TRANSLATIONAL SCIENCE

Senescent cells in giant cell arteritis display an inflammatory phenotype participating in tissue injury via IL-6-dependent pathways

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ABSTRACT

Objectives Age is the strongest risk factor of giant cell arteritis (GCA), implying a possible pathogenetic role of cellular senescence. To address this question, we applied an established senescence specific multimarker algorithm in temporal artery biopsies (TABs) of GCA patients.

Methods 75(+) TABs from GCA patients, 22(−) TABs from polymyalgia rheumatica (PMR) patients and 10(−) TABs from non-GCA/non-PMR patients were retrospectively retrieved and analysed. Synovial tissue specimens from patients with inflammatory arthritis and aorta tissue were used as disease control samples. Senescent cells and their histological origin were identified with specific cellular markers; IL-6 and MMP-9 were investigated as components of the senescent associated secretory phenotype by triple costaining. GCA or PMR artery culture supernatants were applied to fibroblasts, HUVECs and monocytes with or without IL-6R blocking agent to explore the induction of IL-6-associated cellular senescence.

Results Senescent cells were present in GCA arteries at higher proportion compared with PMR (9.50% vs 2.66%, respectively, p<0.0001) and were mainly originated from fibroblasts, macrophages and endothelial cells. IL-6 was expressed by senescent fibroblasts, and macrophages while MMP-9 by senescent fibroblasts only. IL-6(+) senescent cells were associated with the extension of vascular inflammation (transmural inflammation vs adventitia limited disease: 10.02% vs 4.37%, respectively, p<0.0001). GCA but not PMR artery culture supernatant could induce IL-6-associated senescence that was partially inhibited by IL-6R blockade.

Conclusions Senescent cells with inflammatory phenotype are present in GCA arteries and are associated with the tissue inflammatory bulk, suggesting a potential implication in disease pathogenesis.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The role of cellular senescence in giant cell arteritis (GCA) pathogenesis is poorly investigated.

WHAT THIS STUDY ADDS

⇒ Senescent cells are present in GCA arteries bearing an inflammatory phenotype that may mediate IL-6 and MMP-9 depended tissue injury.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Detection of senescent cells with inflammatory phenotype in GCA temporal artery biopsies may serve as a potential biomarker for therapeutic implications.

INTRODUCTION

Giant cell arteritis (GCA) is a chronic autoimmune disease, characterised by remarkable heterogeneity of the clinical phenotype and histological pattern, reflecting the complexity of the underlying pathogenetic mechanisms which remain unclear.[1 2] The histopathological hallmark of the disease is the pronounced inflammation of the vascular wall, mediated by different cell types including dendritic cells, T lymphocytes, monocytes, macrophages, endothelial cells and fibroblasts. The interplay is mediated by a variety of proinflammatory cytokines as well as growth and angiogenic factors that promote inflammation and abnormal tissue remodelling within the vascular wall.[3] Nevertheless, very few cytokines have been proven to play an important role in GCA pathogenesis (eg, IL-6, IL-17A and granulocyte-macrophage colony-stimulating factor (GM-CSF)) as supported by previous randomised control trials.[4 5] However, GCA patients may be refractory or relapse even after the introduction of novel treatments, pointing out that the major players of vascular inflammation have been only partially recognised.

GCA affects adults over 50 years old and among other risk factors, age is the strongest for GCA development.[6 7] Vascular ageing in GCA has been associated with structural changes and altered immune responses,[8] leading to the assumption

that cellular senescence as part of the normal ageing of vessels, is anticipated to be involved in disease pathogenesis. Cellular senescence is a state characterised by cell cycle arrest, deregulated metabolism, macromolecular damage and a specific secretory phenotype termed SASP (senescence associated secretory phenotype).[9 10] The SASP includes proinflammatory cytokines, chemokines, metalloproteinases, angiogenic and growth factors implicated in wound healing, tissue plasticity and chronic inflammation. The inflammatory mediators of SASP (eg, IL-6, IL-1β, TNF-α) may act in an autocrine and paracrine manner to induce and spread senescence,[11] perpetuating the inflammatory process through a local positive feedback loop. Although the phenomenon of senescence has been well studied *in vitro* by detecting SA-β-gal, *in vivo* studies of human tissues require fresh biological material (non-fixed) that renders the SA-β-gal approach ineffectual. On the other hand, the widely used approach to study senescence based on $p16^{INK4A}$ and $p21^{WAF/Cip1}$ molecular pathways has led to misleading results, since several non-senescent conditions may be linked to upregulation of these particular pathways.[12 13] To overcome these obstacles, we developed GL13 (a biotinylated Sudan Black-B) analogue that strongly interacts with lipofuscin, a hallmark of all senescent cells.[9 14 15] GL13 application can identify senescence in any biological material including formalin-fixed and paraffin embedded (FFPE) and is compatible with costaining for other senescence markers.[14] Lipofuscin staining with GL13 has been adopted by the International Cell Senescence Association in the consensus guideline multimarker algorithm.[9 14] According to the proposed algorithm, screening for senescence using lipofuscin staining should precede, followed by verification via absence of key proliferation markers (eg, Ki67) and expression of hallmark senescence markers ($p16^{INK4A}$, $p21^{WAF1/Cip1}$).[9 15] This novel strategy has offered the opportunity to identify senescent cells in human diseases such as Hodgkin's lymphoma and COVID-19.[16–18]

Studies on senescence in GCA are limited, focusing on either autoreactive senescent T cells[19] or relevant molecular pathways while few microRNAs proposed to be involved in inflammatory and senescence processes, are upregulated in tissue artery biopsies (TABs) of GCA patients.[20 21] In a recent study, $p16^{INK4A}$ and $p21^{WAF/Cip1}$ upregulation have been described in tissue specimens of GCA patients, although senescent cells were not detected.[22] The current study aims to identify the various senescent cell types in inflamed temporal arteries of GCA and polymyalgia rheumatica (PMR) patients, by applying the novel multimarker algorithm, define the SASP key molecules and explore possible implications of senescence in GCA pathogenesis.

