of PI3kinase p85 with FAK at VSMC focal adhesions (figure 5D). This interaction was abrogated by both ET, R and ET, R antagonists and by inhibition of FAK kinase activity. Inhibition of PI3kinase by LY294002 prevented formation of fully developed cell protrusions induced by ET-1, but did not prevent ET-1-induced recruitment of p85 and FAK at the focal contacts in nascent, immature buds (figure 5D).

ET-1 induces neointima formation in ex vivo-cultured normal TA

In control arteries, αSMA-expressing quiescent VSMCs were concentrically organised (figure 6A). In contrast, in GCA-involved arteries the muscular layer was disrupted and αSMA-expressing VSMCs were mostly located at the neointima (figure 6A). Treatment of cultured TA explants with ET-1 at concentrations similar to those found in patient plasma or in the coculture supernatants increased αSMA expression (figure 6B,C). Exposure of cultured normal TA to ET-1 also resulted in a striking disruption of the muscular layer and migration of VSMC towards the intima (figure 6B).

ET, R and ET, R antagonists reduce αSMA expression and VSMC outgrowth from ex vivo-cultured arteries from patients with GCA

ERK-receptor antagonists BQ123 and BQ788 dramatically reduced αSMA expression in cultured artery sections for a patient with GCA.
**Figure 3**  Y397 FAK phosphorylation is essential for ET-1-induced TA-derived VSMC migration. (A) Immunoblot and corresponding quantification of total FAK or FAK phosphorylated at the indicated tyrosine residues in lysates of VSMC cultured for 90 min in the presence or the absence of ET-1 with or without pretreatment with ET-1 receptor antagonist BQ123 or BQ788 at the same concentrations as in previous experiments. (B) Immunoblot and corresponding quantification of p-FAK and total FAK in lysates of VSMC cultured for 90 min in the presence of ET-1 (10−9 mol/L) and with or without previous incubation with PT (1 µg/mL). (C) Immunoblot and corresponding quantification of Y397 and total FAK from lysates of VSMC cultured for 90 min with or without ET-1 and with or without previous incubation with PT (1 µg/mL). (D) Immunofluorescence of VSMC cytoskeleton with phalloidin-rhodamine (red) and nuclei (blue). ET-1 (10−9 mol/L) was added at the time of VSMC seeding. When indicated, VSMCs were preincubated with a FAK inhibitor (PF-573228) at 20 µmol/L for 30 min before ET-1 exposure. Representative pictures are displayed. (E) Boyden chamber migration assay of VSMC preincubated with increasing concentrations of FAK inhibitor (PF-573228) with or without subsequent addition of ET-1. **p<0.005 untreated cells versus ET-1-treated cells. #p<0.005 comparing ET-1-treated cells versus ET-1-treated cells preincubated with PF-573228. Cells were counted at 10× magnification. (F) Boyden chamber migration assay of VSMC, 3 days after transfection with empty pcDNA3 vector (MOCK), wild-type FAK (FAK) or FAK mutated at the phosphorylation site Y397F or Y925F. Bars represent the number of migrating cells (mean and SEM) of quadruplicates at 10× magnification. *p<0.05; **p<0.005 untreated cells versus ET-1-treated cells. #p<0.05; ##p<0.005 for the indicated comparisons. Notice that baseline migration in transfected cells is globally inferior than in non-manipulated cells displayed in figure 2. (G) Gelatin zymography of serum-free supernatants of VSMC cultured in the absence or in the presence of ET-1 for 6 hours. A representative experiment out of three is displayed. (H) Gelatin zymography of serum-free supernatants of VSMC cultured in the presence or in the absence of ET-1 (10−9 mol/L) and ET-1 receptor antagonists BQ123 and BQ788 (20 µmol/L) for 6 hours. A representative experiment out of two is displayed. ET-1, endothelin-1; FAK, focal adhesion kinase; MMP2, matrix metalloproteinase 2; PT, pertussis toxin; TA, temporal arteries; VSMC, vascular smooth muscle cells.
Basic and translational research

GCA (figure 6D). Blocking ET\textsubscript{R} with BQ788 remarkably inhibited VSMC outgrowth from GCA-involved arteries (figure 6E).

The effect of blocking ET\textsubscript{R} with BQ123 was less intense but also delayed VSMC outgrowth (figure 6E). Taking together, these data support a seminal role of ET-1 in inducing neointima formation in GCA.

DISCUSSION

Expression of ET-1 was increased in GCA lesions compared with normal arteries. In GCA, infiltrating leukocytes accounted for the majority of ET-1 expression, which was also enhanced in the luminal endothelium. Some ET-1 expression was also observed in remaining VSMC. Coculture experiments supported that, in an

inflammatory microenvironment, ET-1 production is increased in mononuclear cells and decreases in VSMC. ET_R was constitutively expressed by VSMC in normal arteries but, in the context of vascular inflammation, both ET receptors were remarkably increased and expressed by endothelial cells, VSMC and infiltrating leukocytes. As previously reported, the increase in ET_R was much more prominent.17

ET-1 stimulated VSMC migration through FAK activation, revealed by ET-1-enhanced FAK autophosphorylation at Y397, creating binding sites for Src kinase and the p85 regulatory subunit of PI3 kinase, a crucial process in cell motility.29-35 ET-1 promoted colocalisation of activated FAK and p85-PI3 kinase at the focal adhesions. Subsequent signalling cascades participating in cell motility in other cell types, such as Src and ERK, were also slightly activated. Interestingly, while FAK and Src inhibitors strongly reduced both baseline and ET-1-induced migration, PI3 kinase inhibitor selectively inhibited the increase in migration induced by ET-1. Class I PI3 kinases are activated by tyrosine kinases whereas class II PI3 kinases are activated through GPCR.16 36 37 It is likely that ET-1 promotes activation of both classes of PI3 kinases through FAK activation and through GPCR ET_AR and ET_BR. In addition, GPCR-induced heterotrimeric G-protein activation may

Figure 5  ERK, Src and PI3K participation in ET-1 induced VSMC migration. (A) Immunoblot of phospho-Src (Y416)/total Src and phospho ERK/total ERK in lysates of VSMC treated with ET-1 (10−9 mol/L) for 90 min. Graphs show quantification of a representative experiment out of three independent experiments. (B) VSMC migration in Boyden chambers with or without preincubation with Src inhibitor PP2 (10 µmol/L), or PI3K inhibitor LY294002 (20 µmol/L), with or without subsequent addition of ET-1. Bars represent the number of migrated cells (mean and SEM of quadruplicates). **p<0.005 for untreated cells versus ET-1-incubated cells. ###p<0.005 for ET-1-treated cells versus VSMC preincubated with inhibitors. (C) Immunofluorescence staining of f-actin cytoskeleton (red) and nuclei (blue). ET_1 was added at the time of VSMC seeding. VSMCs were preincubated 30 min in suspension with Src inhibitor (PP2), ERK inhibitor (PD98059) or PI3K inhibitor (LY294002) before addition of ET-1 (10−9 mol/L). Representative pictures of each situation are shown. (D) Immunofluorescence staining of f-actin cytoskeleton (blue), p85 (green), total FAK (red) and nuclei (white) in VSMC treated with ET-1, or ET-1 plus ET_AR antagonist (BQ123), ET_BR antagonist (BQ788), PI3K inhibitor (LY294002) or FAK inhibitor (PF-573228) as labelled. Arrows highlight p85 and FAK colocalisation in the cell protrusions of ET-1-treated VSMC or p85/FAK clusters in immature cell protrusions triggered by ET-1 in the presence of PI3 kinase inhibitor LY294002. ET-1, endothelin-1; ET_AR, ET-1 receptor A; ET_BR, ET-1 receptor B; FAK, focal adhesion kinase; VSMC, vascular smooth muscle cells.
Basic and translational research

Also contribute to FAK activation since pertussis toxin abrogated ET-1-induced FAK phosphorylation.

It has been previously shown that FAK coordinates migration with matrix metalloproteinase (MMP) release, which is necessary for cell progression through the extracellular matrix. ET-1 moderately stimulated release of MMP2 by VSMC, a process mostly mediated by ET BR in our experimental conditions. Since MMP2 has elastinolytic activity, ET-1-induced MMP-2 release may be relevant to the disruption of the internal elastic lamina, characteristically observed.

Figure 6  ET-1 induction of VSMC migration in ex vivo TA cultures. (A) Immunofluorescence staining of αSMA (red) and nuclei (blue) in a control TA (left panel) or in a GCA artery with typical lesions (right panel). (B) Immunofluorescence staining of αSMA (red) and nuclei (blue) in a control TA cultured on Matrigel for 5 days in the presence or in the absence of ET-1 (10⁻⁹ mol/L). Arrows indicate αSMA-positive cells migrating from the media to the intimal layer. Pictures are representative of three different control arteries. L, lumen; I, intimal layer; M, media layer. (C) αSMA mRNA expression in three cultured normal arteries in the absence or in the presence of ET-1 (10⁻⁹ mol/L) (upper panel). Immunoblot of αSMA and β-actin, with the corresponding quantifications, in a normal TA cultured for 5 days in the absence or in the presence of ET-1 at the same concentration. The experiment was repeated twice with consistent results. (D) Immunofluorescence staining of αSMA (red) and nuclei (blue) in a control TA cultured in Matrigel for 5 days (left panel) and in a GCA-involved artery (right panel) in the presence or absence of ET-1 receptor antagonists (BQ123 and BQ788) at 20 µmol/L. (E) VSMC outgrowth from three TAs from patients with GCA cultured on Matrigel for the indicated periods of time with or without the presence of ET-1 receptor antagonist BQ123 (20 µmol/L) or BQ788 (20 µmol/L). αSMA, α-smooth muscle actin; ET-1, endothelin-1; GCA, giant-cell arteritis; RU, relative units; TA, temporal arteries; VSMC, vascular smooth muscle cells.
in GCA lesions, allowing VSMC migration from the muscular to the intimal layer. 5,18–21

FAK has received substantial attention in pathological processes where cell migration is seminal including cancer and fibrosis. 18,20,21 Our data suggest that FAK is involved in vascular remodelling. Supporting our findings, a recent study has evidenced Y397 phosphorylated FAK in the resistance arteries undergoing vascular remodelling in hypertension. 19 Selective myeloid deletion of FAK does not influence vascular remodelling in a mouse model, suggesting that expression and activation of FAK in VSMC rather than inflammatory cells may be relevant to vascular occlusion. 46 Moreover, a naturally occurring truncated form of FAK, FRNK, which acts as competitive inhibitor of FAK, inhibits VSMC invasion in a carotid rat injury model. 53 Consequently, our results indicate that ET-1-mediated activation of FAK in VSMC may have a seminal role in vascular remodelling in the context of vascular inflammation where ET-1 is mainly produced by inflammatory cells and their production is amplified through interactions with VSMC. These newly recognised functions of ET-1 on VSMC may have a broader impact and may operate in vascular diseases with prominent vascular remodelling beyond GCA. To date, in the field of vascular biology, attention has mainly focused on the vasoconstriction role of ET-1 and only responses related to vascular reactivity or hypertension have been explored after conditional deletion of ET-R in VSMC. 9,10,41 In a pioneer study performed in mice more than one decade ago, induced overexpression of ET-1 in endothelial cells resulted in increased vascular remodelling. 42 However, this interesting observation has not been further explored.