Patients and methods

Overall, 75 GCA and 22 PMR patients, who were diagnosed and followed up in 3 rheumatology centres from Greece and Italy (University of Athens, Sismanoglio General Hospital, Reggio Emilia Hospital) between 2009 and 2022, were retrospectively included in the current study. All participants underwent temporal artery biopsy as part of standard of care and fulfilled the international classification criteria for each disease.[23 24] Almost all patients were newly diagnosed (94.3%), and all TABs were performed at disease diagnosis or relapse. TABs were stained by immunohistochemistry (IHC) or immunofluorescence (IF) to detect senescent cells, their histological origin and the components of SASP. As control samples, we used synovial tissue from inflammatory arthritis (IA) patients (n=6), non-inflammatory aortic aneurysmal tissue (n=3) and temporal artery biopsies from non-GCA/non-PMR individuals (n=10). To investigate if artery culture supernatant from GCA or PMR patients has the capacity to induce IL-6 associated cellular senescence, an *ex vivo* short-term artery tissue culture system was developed to collect the 24 hours supernatant from GCA or PMR arteries and primary skin fibroblasts, endothelial cells and monocytes were treated for 3 or 5 days with the 24 hours temporal artery culture supernatant with or without blocking of IL-6 or IL-1β signaling.[25] A more detailed description of methods and protocols used in the present study are provided in online supplemental material.

Statistical analysis

For continuous variables, two-tailed Student's t-test or Mann-Whitney test was used, after implementing the Shapiro-Wilk normality test. For comparing more than two groups, one-way analysis of variance with Bonferroni post hoc or Kruskal-Wallis was performed accordingly. Associations between continuous variables were explored by Pearson's correlation and between continuous and categorical by point–biserial correlation. All data were analysed using GraphPad Prism V.9.0 software. Data are presented as mean±SD. Differences were considered statistically significant at $p<0.05$.

RESULTS

Senescent cells are present in GCA arteries

The clinical and laboratory features of GCA, PMR and IA patients are presented in online supplemental table S1,S2. To detect senescent cells in TABs of GCA and PMR patients, the multimarker algorithm that is widely-accepted was applied.[9 15] Initially, GL13 staining was performed immunohistochemically in 75 GCA and 22 PMR tissue specimens, followed by immunofluorescence in 15 GCA and PMR TABs. Liver tissue specimens were used as control samples (online supplemental figure 1A,B). GL13 positive senescent cells were detected in all TABs of GCA patients with both techniques, as opposed to PMR patients, in whom senescent cells were almost absent (figure 1A). To quantify senescent cells, GL13(+) cells were calculated immunohistochemically, as a proportion of the total number of cells within TABs and after careful exclusion of lymphocytes and granulocytes (<1% positivity) in TABs of GCA patients. It was found that TABs of GCA patients (n=75) contained significantly higher proportion of senescent cells (range: 0.8%–25%) compared with PMR patients (n=22, range: 0%–6.8%) (mean proportion of GL13 positive cells: 9.50% vs 2.66%, $p<0.0001$) (figure 1B). Using as control samples synovial tissue from IA patients (n=6), non-inflammatory aortic aneurysmal tissue (n=3) and TABs from non-GCA/non-PMR individuals (n=10), we found that senescent cells were statistically increased only in GCA TABs (figure 1B, online supplemental figure C,D). The finding of senescent cells in GCA arteries was further confirmed, by costaining with GL13 and $p21^{WAF1/Cip1}$ or Ki67 (n=8, for both GCA and PMR group) (figure 1C–F). GL13 (+)/$p21^{WAF1/Cip1}$(+) and GL13(+)/Ki67(−) cells were significantly increased in GCA tissues compared with PMR ($p<0.01$ and $p<0.001$, respectively) (figure 1C–F). Transmission electron microscopy (TEM) analysis in two randomly selected samples from both groups showed that TABs from GCA patients exhibited high lipofuscin accumulation (figure 1G) in contrast to sparse levels in PMR samples (figure 1Hi,ii). Ultrastructural study of the arteries showed in GCA patients that lipofuscin granules were mainly distributed nearly and around the nucleus (figure 1Gi) or scattered in the cytoplasm of cells (fibroblasts) (figure 1Gii). Interestingly, bulk RNAseq analysis of already published data[26] from TABs of GCA and non-GCA controls, was in line with our findings showing

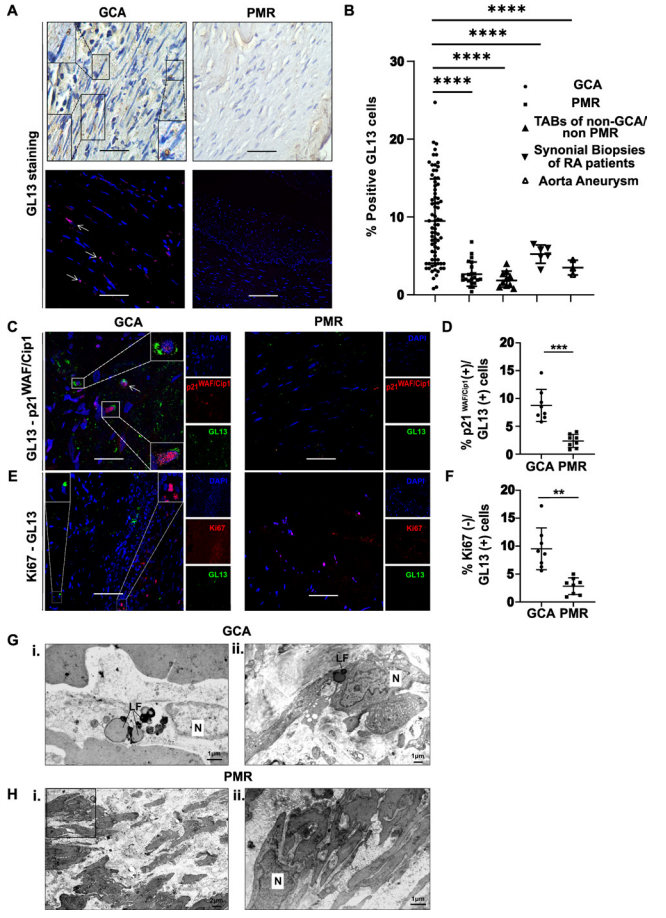

Figure 1 Detection of senescent cells in tissue artery biopsies of GCA patients. (A) Representative images for single GL13 staining with immunohistochemistry and immunofluorescence in TABs of GCA and PMR patients, showing higher proportion of GL13 positive cells adjacent to the inflammatory cells in GCA arteries compared with PMR. (B) Graphical representation and comparison of the proportion of GL13 positive senescent cells, after immunohistochemical staining between 75 GCA and 22 PMR, 10 non-GCA/non-PMR TABs, 6 IA-synovial biopsies and 3 aortic aneurysm tissues (9.50% vs 2.66% vs 2.01% vs 5.23% vs 3.5%, respectively) p<0.0001. (C) Confirmation of senescent cells by costaining for GL13 (cytoplasmic) and $p21^{WAF1/CIP1}$ (nuclear) in a TAB of a GCA compared with a PMR patient (representative image). (D) Graphical representation of the proportion of $p21^{WAF1/Cip1}$/ GL13 double positive senescent cells in 8 GCA and 8 PMR TABs (GCA vs PMR): 9.52% vs 2.837%, p<0.001. (E) Verification of senescent cells by costaining for GL13 and Ki67 in a TAB of a GCA compared with a PMR patient (representative image). (F) Graph shows the percentage of Ki67(–)/GL13(+) positive senescent cells in 8 GCA and 8 PMR TABs (GCA vs PMR): 8,76% vs 2.36%, p<0.001. (G) Electron micrographs of senescent cells in artery of GCA patients showing lipofuscin (LF) granules in their cytoplasm (Gi,ii). (H) Electron micrographs of cells in artery of PMR patient without lipofuscin granules (Hi,ii). For qualitative assessment, 30 fields of each PMR and GCA cases were assessed; in 27 of 30 fields lipofuscin was detected in GCA cases. Only in 2 of 30 fields, lipofuscin was present in these 2 PMR cases. Higher magnification of the area in the black box of (Hi). N: nucleus. Staining: uranyl acetate/ lead citrate. Objectives (A, E) ×20, (C) ×63. Scale bars: 50 µm (A) 10 µm (C) (E) 1µm (Gi,ii, Hii), 2 µm (Ei). **p<0.01, ***p<0.001, ****p<0.0001. Colour code: (A) GL13: magenta, (C) DAPI: blue, $p21^{WAF1/Cip1}$: red, GL13: green, (C) DAPI: blue, Ki67: red, GL13: green. GCA, giant cell arteritis; IA, inflammatory arthritis; PMR, polymyalgia rheumatica; TAB, temporal artery biopsies.

upregulation of genes and molecular pathways linked to cellular senescence. Components of SASP such as MMP-9, CXCL10, TGFB1 were significantly upregulated and positive regulators of cell cycle including HES1, ADAMTS1, SIN3A and KIF23 were significantly downregulated (online supplemental figure 2A–C). However, the fact that in that study TABs were removed postmortem, may have influenced the validity of results. No correlation was found between the proportion of senescent cells in tissue specimens and the levels of CRP, ESR, the histological pattern (transmural vs limited to adventitia), patients' age, disease phenotype (cranial vs extracranial) or the occurrence of ocular manifestations in GCA patients (online supplemental figure 3A–D). Since all TABs were performed within 10 days from the onset of symptoms, no correlation could be made between symptom duration and proportion of senescent cells.

Senescent cells in GCA arteries are mainly originated from fibroblasts, macrophages and endothelial cells