Based on its presumed major function, ET-1 has been considered a therapeutic target for vascular diseases where vasoconstriction is thought to play a major role such as systemic or pulmonary hypertension or, more recently, fibrotic diseases, such as scleroderma or lung fibrosis, according to the newly recognised functions of ET-1 on fibroblasts. 9,10,12,14 However, to date, clinical trials with ET-1 receptor antagonists have shown the best efficacy for diseases with prominent vascular remodelling such as ischaemic ulcers in systemic sclerosis or pulmonary hypertension rather than vasoconstriction or fibrotic diseases. 10,43

There is an unmet need of treatments reducing inflammation-induced vascular remodelling in GCA since patients with systemic vasculitis may develop complications derived from vascular occlusion in spite of glucocorticoid or immunosuppressive therapy. 44 Our data underline an unprecedented and crucial role for ET-1 in inducing vascular remodelling and vascular occlusion in the context of vascular inflammation and point towards endothelin receptor antagonists as potential therapeutic targets to avoid maladaptive vascular remodelling in inflammatory diseases of blood vessels.

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**Contributors** MCC and EPR designed the experiments and wrote the manuscript. EPR, NTG and MCB performed the experimental work. EL and MS contributed preliminary results essential for the development of the study. MAA, GEE, SPG and JHR contributed to clinical selection and TA biopsy collection. SP and RL produced and provided BQ123. All authors read, made improvements and approved the final version.

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Endothelin-1 promotes vascular smooth muscle cell migration across the artery wall: a mechanism contributing to vascular remodelling and intimal hyperplasia in giant-cell arteritis

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**INTRODUCTION**

Giant cell arteritis (GCA) is a chronic inflammatory disease targeting large and medium-sized arteries in aged individuals.\(^1\) In spite of the initial response to high-dose glucocorticoids (GCs), 40–60% of patients relapse when GCs are tapered and prolonged GC treatment results in significant side effects.\(^2\)

Search for new therapeutic options requires better understanding of pathogenesis. GCA has been classically considered a Th1-mediated disease based on the granulomatous nature of inflammatory lesions with the presence of giant cells.\(^1\)\(^3\) Moreover, while transcripts of several cytokines (ie, interleukin (IL)-6, tumour necrosis factor-α (TNFα)) can be detected in unaffected temporal artery biopsies (TABs),\(^4\)\(^5\) IFNγ, the distinctive cytokine produced by Th1 lymphocytes, is selectively expressed in GCA lesions.\(^4\)\(^7\)–\(^12\) Recently, Th17-mediated mechanisms are also emerging as a relevant component of GCA pathogenesis.\(^9\)\(^-\)\(^13\)

IFNγ has important roles in innate and adaptive immunity. It is primarily expressed by Th1 and natural killer (NK) cells and also by plasmacytoid dendritic cells, B cells and macrophages.\(^14\)\(^15\) IFNγ signals by inducing dimerisation of its receptor chains, which, in turn, induces phosphorylation of JAK 1 and JAK 2, creating binding sites for STAT-1.\(^14\)\(^16\)\(^17\) STAT-1 phosphorylation results in binding of STAT-1 to interferon-stimulated responsive elements (ISREs), respectively, in their promoter regions.\(^14\)\(^16\)\(^17\) Some of the STAT-1-induced target genes are themselves transcription factors (ie, IRFs), creating subsequent waves of inflammatory molecule expression.\(^18\) Adding complexity, IFNγ may also induce STAT-3, particularly in conditions of STAT-1 paucity.\(^19\) IFNγ promotes NK cell activity, macrophage activation, Th1 differentiation and expression of class I and class II major histocompatibility complex molecules on antigen-presenting cells.\(^14\)\(^-\)\(^21\) Based on these functions, IFNγ is thought to have a major role in GCA. Its expression by adventitial infiltrates in early GCA lesions suggests a relevant role from the initial steps of vascular inflammation.\(^11\)\(^22\)
To date, no mechanistic studies assessing the role of IFNg in the development of full-blown lesions in GCA have been performed. In this study, we explored functional roles of IFNg on GCA lesions by exposing cultured temporal artery sections from patients with GCA to a neutralising anti-human IFNg monoclonal antibody.

PATIENTS AND METHODS

Patient samples

TABs were performed to 55 patients with suspected GCA for diagnostic purposes. A 5–15 mm segment was saved for the present study. Thirty-four patients had histopathological features of GCA in their TAB. Thirteen of them had started GC treatment 3.6±3.2 days before TAB. Twenty-one patients had no inflammatory infiltrates and served as controls. Clinical data of GCA patients and final diagnosis in control patients are disclosed in online supplementary tables S1 and S2, respectively.

Neutralising, monoclonal antibody A6 against IFNg

A6 is a fully human monoclonal antibody generated in the laboratories of Novimmune (Geneva, Switzerland) from human immunoglobulin libraries using in vitro display technologies. A6 binds to human IFNg and neutralises its bioactivity.

Temporal artery culture

Temporal artery sections from patients with GCA and controls were embedded in Matrigel to ensure prolonged survival, cultured ex vivo as described8 with or without A6 antibody (10 μg/mL), recombinant IFNg at 100 ng/mL (R&D Systems, Minneapolis, Minnesota, USA), human non-immune immunoglobulin IgG1 at 10 μg/mL (Sigma, Ayrshire, UK) as a negative control or dexamethasone (0.5 μg/mL, Sigma). Each condition was tested in 3–4 replicate wells. Biopsies were frozen in TRIzol reagent (Life Technologies) for RNA extraction or in radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors for western blot studies.

Cell culture

Vascular smooth muscle cells (VSMCs) were isolated from TABs as previously described22 and used after 3–8 doubling passages. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood of healthy donors using Lymphoprep (Axis-Shield, Oslo, Norway) (see culture details in online supplementary methods).

In specific experiments, VSMCs were seeded in 12-well plates and cultured alone or with 0.5×10⁶ PBMC/well. At the end of the co-culture period, PBMCs were isolated from supernatants by centrifugation. The underlying VSMCs were separately recovered after gentle treatment with EDTA (Versene, Life Technologies) to remove adherent PBMC.

Gene expression analysis

Total RNA was obtained from cultured arteries or cells and cDNA was obtained by reverse-transcription (see online supplementary methods). Specific pre-developed TaqMan probes from Applied Biosystems (TaqMan Gene Expression Assays) (see online supplementary table S3) were used for PCR amplification. Fluorescence was detected with ABI PRISM 7900 Hardware and results were analysed with the Sequence Detection Software V2.3 (Applied Biosystems). Gene expression was normalised to the expression of the endogenous control GUSb using comparative ΔCt method.4–7 11 mRNA concentration was expressed in relative units with respect to GUSb expression.

Chemokine secretion

CXCL9, CXCL10 and CXCL11 concentrations in supernatants were measured by immunoassay using Quantikine (R&D Systems).

STAT-1 and STAT-3 phosphorylation in cultured arteries

Lysates were obtained from cultured artery sections or VSMC and phospho-STAT-1/total STAT-1 and phospho-STAT-3/total STAT-3 were assessed by western blot (see online supplementary methods).

Immunohistochemistry and immunofluorescence staining

Detailed immunostaining of cultured temporal artery sections or VSMC, as well as primary and secondary antibodies used, are depicted in online supplementary methods.

Chemotaxis assay

PBMC chemotaxis was assessed using Boyden chambers with 5 μm pore polycarbonate filters (see specific details in online supplementary methods).

Cell adhesion assays

VSMCs were grown to confluence in 96-well plates and stimulated with recombinant IFNg. After 24 h 7.5×10⁴ PBMCs per well were added and incubated at 37°C for 30 min. Wells were gently rinsed with phosphate-buffered saline and cells were fixed and stained with 0.2% crystal violet in 20% methanol for 10 min. Plates were extensively washed and crystal violet was solubilised with 1% sodium dodecyl sulfate (50 μL/well). Optical density was assessed by spectrophotometry at 560 nm wavelength.

Statistical analysis

Student’s t test, when applicable, or Mann–Whitney test was applied for independent or paired samples for statistical analysis using SPSS software, PASW V18.0.

RESULTS

Clone A6, a neutralising human monoclonal antibody against IFNg, recognises IFNg in GCA lesions and interferes with IFNg-mediated signalling

Since A6 was screened and selected by its potential to neutralise IFNg in vitro, we assessed its ability to bind IFNg expressed in GCA lesions. As shown in figure 1, biotinylated A6 antibody immunostained GCA-involved temporal artery sections, whereas immunostaining with biotinylated human IgG1 was negative. Moreover, immunostaining of non-inflamed temporal artery sections from a control individual was negative according to the absence of IFNg expression in normal arteries.4–7

Temporal artery culture in 3-D matrix has been shown to be a suitable model to test the effects of easily diffusing molecules such as dexamethasone.7 However, it is not known whether complex molecules such as monoclonal antibodies are able to elicit biological responses in this system. To test this point, we investigated whether A6 was able to interfere with IFNg-mediated signalling in cultured arteries by exploring the phosphorylation status of the transcription factors STAT-1 and STAT-3. As shown in figure 1E, F, normal arteries in culture had a remarkable constitutive expression and phosphorylation of STAT-3 but not STAT-1. Involved GCA arteries in culture had increased expression and phosphorylation of STAT-1 and decreased expression and phosphorylation of STAT-3 compared with cultured normal arteries. A6 antibody decreased expression and phosphorylation of STAT-1, whereas STAT-3 expression/
phosphorylation was not substantially affected. Treatment with recombinant IFNγ strongly increased expression and phosphorylation of STAT-1.

Neutralising endogenous IFNγ production selectively downregulates CXCL9, CXCL10, CXCL11 chemokines and STAT-1 expression in cultured GCA arteries: adding exogenous IFNγ elicits opposite effects

We investigated the effects of neutralising IFNγ with A6 on the expression of a variety of candidate molecules relevant to the pathogenesis of vascular inflammation and remodelling in GCA. Molecules investigated included transcription factors involved in T-cell functional differentiation, proinflammatory cytokines, chemokines/chemokine receptors, adhesion molecules, growth factors, metalloproteinases and their natural inhibitors, and extracellular matrix proteins (table 1 and figure 2). Since the temporal artery culture conveys by itself a downregulation of IFNγ expression with respect to the original fresh arteries, which may have minimised these results,7 we sought to confirm the potential effects of IFNγ revealed by inhibition with A6 antibody by treating cultured GCA arteries with recombinant IFNγ.

Among the molecules tested, neutralising endogenous IFNγ with A6 mainly downregulated STAT-1 and chemokine CXCL9, CXCL10 and CXCL11 mRNAs (figure 2A). As shown in figure 2B, exposure of cultured GCA arteries to IFNγ elicited the expected opposite effects and induced strong expression of STAT-1 and chemokines CXCL9, CXCL10 and CXCL11 (figure 2B). CXCL9 and CXCL10 concentrations in the supernatant fluid were also reduced upon A6 antibody treatment and increased under exposure to recombinant IFNγ (figure 2C). Concentrations of CXCL11 were around the detection level and were not substantially modiﬁed by A6 antibody or recombinant IFNγ, suggesting that CXCL11 is not secreted or is retained in the extracellular matrix.

Table 1 shows the effect of blocking IFNγ with A6, as well as the effect of adding recombinant IFNγ on the additional molecules tested. Neutralising IFNγ with A6 tended to decrease HLA-DRA, TBX21, NOS-2, TNFα, IL-6, CCL2, CXCR3, intracellular adhesion molecule-1 (ICAM-1) and platelet-derived growth factor A mRNAs and, consistently, these tended to increase upon exposure to recombinant IFNγ (figure 2C). Concentrations of CXCL11 were around the detection level and were not substantially modiﬁed by A6 antibody or recombinant IFNγ, suggesting that CXCL11 is not secreted or is retained in the extracellular matrix.