Next, the origin of senescent cells was investigated by carrying out costaining with GL13 and a specific marker for different cell types each time, in TABs of 13 GCA and 13 PMR patients. Double positive GL13/Vimentin (senescent fibroblasts), GL13/CD68 (senescent macrophages) and GL13/CD34 (senescent endothelial cells) cells were mainly observed in TABs of GCA patients compared with PMR patients (figure 2A–C). Additionally, double positive GL13/aSMA cells (senescent smooth cells) were hardly detected exerting very low/sparse labelling indices in GCA and PMR tissue samples (figure 2D). The proportion of senescent cells per cell type was calculated as the fraction of double positive cells over the total population of the specific cell type. The proportion of senescent fibroblasts, senescent macrophages and senescent endothelial cells was higher in TABs of 13 GCA patients compared with 13 PMR patients (29.6% vs 2.16% p<0.001, 16.23% vs 1.7% p<0.01 and 14.53% vs 1.1% p<0.05, respectively) (figure 2E). Smooth muscle (aSMA(+)/Desmin(+)/Vimentin(−)) and endothelial (vWF (+)/CD34(+)) cells were also identified by additional markers in serial section analysis of GCA (n=3) and PMR (n=3) TABs (online supplemental figure 4A,B). The majority of senescent endothelial cells were located mainly in vasa vasorum and fewer in neovessels and luminal endothelium (online supplemental figure B-C). It should be noted that the comparison of senescent macrophages between GCA and PMR TABs is difficult, since non-inflamed tissues typically contain very few macrophages. Interestingly, senescent fibroblasts in TABs of GCA patients were mainly observed in tunica media.

Senescent cells of GCA arteries demonstrate a SASP phenotype encompassing IL-6 and MMP-9

To assess the SASP in TABs of GCA patients, triple staining in tissue specimens from 13 GCA and 13 PMR patients was applied: GL13, a specific cell type biomarker (Vimentin(+), CD68(+), CD34(+) or aSMA(+)) and IL-6 a key molecule in GCA pathogenesis. IL-6 was mainly expressed by senescent fibroblasts and to a lesser extent by senescent macrophages in GCA arteries (figure 3A,B) while senescent endothelial cells exhibited very low expression of IL-6 (figure 3C). IL-6 expression was hardly observed by senescent smooth muscle cells in both GCA and PMR tissue specimens (figure 3D). Quantification of the proportion of IL-6(+) senescent cells in TABs of 13 GCA compared with 13 PMR patients, showed higher rates of IL-6(+) senescent macrophages (7.76% vs 0.33% p<0.05, respectively) and senescent

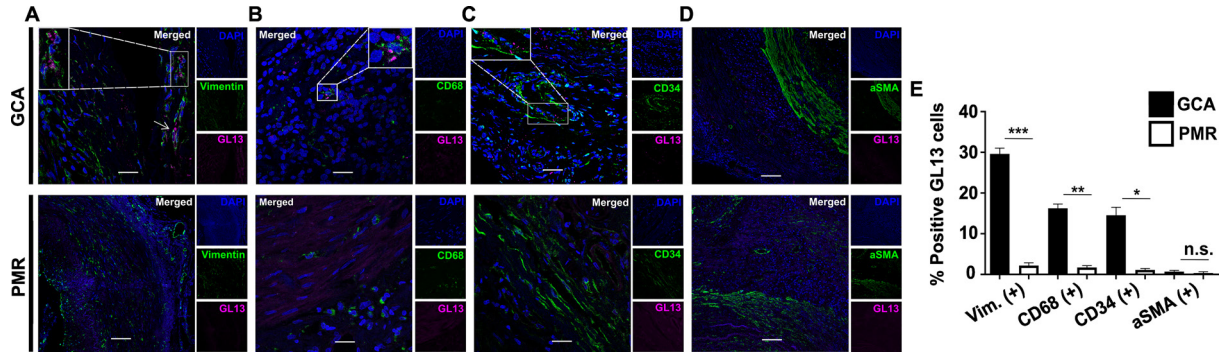

Figure 2 Characterisation of senescent cells per cell type in tissue artery biopsies of GCA patients. Representative images of double positive senescent cells after staining for GL13 and (A) Vimentin(+) (fibroblasts), (B) CD68(+) (macrophages), (C) CD34(+) (endothelial cells) or (D) aSMA(+) (smooth muscle cells) in a TAB of one GCA and one PMR patient (double-staining immunofluorescence). Squares depict magnified cells with presence of double positive staining. (E) Quantification analysis by two independent observers, showing significantly higher proportion of senescent fibroblasts (GL13(+)/Vimentin(+)), macrophages (GL13(+)/CD68(+)) and endothelial cells (GL13(+)/CD34(+)) in 13 GCA compared with 13 PMR biopsies. Objective ×20. Scale bars: 50 µm. n.s. non significant, *p<0.05, **p<0.01, ***p<0.001. Colour code: DAPI: blue, GL13: magenta, Vimentin- CD34-CD68- aSMA: green. GCA, giant cell arteritis; PMR, polymyalgia rheumatica.

fibroblasts (21.96% vs 0.73%, p<0.01, respectively). No statistically significant difference was found between GCA and PMR specimens regarding the proportion of IL-6(+) senescent smooth muscle cells and senescent endothelial cells (figure 3E). Furthermore, serial section analysis in PMR (n=3) and GCA TABs (n=3), revealed coexpression of IL-6 and MMP-9 in GL13(+) cells in GCA as opposed to PMR (online supplemental figure 5A–B). Given the role of metalloproteinases in tissue remodelling, the expression of MMP-9 by senescent fibroblasts only, in three TABs from GCA and three from PMR patients, was explored. Lymph node and liver tissue from a young individual were applied as positive and negative control samples, respectively, for IL-6 and MMP-9 (online supplemental figure 5C–D). Staining of GCA samples for MMP-9 or IL-6 solely revealed a diffused pattern corresponding to many cell types (online supplemental figure 5E). MMP-9 was almost exclusively observed on GL13/Vimentin double positive cells of GCA

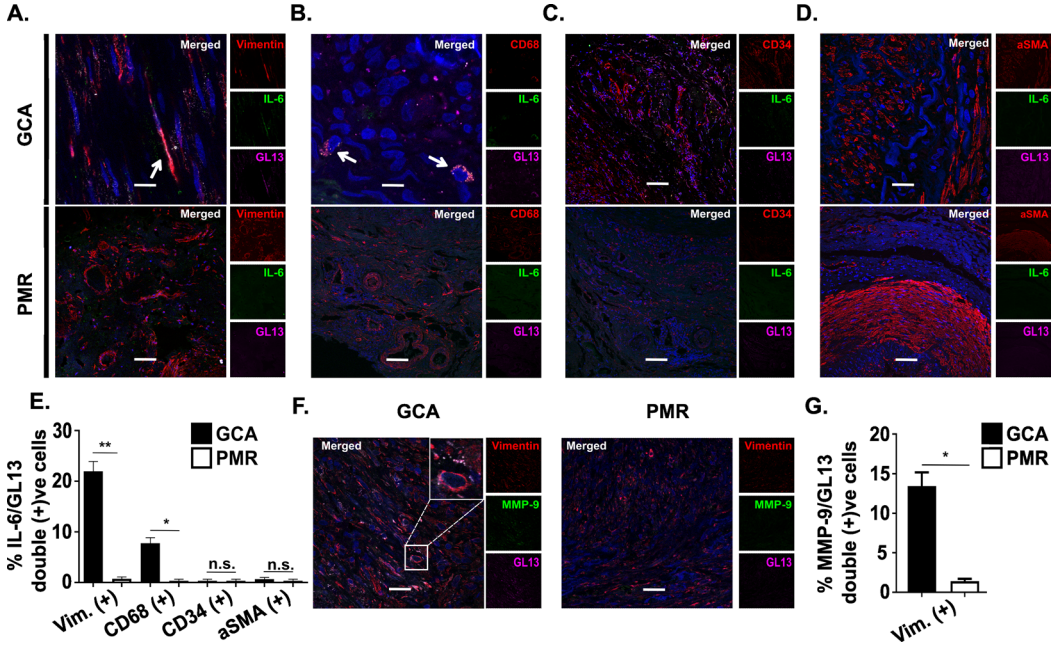

Figure 3 Senescent cells in GCA express SASP that includes IL-6 and MMP-9. Representative images of IL-6 expression by senescent cells of different origin: (A) GL13(+)/Vimentin(+) (senescent fibroblasts), (B) GL13(+)/CD68(+) (senescent macrophages), (C) GL13(+)/CD34(+) (senescent endothelial cells) and (D) GL13(+)/aSMA(+) (senescent smooth muscle cells) in a TAB of n=10 GCA and n=10 PMR patients (triple staining immunofluorescence). (E) Graphical representation of the proportion of IL-6 positive senescent cells in 10 GCA and 10 PMR biopsies, showing significantly higher proportion of IL-6 positive senescent fibroblasts (GL13(+)/Vimentin(+)) and macrophages (GL13(+)/CD68(+)). Representative images of MMP-9 expression by GL13/Vimentin double positive (senescent fibroblasts) cells in a TAB of one GCA and one PMR patient (triple staining immunofluorescence). (F, G) Graphical representation showing higher proportion of MMP-9 positive senescent fibroblast (GL13(+)/Vimentin(+)) in the three GCA compared with three PMR specimens. Objectives: all panels were obtained with x20 except (A) and (B) GCA panels with x63. Scale bars: all panels were 50 µm, except (A) and (B) GCA at 10 µm. n.s.: not significant, *p<0.05, **p<0.01. Colour code: DAPI: blue, aSMA-CD34-CD68-Vimentin: red, IL-6-MMP-9: green, GL13: magenta. Arrows depict cells with co-localisation of the respective markers. GCA, giant cell arteritis; PMR, polymyalgia rheumatica; SASP, senescent-associated secretory phenotype; TAB, temporal artery biopsies.