VSMCs contribute to chemokine production induced by IFNγ

Most of the effects of IFNγ have been investigated in T cells, monocytes and endothelial cells. To mimic vascular inflammatory inﬁltrates, we co-cultured PBMC from healthy donors with human temporal artery-derived VSMC, the main component of the arterial wall. Co-culture induced a variety of chemokines

Figure 1  Interferon γ (IFNγ) expression in arteries with giant cell arteritis (GCA) and the effects of IFNγ on cultured temporal arteries from patients with GCA. (A–D) Immunostaining with A6 or control IgG1 on normal temporal arteries or GCA-involved arteries. (A) Histologically negative temporal artery from a control individual incubated with biotinylated A6 (negative control). (B) GCA-involved artery incubated with biotinylated A6 antibody (brown staining reﬂects IFNγ expression). (C) A GCA-involved artery incubated with biotinylated non-immune human IgG1 (isotype control). (D) A GCA-involved artery incubated with the detection system reagents but no A6 (negative control). (E) Western blot performed to assess phosphorylated or total STAT-1 and STAT-3 in protein extracts of a cultured histologically negative biopsy (Neg Bx) and a cultured GCA-involved biopsy (GCA) untreated (Ø), exposed to A6 (10 μg/mL), IFNγ (100 ng/mL), human IgG1 (IgG; 10 μg/mL) or dexamethasone (Dexa; 0.5 μg/mL) for 5 days. The experiment was repeated twice with consistent results and a representative blot is shown. β-Actin was used as a control for loading. (F) Densitometric analysis of bands obtained in western blot experiments is exempliﬁed in (E).
Table 1 Mean fold change in mRNA of selected representative genes related to T helper functional differentiation, inflammation and vascular remodelling in cultured temporal arteries from 34 patients with GCA and 21 controls subjected to different treatments (control IgG1, A6, recombinant IFNγ or dexamethasone)

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Number of specimens analysed: negative biopsies: 21; untreated GCA: 29; GCA treated with A6: 21; GCA treated with IgG1: 18; GCA treated with IFNγ: 9; GCA treated with dexamethasone: 11.

Bold values indicate consistent opposite results achieved by blocking IFNγ with A6 or by adding recombinant IFNγ.

*p<0.05

Clones A6, anti-human IFNγ monoclonal antibody; GCA, giant cell arteritis; IgG1, isotype-matched control immunoglobulin; ICAM, intracellular adhesion molecule; IFNγ, recombinant interferon γ; MMP, matrix metalloproteinases; PDGF, platelet-derived growth factor.

Not only in PBMC but also in VSMC, indicating that VSMCs are an active source of chemokines in an inflammatory microenvironment (figure 3A). IFNγ was produced and secreted by PBMC, basally and in co-culture (see online supplementary figure S1). In accordance with the previous results, neutralising IFNγ with A6 strongly and selectively inhibited CXCL9, CXCL10 and CXCL11 chemokine expression by all cell types (figure 3A). A slight, non-significant, reduction in STAT-1 and adhesion molecule ICAM-1 was observed. No effects of A6 were observed on the expression of STAT-3, VCAM-1 or other chemokines tested in this multicellular system (figure 3A and online supplementary figure S2).

To confirm that VSMC are an important source of chemokines upon IFNγ influence, we exposed human temporal artery-derived VSMC to IFNγ, which elicited a remarkable increase in STAT-1 (figure 3B). An induction of CXCL9, CXCL10 and CXCL11 and upregulation of constitutive CCL2 was obtained, whereas expression of other chemokines related to Th1 responses (ie, CCL3, CCL4 or CCL5) or CXCL8 was not significantly induced (figure 3B). Promoter analysis (4 kb upstream and 1 kb downstream of the transcription initiating sequence) of chemokine genes was performed using Chip Bioinformatics Mapper (http://snppermapper.chip.org/mapper/mapper-main). The promoters of CXCL9, 10, and 11 as well as CCL2 shared ISRE sequences, whereas the remaining chemokines tested did not, supporting the exquisite sensitivity of these cytokines to IFNγ exposure. However, although IFNγ significantly upregulated constitutive CCL2 expression by cultured VSMC, A6 failed to downregulate CCL2 in multicellular systems such as PBMC/VSMC co-culture or whole GCA arteries where other inducers may be present (table 1 and figure 3A).

As previously demonstrated in other settings, IFNγ also upregulated STAT-1 and adhesion molecule ICAM-1 expression by cultured VSMC and induced a slight expression of VCAM-1 and STAT-3 (figure 3B).

We next cultured temporal artery-derived VSMC from 8 patients and 11 controls and investigated chemokine production at passage zero to avoid phenotypic changes induced by prolonged culture. As shown in figure 3C, patient-derived VSMC conserved IFNγ signature and expressed significantly higher mRNA concentrations of IFNγ-induced chemokine CXCL9 and a tendency to increased expression of CXCL10 and CCL2 than VSMC derived from control individuals. Higher mRNA concentrations of other chemokines not clearly in our experiments, particularly CCL4, were also observed. This may indicate VSMC exposure to stimuli other than IFNγ or to second-wave IFNγ-induced mediators in the complex multicellular and multimolecular microenvironment existing in GCA lesions.

Functional relevance of chemokine and adhesion molecule expression by VSMC

Treatment of cultured temporal artery-derived VSMC with IFNγ resulted in an increase in adhesion to PBMC (figure 4A).

The effect of IFNγ on ICAM-1 expression by VSMC was confirmed in GCA lesions. VSMC expressed ICAM-1 in cultured GCA arteries, particularly in the vicinity of inflammatory infiltrates. ICAM-1 expression was reduced in sections treated with A6 or to second-wave IFNγ-induced mediators in the complex multicellular and multimolecular microenvironment existing in GCA lesions.
Chemokine-rich supernatant of VSMC exposed to IFNγ stimulated PBMC chemotaxis in Boyden chambers (figure 4C), and this increase was abrogated by an antagonist of CXCR3, chemokine receptor common to CCL9, CXCL10 and CXCL11.

Effects of IFNγ neutralisation on infiltrating mononuclear cells in cultured temporal arteries from patients with GCA

The above findings suggest an important role for IFNγ in the initial recruitment of inflammatory cells in temporal arteries from patients with GCA and the participation of VSMC, the major component of normal arteries in this process. To confirm this hypothesis, normal temporal arteries were exposed to recombinant IFNγ and induction of CXCL9, CXCL10, CXCL11 and STAT-1 was confirmed (figure 5A). Incubation of IFNγ-treated normal arteries with PBMC from healthy donors resulted in infiltration of the artery wall by CD68 macrophages, which formed aggregates resembling giant cells (figure 5B). No CD3-positive cells penetrated the artery walls.

We next exposed cultured temporal arteries from patients with GCA to A6 and explored changes in the number of infiltrating T cells (CD3) and macrophages (CD68). Blocking IFNγ did not decrease the number of T cells (data not shown) but reduced the number of CD68-expressing cells and abrogated the presence of giant cells (figures 5C, D).

DISCUSSION

In this study, the first attempt to investigate the functional role of IFNγ in GCA, blocking endogenous IFNγ with a neutralising anti-IFNγ antibody, led to a significant reduction of STAT-1 and chemokine CXCL9, CXCL10 and CXCL11 expression in ex vivo cultured GCA arteries. Moreover, neutralising IFNγ resulted in decreased infiltration by CD68-expressing macrophages and reduced expression of TNFα along with a non-significant trend to decrease inflammatory molecules typical of a proinflammatory (M1-like) phenotype (HLA-DRA and inducible nitric oxide synthase). These molecules, previously known to be expressed in GCA, are modulated by IFNγ in other...
Figure 3  Effect of blocking the spontaneous interferon γ (IFNγ) production by co-cultured vascular smooth muscle cells (VSMCs) and peripheral blood mononuclear cells (PBMCs) on chemokine expression and effect of adding IFNγ on chemokine expression by temporal artery-derived VSMC. (A) VSMCs from normal temporal arteries were incubated alone or with PBMC (healthy donor) per well for 24 h. PBMCs were also cultured alone as a control. Cells were cultured with (filled bars) or without (empty bars) the anti-IFNγ mAb A6 (10 μg/mL). After co-culture, PBMCs (adherent and non-adherent) were separated from VSMC. RNA was extracted from each individualized cell type: PBMC cultured alone, VSMC cultured alone, co-cultured PBMC (adherent and non-adherent) and co-cultured VSMC, and mRNA levels of various chemokines was determined. Notice that the Y scale is different for each molecule. The experiment was repeated three times with consistent results. *Significant reduction by A6 p<0.05 (B) Cultured VSMCs obtained from histologically normal temporal arteries were exposed to increasing concentrations of recombinant IFNγ (0-20-50-100 ng/mL) for 24 h and expression of transcription factors STAT-1 and STAT-3, chemokines and adhesion molecules intracellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1 was assessed by real-time quantitative RT-PCR. All increases in expression of chemokines, ICAM-1, STAT-1 and VCAM-1 were statistically significant (p<0.05) compared to baseline. Increase in STAT-3 was significant (p<0.05) at the highest IFNγ concentration. (C) Spontaneous chemokine mRNA expression (relative units) by primary cultures of VSMC obtained from 11 normal arteries (empty boxes) or giant cell arteritis (GCA)-involved arteries (filled boxes). *p<0.05.
settings. Treatment of GCA arteries with exogenous IFN\(\gamma\) elicited opposite effects and tendencies, supporting the specificity of these findings. Blocking IFN\(\gamma\) in our system led to a highly selective inhibition of ISRE-containing chemokine genes CXCL 9, 10 and 11. Although IFN\(\gamma\) also induced ISRE-dependent CCL2 in isolated VSMC, the effect of blocking IFN\(\gamma\) on CCL2 expression in GCA arteries was not apparent probably due to the remarkable constitutive expression of CCL2 in aged temporal arteries and the presence of potential additional inducers. Since CXCL 9, 10 and 11 are powerful chemoattractants of mononuclear cells and these are able to interact with ICAM-1 expressing microvessels in inflamed arteries, our findings support a relevant role for IFN\(\gamma\) in the development and perpetuation of inflammatory infiltrates.

Based on the potent known effects of IFN\(\gamma\) on macrophages, and their predominance in GCA lesions, we expected that blocking IFN\(\gamma\) would have higher impact in the expression of downstream macrophage inflammatory products such as HLA-DR, NOS-2 and monokines. In our model, IFN\(\gamma\)
neutralisation slightly modified or did not modify at all a number of relevant proinflammatory molecules that were, indeed, suppressed by GC. This may be determined by concomitant activation of IFNγ-independent pathways. In this regard, neutralisation of IFNγ did not substantially reduce expression and activation of STAT-3, which appears to be highly activated in normal arteries and in GCA lesions, in accordance with the remarkable production of IL-6 in normal and inflamed arteries. Concomitant activation of STAT-3 and nuclear factor-xB may sustain expression of many inflammatory molecules in spite of IFNγ blockade.