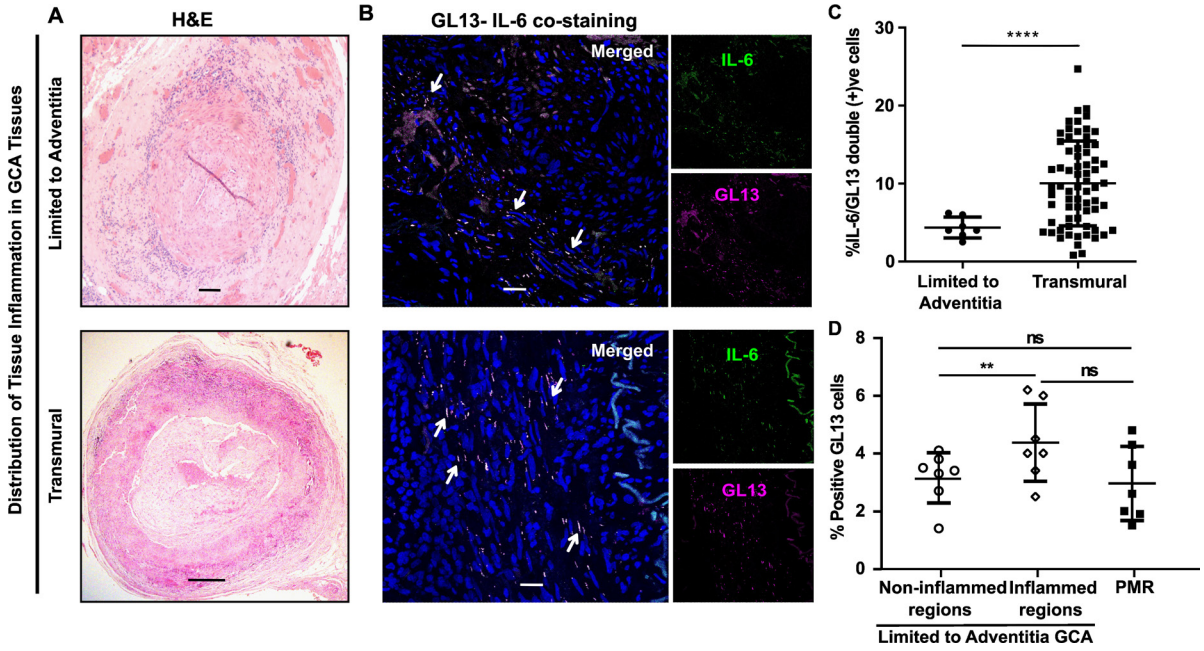

Figure 4 IL-6 positive senescent cells in GCA biopsies of patients with limited or extended disease. (A) Representative images of TAB from GCA patient with disease limited to adventitia (upper panel) and with extended transmural inflammation (lower panel) (H&E). (B) Double positive IL-6/GL13 senescent cells are observed more abundantly in extended disease (lower panel) compared with limited disease (representative images from one GCA patient from each group, immunofluorescence). (C) Graphical representation of the proportion of IL-6 positive senescent cells in 7 GCA patients with limited vs 68 patients with extended disease. (D) Graphical representation of the proportion of senescent cells as indicated by GL13 positivity in seven GCA TABs at the site of inflammation (adventitia), adjacent non-inflamed regions and in seven PMR TABs. Positive staining for each panel was quantified by two independent observers. Objectives (A) ×5 (upper), ×4 (lower), (B) ×20. Scale bars: (A) 100 µm, (B) 50 µm. **p<0.01, ****p<0.0001. Colour code: DAPI: blue, IL-6: green, GL13: magenta. Arrows depict cells with co-localisation of the respective markers. GCA, giant cell arteritis; ns, non-significant; PMR, polymyalgia rheumatica; TAB, temporal artery biopsies.

arteries (figure 3F) and the proportion of MMP-9(+) senescent fibroblasts was higher in GCA specimens compared with PMR (13.43% vs 1.4% p<0.05) (figure 3G).

The presence of IL-6 positive senescent cells is associated with the extent of inflammation in GCA arteries

To explore the association of IL-6 associated senescent cells with the extent of the inflammatory process in GCA arteries, we compared 7 patients with disease limited to the adventitia (limited disease) and 68 patients with transmural inflammation (extended disease) (figure 4A). GL13(+)/IL-6(+) cells were more prominent in GCA patients with extended disease compared with those with limited disease (figure 4B) and after quantification, the proportion of IL6(+) senescent cells was found significantly higher in the former group (10.02% vs 4.37%, p<0.0001) (figure 4C). The non-inflamed regions within the artery wall of GCA patients with limited to adventitia disease, exhibited similar levels of senescence when compared with corresponding tissues from PMR vessels (4.08% vs 2.95, ns) (figure 4D).

GCA artery culture supernatant triggers senescence phenotype in primary fibroblasts, endothelial cells and monocytes: significant inhibition by IL-6 signalling blockade

To investigate if the microenvironment of GCA arteries carries the capacity to induce IL-6-associated cellular senescence, we treated primary skin fibroblasts from young and aged donors for 5 days, endothelial cells (HUVECs-Human umbilical vein endothelial cells) and monocytes for 3 days with 24 hours temporal artery culture supernatant from: three patients with GCA, three with PMR (disease controls) and two normal arteries from healthy individuals served as normal controls (Ctr). Senescent cells were detected by multi staining for GL13, Ki67 or $p21^{WAF1/Cip1}$ and IL-6. Inhibition experiments were also performed by applying IL-6 or IL-1β signalling blocking agents (figure 5A). Time dependent preliminary experiments in which fibroblasts were treated with aorta culture supernatant from one GCA patient with aortitis, showed the optimal time (5 days) to detect cellular senescence (online supplemental figure 6A). Interestingly, fibroblasts treated with sera from GCA or PMR patients for 5 days displayed no cellular senescence (data not shown). GL13(+)/ $p21^{WAF1/Cip1}$(+)/IL-6(+) fibroblasts from young donor, GL13(+)/IL-6(+) monocytes and GL13(+)/IL-6(+)/Ki67(−) HUVECs were mainly observed following treatment with GCA supernatants while cellular senescence was minimal or absent on incubation with PMR or control artery supernatants (figure 5B–G). Senescence was further confirmed by SA-β-Gal staining (online supplemental figure 6B–C). The proportion of GL13(+)/$p21^{WAF1/Cip1}$(+)/IL-6(+) fibroblasts, GL13(+)/IL-6(+) monocytes and GL13(+)/IL-6(+)/Ki67(−) HUVECs after treatment with GCA supernatants was significantly higher compared with PMR supernatants (young donor: 54.2% vs 5%, p<0.001, monocytes: 41.16% vs 14.14%, p<0.1, HUVECs: 42.2% vs 10.33%, p<0.01) (figure 5C, E, G). The 24 hours temporal artery culture supernatant from GCA and PMR had comparable levels of IL-6 but much higher compared with IL-6 serum levels, respectively (online supplemental figure 6D–E). Next, we blocked IL-6 and IL-1β signalling with tocilizumab and anakinra as described previously,[27 28] before treating fibroblasts from young and aged donor as well as HUVECs with GCA temporal artery culture supernatants. Induction of IL-6 associated cellular senescence was significantly inhibited by IL-6 receptor blockade (12.00% vs 32.17%, p<0.001) and to a lesser extent by IL-1β

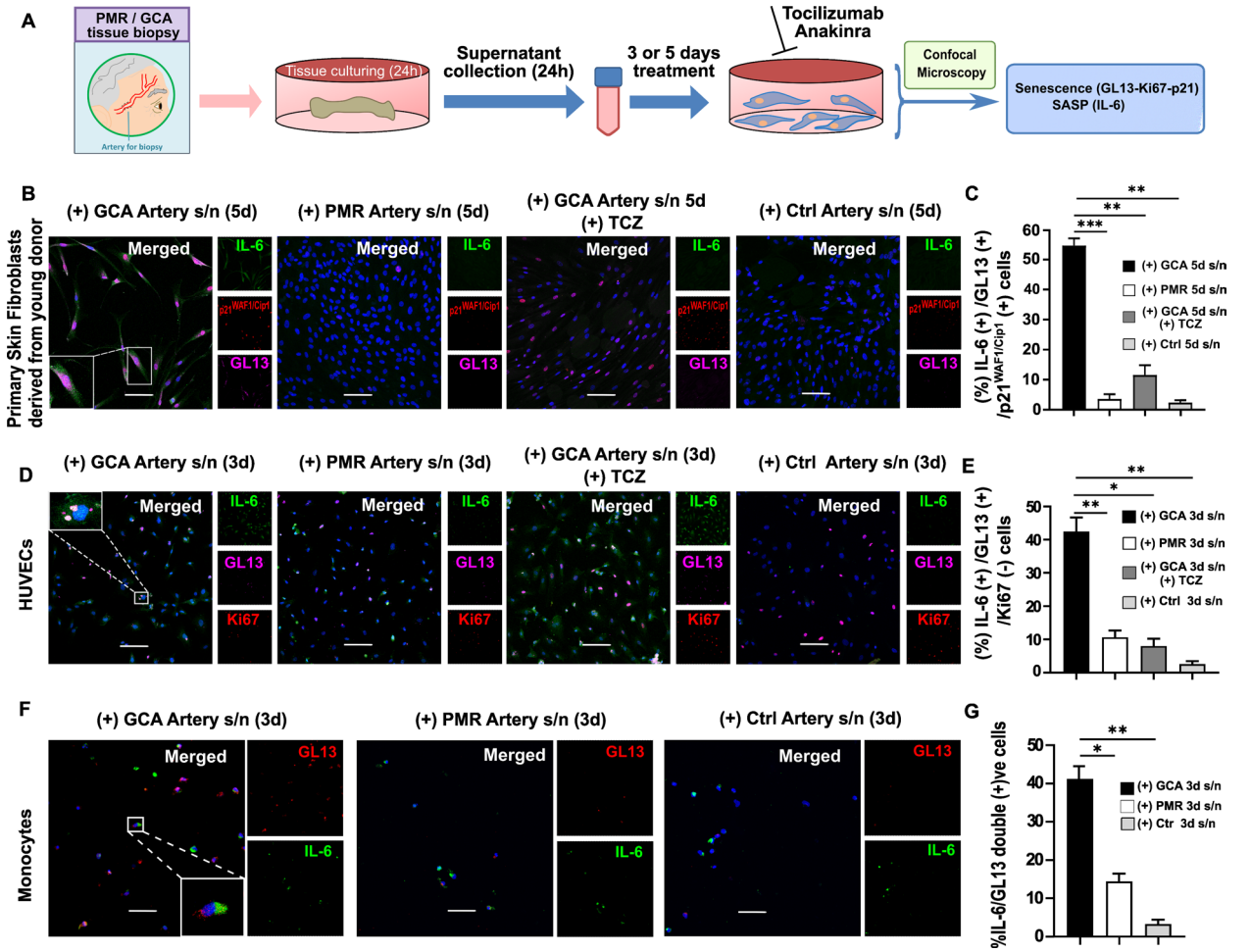

Figure 5 IL-6 associated cellular senescence induced by GCA temporal artery culture supernatant and partial inhibition by IL-6 blockade. (A) Workflow of experimental procedure. (B) Representative images from GL13/IL-6/p21$^{WAF1/Cip1}$ costaining of primary skin fibroblasts derived from young donor treated with GCA, GCA (+) TCZ, PMR and healthy artery tissue culture supernatants for 5 days, respectively. GL13/IL-6 positive senescent fibroblasts are mainly evident following treatment with GCA supernatant. (C) Graphical representation of the percentage of triple positive GL13/IL-6/p21$^{WAF1/Cip1}$ fibroblasts treated with GCA (n=3) (53% of total senescent fibroblasts), PMR (n=3) or control supernatants (n=2) (Kruskal-Wallis analysis). Administration of tocilizumab for 2 hours prior treatment with GCA tissue culture supernatant for 5 days resulted in partial inhibition of IL-6 associated senescence of treated fibroblasts. (D) Representative images from IL-6/GL13/Ki67 costaining of HUVECs treated with GCA (±tocilizumab, n=3), PMR (n=3), healthy artery tissue culture supernatants (n=2) for 3 days, respectively, demonstrating IL6(+)/GL13(+)/Ki67(–) staining associated senescent HUVECs, mainly after treatment with GCA supernatant. Tocilizumab resulted in partial inhibition of IL-6 associated senescence of treated endothelial cells. (E) Graphical representation of the percentage of IL-6 (+)/GL13(+)/Ki67(–) stained senescent HUVECs, showing that tocilizumab has significant inhibitory capacity. (F) Representative images from IL-6/GL13 costaining of monocytes treated with GCA (n=3), PMR (n=3), healthy artery tissue culture supernatants (n=2) for 3 days, respectively. (G) Graphical representation of the percentage of IL6/GL13 positive costaining associated senescent monocytes cells, were mainly observed following treatment with GCA supernatant. Positive events for each staining were quantified by two independent observers). Objective (B, D) ×63 (F) ×20. Scale bar: (B, D) 20 µm, (F) 50 µm. *p<0.05, **p<0.01, ***p<0.001. Colour code: (B) IL-6: green, p21$^{WAF1/Cip1}$: red, GL13: magenta, (D) IL-6: green, Ki67: red, GL13: magenta, (F) IL-6: green, GL13: red. GCA, giant cell arteritis; PMR, polymyalgia rheumatica; TCZ, tocilizumab.