Most of the studies investigating IFNγ proinflammatory functions have explored its effects on macrophages and endothelial cells. In vascular biology, the effects of IFNγ have been

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**Figure 5** Effect of interferon γ (IFNγ) on chemokine expression and macrophage infiltration of histologically normal temporal arteries and reduction of macrophage infiltration in giant cell arteritis (GCA)-involved arteries following IFNγ blockade. (A) Histologically normal temporal arteries (N=6) were cultured on Matrigel with or without IFNγ (100 ng/mL) for 5 days and chemokine mRNA expression was assessed (relative units) by RT-PCR. (B) Histologically normal temporal arteries cultured in 24-well plates as above were incubated in medium alone or in medium containing IFNγ (100 ng/mL) for 4 days and were subsequently exposed to PBMC from a healthy donor (0.25×10⁶/well) for 5 days. Cryosections of the retrieved arteries were processed for immunofluorescence, sectioned and immunostained with an anti-CD68 mAb (red). Nuclei were stained with DAPI (blue). (C) GCA-affected temporal arteries were cultured in medium alone or in medium containing A6 antibody (10 μg/mL), control IgG1 (10 μg/mL) or recombinant IFNγ (100 ng/mL) for 5 days, washed and processed for immunofluorescence and immunostained with anti-CD68 mAb as in (B). Nuclei were stained with DAPI (blue). (D) Cryosections of GCA-affected temporal arteries, cultured as in (C), were immunostained with an anti-CD68 mAb. Notice reduction in immunostained CD68 cells and disappearance of giant cells (arrows) following anti-IFNγ or dexamethasone (Dexa) (0.5 μg/mL) treatment. Graph shows number of CD68+ cells/field (×100) in cultured GCA-involved temporal arteries untreated or exposed to A6, control IgG1 or dexamethasone (Dexa). Twelve fields/condition were assessed. *p<0.05.
essentially investigated in the setting of atherosclerosis and graft vasculopathy. In these models, IFNγ is expressed in lesions and production of IFNγ induced chemokines have been attributed to endothelial cells and inflammatory cells and only occasionally related to myofibroblasts. In VSMC, the effects of IFNγ have been mainly related to vascular remodelling and neo-intima formation. In recent years, it has become apparent that VSMC may acquire a strong proinflammatory phenotype in the appropriate context. Our findings indicate that, in GCA, VSMC are also important targets for IFNγ, which renders them active producers of chemokines and adhesion molecules, especially ICAM-1. Consequently, VSMCs likely contribute to the progression of inflammatory infiltrates through the medial layer of the artery wall and to the development of full-blown granulomatous lesions in GCA.

GC, the cornerstone of current GCA treatment, rapidly downregulate the expression of a variety of inflammatory cytokines (ie, IL-1β, TNFα, IL-6, IL-17), adhesion molecules (ie, ICAM-1) and matrix metalloproteinases (ie, MMP-9). The known role of IFNγ in the progression of in granulomatous lesions in GCA.

However, GC do not repress IFNγ transcription. Although prolonged GC treatment eventually results in decreased IFNγ expression by other mechanisms, IFNγ expression in GCA lesions is less dramatic than that observed with other cytokines. This has led to the hypothesis that incomplete suppression of IFNγ accounts for GCA relapses during GC tapering or withdrawal, and IFNγ has been considered a potential therapeutic target. However, our findings indicate that reducing STAT-1 expression might be useful in preventing relapses, given the relevant role of IFNγ in the recruitment of inflammatory cells since the very early inflammatory stages.

In considering IFNγ as a potential therapeutic target, it is important to consider that IFNγ may have a protective role by limiting tissue injury. Blocking IFNγ worsens, indeed, experimental arthritis by promoting Th17 differentiation and exacerbation of IL-17-mediated inflammatory responses. In addition, an infectious trigger of GCA has been postulated, although no causal agents have been consistently identified. In this regard, IFNγ-deficient mice infected with murine herpesvirus HV68 develop necrotising large-vessel vasculitis, supporting the well-known role of IFNγ in host defence against viruses but also suggesting a role in limiting vascular injury. Moreover, in some experimental settings, but not in others, IFNγ deficiency exacerbates aortic aneurysm development, which is one of the important delayed complications of GCA.

Our study has several limitations. On the one hand, it explores functional activities of IFNγ in a target organ isolated from a functional immune system and variations in chemokine and adhesion molecule expression could not result in effective changes in leucocyte recruitment. It is likely that, in vivo, inhibition of lymphocyte and monocyte recruitment and subsequent macrophage activation would result in greater impact on the generation of downstream inflammatory products. In addition, as mentioned, the culture itself downregulates IFNγ expression, which may have minimised the effect of IFNγ neutralisation in our model. GC treatment of some patients prior to the TAB may also have influenced results.

In spite of these limitations, our findings indicate an important role for IFNγ in the recruitment and activation of macrophages, which may sustain and amplify subsequent waves of proinflammatory cascade in GCA. Moreover, our findings support the suitability of the temporal artery culture model to test functional activities not only of pharmacological agents or chemicals but also of complex molecules such as biological agents.

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Contributors MCC and MC-B designed the study. MHK-V and J-MD contributed important input to its design. MC-B, EP-R, EL and NT performed the experimental work. MAA, SP-G, AG-M, GE-F and JH-R contributed to clinical selection and treatment of the experimental work. RA and AE supervised in silico promoter regions. All authors evaluated and criticised the data and PR-L and MHK-V provided important contributions to their interpretation. MC-B and MCC wrote the manuscript. All authors read, made improvements and approved the final version.

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Competing interests MHK-V and WGF are full employees by Novimmune. MCC, J-HR, GE-F and SP-G are currently participating in a clinical trial of tocilizumab (anti-IL-6) in giant cell arteritis sponsored by Hoffmann-La Roche.

Patient consent Obtained.

Ethics approval Ethics committee of Hospital Clinic of Barcelona.

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Basic and translational research
Basic and translational research


Blocking interferon γ reduces expression of chemokines CXCL9, CXCL10 and CXCL11 and decreases macrophage infiltration in ex vivo cultured arteries from patients with giant cell arteritis


Pathogenesis of giant-cell arteritis: how targeted therapies are influencing our understanding of the mechanisms involved

Nekane Terrades-Garcia and Maria C. Cid

Abstract

GCA is a chronic granulomatous vasculitis that affects large- and medium-sized vessels. Both the innate and the adaptive immune system are thought to play an important role in the initial events of the pathogenesis of GCA. Amplification cascades are involved in the subsequent development and progression of the disease, resulting in vascular inflammation, remodelling and occlusion. The development of large-vessel vasculitis in genetically modified mice has provided some evidence regarding potential mechanisms that lead to vascular inflammation. However, the participation of specific mechanistic pathways in GCA has not been fully established because of the paucity and limitations of functional models. Treatment of GCA is evolving, and novel therapies are being incorporated into the GCA treatment landscape. In addition, to improve the management of GCA, targeted therapies are providing functional proof of concept of the relevance of particular pathogenic mechanisms in the development of GCA and in sustaining vascular inflammation.

Key words: targeted therapy, biologic therapy, giant cell arteritis, pathogenesis, treatment, inflammation, angiogenesis, vascular remodelling

Introduction

GCA is a chronic granulomatous vasculitis with a tropism for large- and medium-sized vessels, particularly the carotid and vertebral arteries [1, 2]. Epidemiological studies report an estimated annual GCA incidence ranging from 1.1 to 32.8 cases per 100,000 individuals aged ≥50 years; incidence varies according to geographic location [3, 4]. However, GCA patients with disease restricted to the large vessels may not have been identified in these studies because of the absence of systematic, cross-sectional imaging modalities. As a result, these epidemiological figures are likely to underestimate the true incidence of GCA.

Histological examination of temporal artery biopsy (TAB) can often be used to identify GCA [5] (Figs 1 and 2), given the common involvement of the superficial temporal artery and its ease of access [6, 7]. Although imaging methods are important tools and are widely used for the diagnosis of GCA, abnormal TAB findings provide the best diagnostic specificity [8], and TAB samples may also provide a valuable source of tissue for investigating the pathogenesis of GCA. To date, our understanding of GCA pathogenesis is largely based on immunopathological and molecular studies performed with TAB samples. However, the majority of these studies are observational in nature, and conclusions are based mainly on the previously known functions of the molecules identified and their correlation with clinical or histological abnormalities.
Functional studies evaluating mechanistic pathways in GCA are scarce.

Advances in the treatment of GCA include testing of new targeted therapies. In addition to broadening the therapeutic armamentarium for this disease, the efficacy or inefficacy of novel therapies provides important functional proof of concept for the specific pathways involved in sustaining vascular inflammation in GCA.

Current understanding of GCA pathogenesis

Predisposing factors

Epidemiological studies report differences in the incidence of GCA among ethnic groups, a higher risk in the ageing population (>50 years of age) and a female predominance; this suggests that GCA pathogenesis is driven by multiple factors, including genetic substrate, sex and alterations of the immune and arterial systems related to ageing [4, 9]. However, the role of age and sex in the development of GCA remains elusive.

A genetic component in the pathogenesis of GCA is supported by observations of sporadic family clustering of affected members, along with the predominance of the disease in whites, particularly those from northern Europe or of northern European descent [10, 11]. Indeed, an increased risk of GCA is associated with polymorphisms in a variety of genes that mediate immune, inflammatory and vascular responses [11, 12].

Candidate gene studies have shown an association between GCA and genetic variants in the MHC region, particularly with class II HLA-DRB1*04 alleles (usually DRB1*0401, but also DRB1*0404) [11, 13]. Recently, large-scale fine mapping of genes related to immune responses confirmed a strong association between variants in the class II MHC region and GCA susceptibility [14]. The amino acids resulting from these risk variants are located in the antigen-binding cavity of HLA molecules. This finding suggests that GCA may be an antigen-driven disease, supporting the role of the adaptive immune system in development of the disease.

A recent genome-wide association study has also revealed that, outside the MHC region, variants in genes related to vascular response to inflammation and remodelling, such as plasminogen and prolyl 4-hydroxylase subunit alpha 2, are also associated with GCA risk [15].

Initial events of GCA

The initial triggering agent(s) in GCA has not been consistently identified. Several pathogens have been proposed as aetiological agents in GCA, but no definitive causal relationship with a particular microorganism or viral agent has been demonstrated [16, 17], and none of the pathogenic sequences detected in temporal arteries have been unequivocally associated with GCA [18]. However, these
pathogenic sequences may play a role in the activation of pathogen sensing receptors given that innate immune mechanisms may also contribute to GCA [19].

**Role of the innate immune system**

Cells of the innate immune system appear to play a role in the pathogenesis of GCA (Fig. 3A). Dendritic cells have been detected in normal or early inflamed large and medium-sized arteries [20-23] and may play an important role in the pathogenesis of GCA. The maturation of dendritic cells from a non-stimulatory to a T cell activating state in the arterial adventitia is thought to be a critical event in the initiation of GCA [22]. Dendritic cells can be activated via toll-like receptors, resulting in the production of cytokines and chemokines that attract and activate other immune cells.
of chemokines (e.g. CCL19 and CCL21) that attract and retain additional dendritic cells [22, 23]. In addition, dendritic cells express activation (CD83) and co-stimulatory (CD86) molecules that are responsible for the activation of T cells [21, 22], which in turn are modulated by immune checkpoints. In GCA, inefficiency of the programmed death 1 (PD-1) receptor/programmed death ligand 1 (PD-L1) immune checkpoint has been observed in GCA-affected temporal arteries; this is thought to contribute to the excessive infiltration of activated T cells into affected medium- and large-sized blood vessels [24].

Role of the adaptive immune system

Involvement of the adaptive immune system appears to be critical to the initial development of vascular inflammation in GCA-involved vessels (Fig. 3A). Observed oligoclonal T cell expansion in GCA lesions supports the participation of antigen-specific adaptive immune responses in GCA [25]. Pathogenic pathways mediated by both IFN-γ-producing Th1 and IL-17-producing Th17 cells are thought to play a role in the pathogenesis of GCA, contributing to systemic and vascular manifestations of the disease (Fig. 3A and B) [26]. Consistent with the relevant role of T cells in GCA, DNA methylation analysis of the temporal artery microenvironment in GCA has revealed that genes related to T cell activation and Th1/Th17 differentiation were hypomethylated in GCA lesions [27]. Increased production of the pro-inflammatory cytokine IFN-γ has been shown in GCA-involved arteries [28, 29], resulting in the expression of IFN-γ-induced products in lesions, including class II MHC antigens [30], endothelial adhesion molecules [31], inducible nitric oxide synthase [32] and chemokines [29, 33, 34]. IFN-γ is a potent activator of macrophages, the predominant cell population in GCA lesions, and is thought to drive the granulomatous reaction and transformation of macrophages to giant cells in these lesions [30]. More recent studies also suggest the involvement of Th17-mediated mechanisms in the development of GCA [35, 36]. Th17 cells produce the pro-inflammatory cytokine IL-17A, which has pleiotropic effects on a variety of cells, including macrophages, neutrophils, endothelial cells and fibroblasts, and actively contributes to inflammatory cascades [37]. Th1 and Th17 precursor cells (CD161+ CD4+ T lymphocytes) have been identified in the inflammatory infiltrates of TAB specimens from patients with GCA [38], and pro-inflammatory cytokines that promote Th17 differentiation have been observed in patients with GCA, including IL-1β, IL-21, TGF-β and IL-6 (Fig. 3C) [25, 32, 39, 40]. IL-12/23p40 and IL-23p19 subunits are expressed in GCA lesions [35], and the resulting cytokine, IL-23, is pivotal in maintaining Th17 differentiation. As a result, IL-17A expression is increased in GCA lesions [36]. These elevated levels of IL-17A are rapidly reduced in biopsies obtained from patients with GCA following treatment with glucocorticoids [36], suggesting that IL-17A suppression may contribute to the dramatic symptomatic improvement in patients with GCA who receive high-dose glucocorticoid therapy. Interestingly, strong expression of IL-17A in the involved arteries of patients with GCA was associated with a better response to glucocorticoid therapy with few relapses [36].

Regulatory T cells, which limit activation of the immune system and the accompanying inflammatory response, are also present in vascular lesions and are decreased in peripheral blood of patients with GCA [36, 38]. Given the well-recognized plasticity of T cell subsets, regulatory T cells may transiently lose their suppressive state and may themselves produce IL-17A in a strongly inflammatory microenvironment with abundant production of cytokines (e.g. GCA lesions) [36]. These abnormalities are reversed in peripheral blood regulatory T cells in patients with GCA treated with the anti-IL-6 receptor mAb, tocilizumab, highlighting the role of IL-6 in promoting a pro-inflammatory phenotype in regulatory T cells [41].

Although B cells are not abundant, their presence in GCA lesions has been observed [20, 42, 43], sometimes forming tertiary lymphoid structures [44]. While GCA has been primarily considered a T cell-mediated disease, it is important to note that B lymphocytes play a crucial role in T cell activation. In patients with active GCA, circulating levels of B cells are decreased, but recover following glucocorticoid treatment and are thought to be recruited into inflamed vessels [42]. In addition, IL-6 production by B cells is enhanced and B cell-activating factor is associated with disease activity in GCA [42, 45]. Additional evidence supporting the involvement of B cells in GCA includes scattered reports of therapeutic benefit following B cell depletion therapy with rituximab in relapsing patients [46, 47]. However, further clinical research to confirm the benefit of B cell-targeted therapy in GCA is currently lacking.

Amplification cascades

Following the initiating events of GCA, amplification cascades play an important role in the development and progression of inflammatory infiltrates, the development of full-blown transmural inflammation, vascular wall injury and remodelling, the pathological substrate of clinical symptoms and complications of GCA [5, 19, 20, 29, 30, 32]. Macrophages play an important role in this process. Both pro-inflammatory (M1-like) and reparative (M2-like) macrophages are abundant in GCA vascular lesions, and appear to promote neovascularization and several mechanisms of arterial wall damage (e.g. reactive oxygen species, matrix metalloproteinase (MMP)-2 production; Fig. 3D) [32, 39, 48-50].

The production of cytokines by pro-inflammatory macrophages has prominent local and systemic effects, with a potential impact on disease manifestations and outcome in GCA. The intensity of the systemic inflammatory response in GCA correlates with expression of TNF-α, IL-1β, IL-6 and IL-33 (Fig. 3C) [39, 48]. Moreover, circulating TNF-α and IL-6, along with tissue expression of TNF-α, have been shown to correlate with relapses and disease persistence [39]. Inflammatory loops associated with GCA may be further reinforced by the upregulation of chemokines, endothelial adhesion molecules and colony-stimulating factors in lesions, resulting in the continuous
recruitment and expansion of additional inflammatory cells [28, 29, 31, 33]. The formation of new vessels in vascular lesions of GCA (Fig. 2) may be promoted by macrophage production of angiogenic factors, such as VEGF, fibroblast growth factor-2 and PDGFs (Fig. 3D) [30, 49, 51]. Acute phase proteins, typically increased in patients with GCA, may also be angiogenic [52, 53]. The expression of endothelial adhesion molecules by neovessels facilitates the recruitment of additional leukocytes [30, 31, 54]. While angiogenesis is an important process in the progression and maintenance of chronic inflammatory diseases, such as GCA, inflammation-induced angiogenic activity may also play a compensatory role for ischaemia at distal sites in patients with GCA, thus protecting against ischaemic complications [55, 56].

The role of inflammation in arterial damage

Damage of GCA-involved arteries may in part be related to the presence of cytotoxic lymphocytes in advanced lesions, which might contribute to the depletion of vascular smooth muscle cells (VSMCs) [57]. Oxidative damage and vessel wall injury may also arise as a result of reactive oxygen species produced by activated macrophages [32]. The destructive role of proteases in inflamed arteries is evidenced by upregulation of MMPs, MMP-9 and MMP-2, which have elastolytic activity and are up-regulated in GCA lesions, whereas their natural inhibitors, tissue inhibitor of metalloproteinases (TIMP)-1 and -2, are down-regulated, yielding an increase in proteolytic balance [32, 50]. Indeed, increased MMP-9/MMP-2 proteolytic activity has been observed in GCA lesions and may contribute to the disruption of elastic fibres and abnormal vascular remodelling [50, 58] (Fig. 3D). Furthermore, the disruption of elastic fibres may favour aortic dilatation, which is an increasingly recognized and delayed complication of GCA [58-61].

Vascular remodelling and occlusion

Patients with GCA may experience symptoms of vascular insufficiency and ischaemic complications due to vascular remodelling through intimal hyperplasia and vessel occlusion (Fig. 1B). Activated macrophages or injured VSMCs produce growth factors that trigger a vascular remodelling process leading to myofibroblast differentiation of VSMCs, migration towards the intimal layer and deposition of extracellular matrix proteins. Several of these factors are expressed in GCA lesions, including PDGFs, TGF-β and ET-1 (Fig. 3D); these factors may contribute to vascular remodelling by inducing myofibroblast activation and the production of matrix proteins [25, 62-64]. Indeed, blockade of the PDGF receptor by imatinib mesylate or blocking ET-1 receptors results in reduced myointimal cell outgrowth from cultured temporal arteries of patients with GCA [49, 51, 64]. Circulating concentrations of ET-1 are elevated in patients with GCA who have neuro-ophthalmic ischaemic complications, highlighting their potential role in vasospasm or vascular occlusion [63]. The participation of neurotrophins, such as nerve growth factor and brain-derived neurotrophic factor, in the generation of intimal hyperplasia has been proposed given that they are expressed in GCA lesions and promote the proliferation and migration of VSMCs [65]. A number of microRNAs that regulate the functions of VSMCs are up-regulated in GCA lesions, further supporting their involvement in the generation of intimal hyperplasia [66]. Unfortunately, these vascular remodelling factors do not appear to be substantially down-regulated in GCA lesions following glucocorticoid therapy, suggesting that modulation of their potential impact in vessel stenosis and occlusion may require specific therapeutic approaches in large-vessel vasculitis [29, 35].

Functional models

Several animal models of large-vessel inflammation have been generated and provide important clues about triggers and mechanisms potentially involved in vascular inflammation. IFN-γ-deficient mice infected with murine herpesvirus HV68 develop necrotizing large-vessel vasculitis [67, 68], suggesting that herpesvirus members can induce vascular inflammation. On the other hand, this evidence underlines the protective role of IFN-γ in maintaining virus latency and possibly in avoiding excessive vascular destruction [67, 68]. A mouse model of large-vessel arteritis demonstrated that mice deficient in the gene encoding the anti-inflammatory cytokine IL-1 receptor antagonist developed lethal arterial inflammation, thus suggesting a role of the IL-1 receptor antagonist in protecting the vessel wall from inflammatory stimuli [69]. Mice deficient in interferon regulatory factor 4 binding protein have increased expression of IL-21 and IL-17A, along with subsequent development of large-vessel vasculitis, which supports the hypothesis that IL-17 is involved in vascular inflammation [70]. Taken together, these models demonstrate that these molecules and their downstream pathways are relevant to vascular inflammation, but do not completely recapitulate the clinical, anatomical and histopathological features of GCA.

Subcutaneous engraftment of GCA-involved temporal artery fragments into mice with severe combined immunodeficiency has been used for functional studies. In this model, T cell depletion with T cell-specific antibodies reduced T cell-dependent cytokines [71], dendritic cell depletion reduced inflammation in the explant [22] and depletion of tissue-infiltrating macrophages resulted in the production of reactive oxygen species [72]. Blockade of PD-1 has also been shown to exacerbate adoptively transferred vascular inflammation in engrafted normal arteries. Infiltrates are enriched in PD-1+ T cells, with enhanced production of multiple cytokines, including IFN-γ, IL-17 and IL-2, in vascular tissue [24].

Temporal artery culture in 3D matrix has been recently introduced to investigate pathogenic pathways. In this model, it has been shown that glucocorticoids decrease production of inflammatory cytokines but do not influence factors involved in vascular remodelling [29]. The induction of a pro-inflammatory phenotype in VSMCs by IFN-γ and their active role in recruiting monocytes has been
demonstrated in this model [34]. Blocking PDGF receptor signalling with imatinib or endothelin-1 signalling with receptor antagonists has been shown to reduce myointimal cell outgrowth [51, 64].

These models have provided interesting insight into some relevant mechanisms of vascular inflammation and remodelling. However, they only examine target tissue isolated from a functional immune system to investigate the pathogenesis of vascular inflammation. Moreover, these models only allow assessment of changes in biomarkers given that clinically relevant disease outcomes such as pain, systemic symptoms, ischaemic complications and aortic dilatation cannot be investigated.

**Targeted therapies shed light on the pathogenic mechanisms of GCA**

Even if unsuccessful, research and development of novel targeted therapies provide unique information regarding the participation or irrelevance of specific pathways in disease pathogenesis. For example, investigation of immune checkpoints for cancer immunotherapy has provided interesting lessons. GCA has developed in some patients with malignant melanoma after blocking cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) with ipilimumab [73, 74], underlining the relevance of T cells and the immunological synopsis in triggering immune activation leading to GCA. Accordingly, abatacept, a recombinant Ig-CTLA-4 molecule, has demonstrated efficacy in maintaining remission in a recent randomized controlled trial [75]. Therapeutic interventions according to pathogenic mechanisms are summarized in Table 1.

TNF-α is a potent, multifunctional, pro-inflammatory cytokine that promotes infiltration of leucocytes via the production of chemokines, the induction of adhesion molecule expression (E-selectin, intercellular adhesion molecule 1 and vascular adhesion molecule 1), and the production of MMPs [76]. The gene encoding TNF-α is hypomethylated in GCA lesions where it is highly expressed, and the association between increased expression of TNF-α and persistent disease activity has been observed in various studies [39], along with the benefit obtained with TNF blockade in other chronic inflammatory or granulomatous diseases. This evidence provided the rationale for conducting clinical trials to investigate TNF inhibition with infliximab, etanercept or adalimumab in GCA. Unfortunately, TNF-α blockade did not provide an advantage over placebo in maintaining remission in newly diagnosed patients [76–79]. The disappointing experience with TNF-α blockade underlines the fact that a biomarker of inflammation is not necessarily a therapeutic target and suggests that TNF-α functions may not be essential for the maintenance of vascular inflammation, or can be supplied by redundant pathways.

IL-6 is a multifunctional cytokine secreted by numerous immune cells (e.g. macrophages, neutrophils, dendritic cells) and exerts pleiotropic effects on a variety of cell types [80]. The effects of IL-6 on the immune system include the activation of macrophages and neutrophils, differentiation of Th17 cells, inhibition of the suppressive activity of regulatory T cells, promotion and differentiation of B cells and stimulation of endothelial cells [80]. Furthermore, IL-6 is thought to play an important role in the switch from acute to chronic inflammation [80]. IL-6 transcripts are abundant in GCA lesions but are also present in normal temporal arteries, indicating a potential role in vascular homeostasis [29, 39]. IL-6 expression in lesions is also significantly higher in patients with GCA with a strong systemic inflammatory reaction [39]. Serum IL-6 is elevated in patients with GCA and correlates with disease activity [45, 81–83]. Moreover, persistently increased serum IL-6 is found in patients with relapsing disease [84]. Recently, IL-6 receptor blockade with tocilizumab was shown to be superior to placebo in maintaining remission and sparing glucocorticoids in phases 2 and 3 clinical trials, both in newly diagnosed and relapsing patients [85, 86]. These trials indicate that IL-6-dependent inflammatory pathways are highly relevant in maintaining inflammatory activity in GCA. Short-term clinical outcomes seem to be clearly improved by tocilizumab. Tocilizumab strongly inhibits the systemic inflammatory response, which is an important burden in patients with GCA, as well as cranial and polymyalgic clinical symptoms. However, the impact of tocilizumab on vascular inflammation and vascular remodelling, along with their associated vascular complications, needs to be evaluated; this will provide unique insights into pathogenic mechanisms of vascular inflammation and repair.

**Conclusions**

To date, our understanding of GCA pathogenesis is largely based on evidence from histopathological characteristics, the identification of cell populations and subpopulations in affected vessels or peripheral blood, the expression of activation and differentiation markers by these cells and the production of certain inflammatory molecules in GCA lesions. The role that infiltrating cells and their products play in the development of GCA is primarily based on the assumption of their known biologic functions and correlation with relevant histopathological features (e.g. neovascularization, intimal hyperplasia, giant-cell formation), clinical phenotypes or disease outcomes [19, 30]. Several animal models of large-vessel inflammation have provided evidence regarding potential mechanisms involved in vascular inflammation. However, the pathogenesis of GCA remains incompletely understood because of the scarcity of functional studies demonstrating the involvement of specific pathways.

The recent introduction of targeted therapies into the treatment landscape for GCA may shed light on the participation of specific pathways in pathogenesis of the disease. In particular, research surrounding immune checkpoint inhibition and cytokine blockade (TNF-α and IL-6) has provided important insights into the roles that the immune system and vascular inflammation play in the development of GCA. Future research into current and novel targeted agents is needed to expand our knowledge.
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Potential pathological effect</th>
<th>Potential intervention</th>
<th>Potential drugs</th>
<th>Investigation status</th>
<th>Results</th>
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</thead>
<tbody>
<tr>
<td>Dendritic cell activation</td>
<td>Attracts and retains additional dendritic cells and activates T cells</td>
<td>Blocking TLR receptors</td>
<td>NC</td>
<td>NP</td>
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<td>T cell activation (Th1 and Th17 cells)</td>
<td>Highly activated T cells, modulated by immune checkpoints, promote excessive infiltration of activated T cells into affected medium- and large-sized blood vessels [21-24]</td>
<td>Interfering with CD28-mediated activation</td>
<td>CTLA-4-Ig (Abatacept)</td>
<td>Phase 2 RCT (NCT00556439)</td>
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<td>B cell differentiation, B cell co-stimulatory signals or other B cell functions</td>
<td>Forms tertiary lymphoid structures [42, 44] and activates T cells</td>
<td>B cell depletion</td>
<td>Rituximab and others</td>
<td>Few case reports</td>
<td>Very low evidence [46, 87, 88]</td>
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<td>Th1 differentiation/effecter pathways</td>
<td>Production of IFN-γ and IL-17 promoting systemic and vascular inflammation [26]</td>
<td>Blocking IL-12</td>
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<td>NP</td>
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<td></td>
<td>Drives granulomatous reaction and transformation of macrophages to giant cells [30]</td>
<td>Blocking IFN-γ</td>
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<td>NP</td>
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<td></td>
<td>Contributes to systemic and vascular manifestations of GCA [28]</td>
<td>Blocking TNF</td>
<td>Infliximab</td>
<td>Phase 2 RCT (NCT0076726)</td>
<td>Negative [77]</td>
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<td>Adalimumab</td>
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<td>Th17 differentiation/effecter pathways</td>
<td>Induces chronic inflammation and activates dendritic cells, endothelial cells and smooth muscle cells involved in arterial tissue damage [37, 38]</td>
<td>Blocking IL-6 receptor</td>
<td>Tocilizumab</td>
<td>Phase 2 (NCT01450137) and 3 (NCT01791153) RCT Ongoing, open-label phase 4 (NCT03244709)</td>
<td>Positive [85, 86]</td>
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<td>Ongoing, open-label phase 3 RCT (NCT02531633)</td>
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<th>Results</th>
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<td>Both Th1 and Th17 differentiation pathways</td>
<td>Interaction between Th1 and Th17 pathways involved in promoting systemic and vascular inflammation [26]</td>
<td>Blocking IL-12/23p40</td>
<td>Ustekinumab</td>
<td>Open-label observational</td>
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<td>Blocking JAK1 and JAK2</td>
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<td>Ongoing phase 4 (NCT03244709)</td>
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<td>Treg function</td>
<td>Usually Tregs have suppressive functions but, under the effects of IL-6, they become pro-inflammatory and contribute to systemic and vascular manifestations of GCA [36]</td>
<td>Blocking IL-6 receptor</td>
<td>Tocilizumab</td>
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<td>Blocking IL-6</td>
<td>Sirukumab</td>
<td>Ongoing phase 3 RCT (NCT02531633)</td>
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<td>Macrophage survival/activation</td>
<td>Oxidative damage and vessel wall injury [30, 32]</td>
<td>Blocking IFN-γ</td>
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<td>Ongoing phase 3 RCT (NCT02531633)</td>
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<tr>
<td>Tissue disruption</td>
<td>Production of matrix proteins [62]</td>
<td>Matrix metalloproteinase inhibitors</td>
<td>NC</td>
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<td>Abnormal vascular remodelling</td>
<td>Promotion of myo-intima proliferation and migration and ECM production leading to hyperplasia and vessel occlusion [30, 51, 62-64]</td>
<td>ROS scavengers</td>
<td>NC</td>
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<td>Endothelin receptor antagonists</td>
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<td>PDGF receptor blockade</td>
<td>NC</td>
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</table>

BAFF/BLyS: B cell activating factor/B lymphocyte stimulator; CD28: cluster of differentiation 28; CSF-1: colony-stimulating factor 1; CTLA-4: cytotoxic T-lymphocyte-associated antigen 4; ECM: extracellular matrix; IL-1RA: IL-1 receptor antagonist; JAK: janus kinase; NC: not currently under consideration; NP: not performed; PDGFs: platelet-derived growth factors; RCT: randomized controlled trial; ROS: reactive oxygen species; TLR: toll-like receptor.
regarding specific disease pathways involved in GCA-associated vascular inflammation and repair.

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Invited Article

Biological treatments in giant cell arteritis & Takayasu arteritis

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A B S T R A C T

Giant cell arteritis (GCA) and Takayasu arteritis (TAK) are the two main large vessel vasculitides. They share some similarities regarding their clinical, radiological and histological presentations but some pathogenic processes in GCA and TAK are activated differently, thus explaining their different sensitivity to biological therapies. The treatment of GCA and TAK essentially relies on glucocorticoids. However, thanks to major progress in our understanding of their pathogenesis, the role of biological therapies in the treatment of these two vasculitides is expanding, especially in relapsing or refractory diseases. In this review, the efficacy, the safety and the limits of the main biological therapies ever tested in GCA and TAK are discussed. Briefly, anti-TNF-α agents appear to be effective in treating TAK but not GCA. Recent randomized placebo-controlled trials have reported on the efficacy and safety of abatacept and mostly tocilizumab in inducing and maintaining remission of GCA. Abatacept was not effective in TAK and robust data are still lacking to draw any conclusions concerning the use of tocilizumab in TAK. Furthermore, ustekinumab appears promising in relapsing/refractory GCA whereas rituximab has been reported to be effective in only a few cases of refractory TAK patients. If a biological therapy is indicated, and in light of the data discussed in this review, the first choice would be tocilizumab in GCA and anti-TNF-α agents (mainly infliximab) in TAK.

1. Introduction

Giant cell arteritis (GCA) and Takayasu arteritis (TAK) are the two main large vessel vasculitides [1]. It has been suggested that TAK and GCA may be different phenotypes of a single disease [2–4] since they share some similarities regarding their clinical, radiological and histological presentations, both being granulomatous vasculitides involving the aorta and its major branches [1]. However, some pathogenic processes in GCA and in TAK are activated differently [5–7]. Furthermore, GCA can be distinguished from TAK by several epidemiological, clinical, arterial distribution and therapeutic features [2]. GCA occurs in people over 50 years and its incidence increases progressively after 50 years with a peak occurring between 70 and 80 years [8,9]. Women are affected two to three times more frequently than men. GCA is very rare in African, Arabic and Asian countries [10–14], whereas the highest prevalence is observed in Caucasian people, especially in Scandinavian countries and in Olmsted County, Minnesota, where the population has a similar ethnic background [15]. Assessment of GCA activity usually relies on clinical symptoms, erythrocyte sedimentation rate (ESR) and acute-phase reaction proteins – mainly C-reactive protein (CRP) and fibrinogen – which are increased in > 95% of cases and closely related to disease activity [16]. By contrast, TAK occurs in patients < 40 year old and is much rarer than GCA, accounting for 1–3 cases per million per year. In 90% of cases, patients affected by TAK are women. TAK has been described in all ethnic groups around the world but is more frequent in Asian countries and in northwest Turkey [17]. It is more challenging to assess disease activity in TAK than in GCA since TAK seems to be a more chronic and insidious disease. Particularly, a substantial number of patients with active disease have normal levels of acute phase reaction proteins and ESR. This situation has led to the proposal of scores to assess TAK activity: the National Institute of Health (NIH) criteria (or Kerr criteria) [18] and more recently the Indian Takayasu Clinical Activity Score (ITAS2010) [19].

In both diseases, glucocorticoids (GC) remain the cornerstone of treatment. They are very effective in inducing remission but relapses are common when doses are tapered. Immunosuppressive drugs,
especially methotrexate, are therefore used to spare GC and/or prevent further relapse(s), but have not shown a major benefit [20]. Therefore, biologics are often used in GCA or TAK after failure of GC tapering despite the use of conventional immunosuppressive drugs. This review focuses on recent data available about the use of biologics in large-vessel vasculitis, and highlights the differences between GCA and TAK with regard to these treatments.

2. Anti-TNF-α drugs (Fig. 1)

a. GCA

By comparing the mRNA levels of interleukin-1beta (IL-1β), tumour necrosis factor-alpha (TNF-α) and IL-6 in temporal artery samples from 36 patients with biopsy-proven GCA and 11 controls, Hernández-Rodríguez et al. demonstrated that the tissue expressions of these three
pro-inflammatory cytokines were high in GCA patients, and notably that a high production of TNF-α was associated with longer GC requirements [21]. These data, together with results of several case series reporting the success of anti-TNF-α drugs in GCA [22,23], led researchers to conduct three prospective clinical trials.

The first trial included 44 patients who were randomized to receive infliximab (5 mg/Kg at week 0 [W0], W2, W6 and then every 8 weeks; n = 28 patients) or its placebo (n = 16 patients) in association with GC which were discontinued before W24. Infliximab therapy failed to increase the proportion of patients without relapse at W22 compared with placebo (43% vs. 50%, respectively) or the proportion of patients whose GC dosages were tapered to 10 mg/day without relapse (61% vs. 75%, respectively). Furthermore, infection occurred in 71% of cases in patients receiving infliximab compared with 56% for those receiving placebo. A double-blind placebo-controlled trial assessed etanercept (17 patients) to draw any conclusions about the efficacy of this drug in this disease [24]. More recently, adalimumab (40 mg/2 weeks from diagnosis to W10) was evaluated in a randomized controlled trial involving 70 GCA patients and did not show any efficacy of adalimumab when compared with placebo [25].

b. TAK

Even though no randomized controlled trials have supported the use of anti-TNF-α drugs in TAK, increasing evidence suggests that TNF-α is an attractive therapeutic target in this vasculitis. By contrast with GCA [26], mRNA expression and intracellular production of TNF-α by T cells are higher in active TAK patients than in inactive TAK patients and controls [27,28]. A review published in 2014 included 120 TAK patients treated with anti TNF-α agents in 20 observational studies: 109 received infliximab, 17 etanercept and 9 adalimumab [29]. In the majority of cases, the disease was still active or relapsing despite prior treatment with prednisone and a second agent. Overall, remission was obtained with anti-TNF-α drugs in 70 to 90% of patients and importantly, GC were stopped in 40% of cases. In the majority of cases, a non-biologic immunosuppressive drug was maintained with the anti-TNF-α drugs [29]. Despite treatment, 37% of patients relapsed and about 50% of patients required an increasing dose or a switch in the anti-TNF-α (usually to infliximab) to maintain remission [29,30]. Even though these data support the efficacy of anti-TNF-α agents in TAK, it has to be highlighted that the great majority of these patients received infliximab and that the experience with etanercept and adalimumab is more limited. Concerning golimumab and certolizumab, no study reporting their use in TAK has been published. Furthermore, two cases of TAK occurring under anti-TNF-α therapy (adalimumab and golimumab, respectively) have been reported [31].

The French Takayasu Network recently reported on 49 TAK patients treated with biologics, of whom 35 received anti-TNF-α agents (28 infliximab [3-7 mg/Kg at W0, W2, W6 and then every 4-8 weeks], six received etanercept [25 mg twice a week], one received adalimumab [40 mg/2 weeks]) and 14 received tocilizumab (8 mg/Kg monthly). Prior treatment with biologic agent, 88% of patients were inadequately controlled with or were intolerant to conventional immunosuppressive therapy. After introduction of biologic agents, a complete response, defined by NIH scale < 2 and prednisone < 10 mg/day, was achieved in 35%, 61% and 74% at 3, 6 and 12 months, respectively. During the follow-up, 40% of the patients switched to another biologic. Importantly, 3-year relapse-free survival was better with biologics than with conventional immunosuppressive therapy (91±59%; P = 0.009). However, this result has to be balanced by the fact that biologics were associated with conventional immunosuppressants (mainly methotrexate) in 76% of cases and the time origin for each treatment in survival analyses was the time at which the patients started this regimen. Therefore, this study does not report a direct comparison between biologics and conventional immunosuppressive drugs but rather outcomes in TAK patients after add-on therapy with anti-TNF-α agents or tocilizumab [32].

c. Conclusion

Regarding the data available in the literature, it can be concluded that anti-TNF-α agents do not effectively prevent relapse or spare GC in GCA. In TAK, no randomized controlled trials are available but there is increasing evidence that anti-TNF-α agents, especially infliximab, are effective in inducing and maintaining remission as well as sparing GC.

3. Blockade of the IL-6 pathway: tocilizumab and sirukumab (Fig. 1)

IL-6 is a pro-inflammatory cytokine produced by many cell types, especially monocytes and macrophages. IL-6 signalling depends on the membrane protein gp130, whose activation leads to the phosphorylation of STAT3. Gp130 is activated after its linkage to a complex composed of IL-6 and its receptor, which is either a transmembrane protein (mIL-6R, expressed by hepatocytes, monocytes, macrophages, some B and T cells, megakaryocytes and endothelial cells) or a soluble protein (sIL-6R). Signalling through sIL-6R is called transsignalling and is more implicated in the pathological features related to IL-6 than is classical signalling, which uses mIL-6R [33].

a. GCA

The concentration of IL-6 is dramatically increased in the serum of patients affected by GCA, and its concentration is closely related to disease activity and the CRP level [34,35]. Furthermore, the Th17/Treg balance, which is disturbed in GCA [35], is mainly controlled by IL-6, which increases Th17 at the expense of Treg polarization [36]. Along this line, it has been demonstrated that blockade of the IL-6 pathway restores the Th17/Treg imbalance in both rheumatoid arthritis [37-39] and GCA [40]. Furthermore, IL-6 induces the recruitment of leukocytes in the arterial wall. After activation with IL-6, endothelial cells – which express IL-6R and gp130 – increase their level of expression of adhesion molecules like VCAM-1 and ICAM-1, which leads to the recruitment of leukocytes expressing their ligands (VLA-4 and LFA-1) [41]. IL-6 could also be implicated in vascular remodelling processes since a recent study demonstrated that serum amyloid A protein, which is produced by IL-6 activated hepatocytes, increases the production of VEGF and MMP9 and the migration and proliferation of vascular smooth muscle cells in an in vivo model of cultured temporal arteries [42].

Tocilizumab is a humanized anti-IL-6 monoclonal antibody directed against mIL-6R and sIL-6R that blocks IL-6 classical signalling and transsignalling. In line with the role of IL-6 in GCA pathogenesis, the first case series suggested that IL-6 is a very attractive therapeutic target in GCA. This led to two randomized placebo controlled trials. The first included 30 patients (23 new-onset GCA and seven relapsing GCA) of whom 20 were treated with prednisone and tocilizumab (8 mg/Kg/4 weeks IV during 52 weeks) and 10 with prednisone and placebo. In both arms, prednisone was started at 1 mg/Kg/day and then rapidly tapered to be stopped after 36 weeks of treatment. This point is crucial for the external validity of this trial since 6 months of GC-therapy is not usually recommended in GCA because it increases the risk of relapse [43]. After 52 weeks of treatment, relapse-free survival was dramatically better in the group of patients treated with tocilizumab [44]. However, it has to be highlighted that, unlikely to the GiACTA study [45], CRP was not blinded in this study [44], and an increase in CRP was considered in the definition of relapse. This may have challenged the double-blind of the trial and may have overestimated the effect of tocilizumab in GCA.

Nevertheless, the efficacy of tocilizumab for the treatment of GCA has recently been confirmed in a larger randomized double-blind placebo-controlled study involving 251 patients (47% new-onset and 53% relapsing GCA) [45]. In order to prevent unblinding that could occur because of biological modifications – mainly suppression of CRP – induced by tocilizumab, CRP levels were blinded and a laboratory
assessor monitored all other laboratory variables independently of the efficacy assessor. In this trial, patients were randomly assigned to four different groups: subcutaneous (SC) tocilizumab (162 mg/week for 52 weeks) combined with a 26-week prednisone taper, subcutaneous (SC) tocilizumab (162 mg/2 weeks for 52 weeks) combined with a 26-week prednisone taper, or placebo combined with a prednisone taper over a period of either 26 or 52 weeks. The primary endpoint was the rate of sustained remission, defined by remission without relapse or deviation from the prednisone regimen, in each group. At 52 weeks, sustained remission was achieved in 56% of patients treated with tocilizumab weekly and 53% in those treated with tocilizumab every 2 weeks in comparison with 14% and 18% in those treated with placebo and 26 or 52 weeks of prednisone, respectively (P < 0.001). Furthermore, tocilizumab resulted in a strong GC-sparing effect of almost 2000 mg when compared with the 52 weeks of prednisone regimen. The safety of tocilizumab was good since serious adverse events occurred in only 14% of patients treated with tocilizumab every 2 weeks and in 15% of patients treated with tocilizumab weekly in comparison with 22% and 25% in those treated with placebo and 26 or 52 weeks of prednisone, respectively. The most common serious adverse events were infections and vascular disorders [45].

However, it has to be assumed that these two trials did not provide data after tocilizumab discontinuation [44,45]. Because tocilizumab blocks the IL-6 receptor, it triggers an increase in levels of IL-6 and sIL-6R, which could lead to relapse after tocilizumab discontinuation. In a phase II trial conducted by our team, in which 20 GCA patients received 4 monthly infusions of tocilizumab starting at GCA diagnosis in association with 9–10 months of prednisone, we observed that 50% of patients experienced relapse 9 months after tocilizumab discontinuation, especially those who had aortitis at diagnosis [46].

Another matter of concern with treatments blocking the IL-6 pathway is related to their ability to trigger a dramatic decrease in acute phase reaction proteins (especially CRP and fibrinogen) as well as ESR, making it particularly difficult to monitor these patients. Therefore, and as reported in TAK [47–50], it cannot be excluded that the disease in GCA patients treated with tocilizumab continues to progress even though inflammatory markers are normal.

As a consequence, even though these two recent randomized controlled trials confirmed the dramatic efficacy of tocilizumab in GCA, several issues need to be addressed before generalizing its use in GCA. In particular, future studies need to determine which GCA patients would really benefit from tocilizumab, how to monitor GCA activity during this treatment and how to stop tocilizumab without increasing the risk of relapse [51].

Sirukumab is a fully human anti-IL-6 IgG1 antibody that blocks the IL-6 pathway and has been shown to be effective in rheumatoid arthritis [52–55]. A phase-3 randomized, controlled, double-blind study was started to determine its efficacy and safety in GCA (NCT02531633) but has been recently cancelled.

b. TAK

As for GCA, several studies suggested that IL-6 plays a crucial role in TAK pathogenesis [5,6,56]. Particularly, IL-6 serum levels are higher in TAK patients than in controls and its concentration correlates with disease activity [57]. Furthermore, IL-6 expression is increased in TAK lesions and Th1 and Th17 cells are implicated in TAK pathogenesis [5,58]. The first description of the clinical efficacy of tocilizumab in TAK was reported in 2008 [59]. Among the 49 TAK patients reported by the French Takayasu Network, 14 received tocilizumab, 11 as the second-line therapy after failure of conventional immunosuppressants and three as the first-line therapy. During the follow-up, at least one switch to another biologic agent was required in 29% of cases. Even though the low number of patients and the retrospective design limited the power of this study, the authors found that the proportion of responders, the vascular complications and interventions, and relapse-free survival were similar between patients treated with tocilizumab and those receiving anti-TNF-α agents [32].

In a review published in 2016 [60], Koster et al. reported 70 cases of TAK patients treated with tocilizumab. The vast majority were treated with IV tocilizumab (8 mg/Kg every 4 weeks) for relapsing or refractory disease. Overall, 80% of patients showed a clinical and laboratory improvement at 3 months and < 20% experienced relapse during the treatment [60]. Importantly, several case series reported relapses occurring during treatment with tocilizumab characterized by radiographic worsening in the setting of normal acute-phase reactants [47–50]. These data are very important to highlight as they demonstrate that vascular lesions may progress despite normal acute-phase reactants in patients treated with tocilizumab in TAK. By blocking IL-6R, tocilizumab inhibits the IL-6-driven production of inflammatory proteins (especially fibrinogen and CRP) by hepatocytes. Therefore, CRP and fibrinogen are usually maintained at a very low level despite the fact that inflammation may still occur through other pathways. It is therefore crucial to find accurate biomarkers to monitor patients treated with tocilizumab. Today, these patients have to be closely monitored using clinical and vascular imaging [61]. Another challenge concerning the use of tocilizumab is related to its discontinuation. As in GCA and similarly to many other biologic agents, tocilizumab is not curative since relapses are frequent after its discontinuation [47,62].

The first results of a prospective randomized placebo-controlled trial evaluating tocilizumab in refractory TAK were reported in 2016 [63]. Thirty-six patients were included and randomized into two groups: treatment with tocilizumab (162 mg/week subcutaneously) or its placebo. For inclusion, patients had to be receiving a stable GC dose at ≥ twice the dose at relapse and to be in remission for 1 week before randomization. In both groups, prednisone was tapered by 10%/week from week 4. The primary endpoint was the time to occurrence of the first relapse, as defined by Kerr’s criteria [18]. After 1 year of follow-up, relapse-free survival tended to be improved in patients treated with tocilizumab but without reaching the level of significance (P = 0.596). The results of this first randomized controlled trial evaluating the efficacy of tocilizumab in TAK are therefore disappointing. However, they may be tempered by the fact that this trial involved only refractory TAK patients and was probably underpowered because of the low number of patients included [63].

Contrary to GCA [44–46], experience regarding the use of tocilizumab in newly diagnosed TAK is very limited [32,64,65]. A phase III, open-label study evaluating tocilizumab as a first-line therapy in TAK is currently recruiting (NCT02101333).

c. Conclusion

For the time being, blockade of the IL-6 pathways essentially relies on the use of tocilizumab, whose efficacy has been confirmed in GCA and needs more investigations in TAK. Even though tocilizumab appears to be extremely effective in GCA, several questions must be answered: which GCA patients would really benefit from tocilizumab, how to monitor GCA activity during treatment and how to stop tocilizumab so as to minimize relapses?

4. Abatacept (Fig. 1)

Before being polarized into Th1, Th2 or Th17 cells, CD4+ T cells are activated by antigen presenting cells (APC), which are mainly dendritic cells. T cell activation relies on the addition of several signals. The first signal is the result of the interaction of the T cell receptor (TCR) and the major histocompatibility complex (MHC)-II/peptide complex which is presented by APC. Then, the second signal depends on other molecular interactions between APC and T cells, in particular through co-stimulation molecules like CD80 and CD86, which are expressed by APC and link to CD28, which is expressed by T cells. This second signal leads to an autocrine production of IL-2 and expression of the high-affinity α-chain of the
IL-2 receptor (CD25) by T cells, which results in T-cell proliferation [66]. In order to physiologically control T cell activation, CTLA-4 is expressed by effector T cells 72 h after their activation. The affinity of CTLA-4 for CD80/86 is higher than that for CD28 and leads to T-cell inhibition and contraction. Abatacept is a fusion protein composed of the crystallizable fragment of a human IgG1 and the extracellular domain of CTLA-4. Abatacept therefore binds to CD80/86, thus blocking its interaction with CD28, which decreases T cell activation.

a. GCA

A multicentre, double-blind, randomized placebo-controlled trial evaluated abatacept for the treatment of GCA. In this study, all patients received remission induction therapy with prednisone and abatacept (10 mg/Kg intravenously at day 1, 15, 29 and week 8). After 3 months of treatment, patients who achieved remission continued with a standardized prednisone taper (prednisone was stopped at week 28) and were randomized to receive either abatacept (10 mg/Kg/28 days) or its placebo. A total of 41 patients (44% relapsing GCA) were randomized. Relapse-free survival at 12 months was better in patients receiving abatacept than in those receiving placebo (48% vs. 31%; P = 0.049). Furthermore, no difference was observed between the treatment arms concerning the frequency or severity of adverse events [67]. Although significant in this study, the effect of abatacept seems to be moderate and should be confirmed in larger studies.

b. TAK

A multicentre, double-blind, randomized placebo-controlled trial evaluated abatacept for the treatment of TAK [68]. The study design was similar to the one for the study involving GCA patients [67]. A total of 26 TAK patients, including four newly diagnosed patients (all in the placebo group) were randomized 3 months after induction therapy with prednisone and abatacept. At 12 months, relapse-free survival was similar in patients treated with abatacept and those receiving placebo (22% vs. 40%, respectively; P = 0.853) [68], which does not support the use of abatacept in TAK.

5. Other biologics (Fig. 1)

a. Ustekinumab

Recent advances in our understanding of the pathophysiology of GCA showed that after dendritic cell activation in the adventitia, CD4+ T cells are recruited in the arterial wall and are critical for vasculitis induction [69]. T cells are polarized into Th1 and Th17 cells, which produce IFN-γ and IL-17, respectively [35,70,71]. IFN-γ triggers the recruitment and activation of macrophages and CD8+ T cells [72,73], and could be implicated in the occurrence of relapses when GC are tapered [74]. In addition, IL-17 and IFN-γ activate macrophages, giant cells and smooth muscle cells thereby inducing vascular remodelling and leading to ischemic manifestations of GCA [7]. It is thus necessary to target both Th17 and steroid-resistant Th1 responses to totally resolve chronic smouldering vasculitis.

IL-12 and IL-23 are two key cytokines involved in Th1 and Th17 polarizations, respectively [75-77]. Furthermore, these two cytokines share a common subunit (p40), which allows ustekinumab, a humanized anti-p40 monoclonal antibody, to target both IL-12 and IL-23 pathways, thus disrupting the Th1 and Th17 immune responses [78,79]. An open-label study recently reported on the efficacy of ustekinumab for the treatment of 14 patients with refractory GCA, which was defined as an inability to taper GC to < 10 mg/day due to symptoms of active GCA with a minimum of two relapses. In this study, ustekinumab was prescribed at 90 mg subcutaneously at week 0, week 4 and then every 12 weeks and allowed a significant decrease in the dose of prednisolone (from 20 to 5 mg/day; P = 0.001). Six adverse events occurred during the study: one case each of urinary tract infection, tinea pedis, dental abscess, lower respiratory tract infection, alopecia and non-dermatomal limb paraesthesia. Three patients stopped ustekinumab due to adverse events, two of whom subsequently had flares of polymyalgia rheumatica [80].

A prospective comparative study is thus necessary to conclude about the efficacy and safety of ustekinumab in GCA.

The role of the Th1 and Th17 axis in TAK [58] and the identification of the IL-12B gene region as a TAK susceptibility gene [81,82] suggest that IL-12 and IL-23 are implicated in the pathogenesis of TAK [5,6]. The use of ustekinumab in TAK was reported in three patients with active TAK despite treatment with GC and immunosuppressants. Ustekinumab was administered at 45 mg subcutaneously at day 0 and day 28. The authors evaluated clinical, laboratory and MRI findings at day 0 and day 84, and showed an improvement in inflammatory markers but without suppression of vascular lesions, which could actually be related to the low dose and short duration of the treatment. Further studies are needed to decipher how effective ustekinumab could be in TAK.

b. Anti-IL-1β therapies

IL-1β mRNA expression is increased in temporal arteries from GCA patients with a strong inflammatory syndrome [21]. Besides, IL-1rn−/− mice, which lack the antagonist of IL-1β (IL-1Ra), show a marked increase in concentrations of IL-1β and suffer from arthritis and arteritis with vasculitis lesions that are close to those observed in GCA, but without giant cells [83].

Anakinra (IL-1Ra) is an IL-1β antagonist which has been successfully used at a dose of 100 mg/day in three refractory GCA patients [84]. A phase-III study will be conducted to address the efficacy of this treatment in GCA (NCT02902731).

Gevokizumab is a recombinant humanized anti-IL-1β antibody, which is also currently being tested in GCA (European Clinical Trials Database identifier 2013–002778–38).

Concerning TAK, the use of anakinra or gevokizumab has never been reported in the literature.

c. Rituximab

Rituximab is a chimeric anti-CD20 monoclonal antibody that induces a depletion of B cells. Experience with this treatment in GCA is limited to two case reports [85,86]. Furthermore, considering that B cells may not play a major role in GCA pathogenesis [7] and that other biologics are more attractive, rituximab does not seem to be an appropriate therapy for GCA.

The pathogenesis of TAK involves different pathways from those implicated in GCA, maybe with a greater involvement of B cells [6]. Along this line, rituximab has been used in a few patients with active TAK. Pazzola et al. recently reported on seven patients (six refractory TAK and one newly diagnosed TAK) treated with rituximab (two infusions of 1000 mg, 15 days apart). Follow-up included a clinical evaluation, laboratory tests and imaging (CT or MR-angiography, and 18fluorodeoxyglucose PET/CT) at the first rituximab administration and then every 6 months. Disease activity was assessed using Kerr's index. Patients received one to four courses of rituximab and follow-up ranged from 12 to 60 months. Despite rituximab therapy, 4/7 patients had evidence of persistent disease activity and/or radiographic disease progression, whereas the remaining three patients achieved complete remission [87]. In this paper, the authors also provided a literature review identifying five papers describing nine additional patients treated with rituximab with a good response in eight cases [87]. They thus concluded that rituximab can be discussed as a second- or third-line biologic therapy.
6. Other therapeutic targets (Fig. 1)

a. Baricitinib

Baricitinib is an orally administered inhibitor of Janus kinase (JAK) 1 and JAK2 [88], which are tyrosine kinases involved in the signalling of many cytokines [89]. Baricitinib is thus a potential inhibitor of the Th17 (IL-6, IL23) and Th1 (IL-12, IFN-γ) pathways, which could be of particular interest for the treatment of GCA. A phase-II open-label pilot study is currently recruiting relapsing GCA patients (NCT030226504).

b. Inhibition of vascular remodelling

In contrast to inflammation, vascular remodelling is rarely targeted by conventional GCA therapy. However, vascular remodelling is a key process in the pathogenesis of both GCA and TAK. It induces progressive stenosis and occlusion of the vascular lumen, which is responsible for the ischaemic complications of these diseases [5,7]. Vascular remodelling is therefore the main source of morbidity and mortality in GCA and TAK and may need to be blocked in addition to anti-inflammatory treatments. The blockades of the PDGF pathway by imatinib [90] and of endothelin-1 receptors are therapies that have been shown to inhibit vascular remodelling. However, their use is limited to in vitro or ex vivo studies [91–94].

7. Conclusion (Fig. 2)

GC remain the cornerstone of the treatment of GCA and TAK. Conventional immunosuppressive drugs (methotrexate in GCA; methotrexate, azathioprine, mycophenolate mofetil or leflunomide in TAK) are still used as second-line therapies if severe GC-related side-effects are expected or in cases of corticosteroid dependence or relapse[6]. Therefore, biologics often remain as second- or third-line therapies in GCA. Tocilizumab and abatacept are effective, but more data are required for ustekinumab and anti-IL-1β therapies, and anti-TNF-α agents are not effective. In TAK, data from prospective controlled studies are lacking but anti-TNF-α agents, mainly infliximab, is probably the best choice. Abatacept is not effective and further data are required to draw any conclusions concerning the efficacy of tocilizumab.

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References


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Sovint quan un intenta reproduir un experiment en base a la metodologia d’un article les coses no acostumen a sortir com un esperaria. Possiblement perquè en l’estalvi de paraules s’han perdut també tots els tips i recomanacions imprescindibles per tenir èxit experimental. Aquesta secció conté precisament això, el que realment hi ha sota les gràfiques i resultats, tots els trucs que han fet possible el desenvolupament d’aquesta tesi. En la meva sort aquests “trucs” tenen nom.

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