receptor inhibition (25.37% vs 32.17%, p<0.05) (online supplemental figure 5F–G). IL-6 and IL-1β receptor blockade, inhibited IL-6 associated senescence by >60% and 10%, respectively (figure 5B,online supplemental figure 5F, G). Moreover, the inhibition of IL-6 associated cellular senescence observed by IL-6 receptor blocking was statistically higher compared with IL-1β blocking (12.00% vs 25.37%, p<0.01) (online supplemental figure 6G). In this context, GCA supernatants and tocilizumab cotreatment in HUVECs and fibroblasts from both donors inhibited senescence as compared with controls (7.3% and 7.8%, respectively, p<0.01) (figure 5D–E, online supplemental figure 6H–I).

DISCUSSION

To our knowledge, this is the first study that senescent cells are directly identified in the diseased tissues of a non-neoplastic, inflammatory autoimmune disease, following an established multimarker algorithm.[16] This particular approach represents a novelty in the field of systemic inflammatory diseases, as opposed to the usual strategy of applying solely p16^{INK4A} and/or p21$^{WAF/Cip1}$ staining for the identification of senescence, leading in many occasions to misinterpretations.[9 13 15 22 29] By employing the multimarker approach, senescent cells can be strictly identified, offering a unique scientific 'tool' to understand and evaluate important biological properties such as the exact location and quantitative

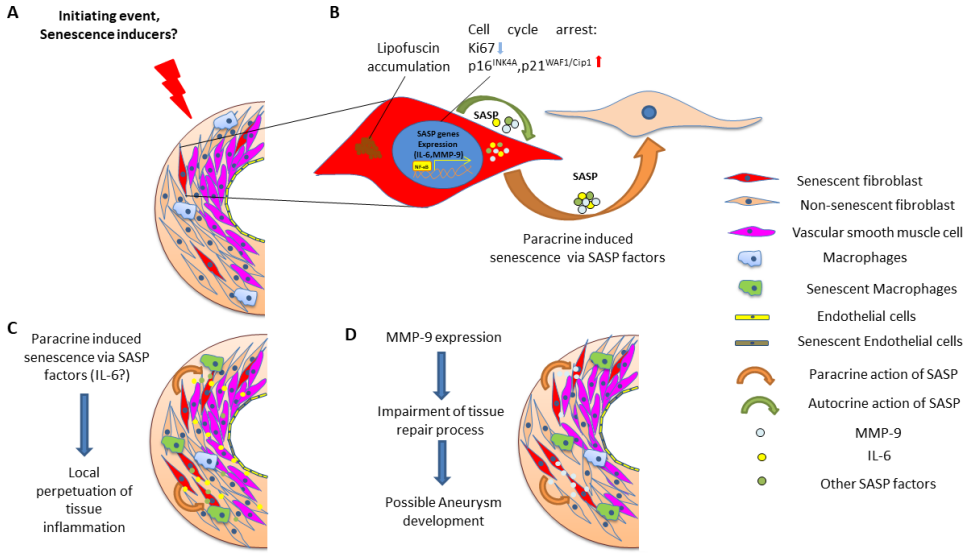

Figure 6 Suggested model for the role of cellular senescence in giant cell arteritis. (A) Diverse initiating events may induce cellular senescence mainly in temporal artery fibroblasts and to lesser extent in macrophages and endothelial cells. (B) Senescent fibroblasts (also the other senescent cell types) accumulate lipofuscin, exert cell cycle withdrawal as detected by overexpression of key cell cycle inhibitors such as $p21^{WAF/Cip1}$ and/or absence of proliferation markers (Ki67). SASP factors including IL-6, MMP-9 and others are produced, inducing senescence in an autocrine or paracrine manner, creating a vicious cycle. (C) The high burden of senescent fibroblasts, macrophages and endothelial cells promotes the local perpetuation of tissue inflammation via SASP. (D) MMP-9 expression and production by senescent fibroblasts may adversely affect tissue repair processes and arterial integrity, possibly leading to aneurysm development. SASP, senescent-associated secretory phenotype.

burden of senescent cells, their origin in terms of cell type and their SASP components that define their pathogenetic potential.[9 15] The application of the multimarker algorithm in the present study, led to significant and novel findings. First, we showed that although senescent cells could be also detected in age and sex matched PMR TABs, their proportion is significantly increased in GCA TABs. The lack of inflammatory infiltrate in PMR TABs, suggests an association between the inflammatory process within the arteries and the occurrence of senescent cells, supporting the notion that in GCA arteries there are both pre-existing and disease associated senescent cells. Indeed, senescent cells in TABs of GCA patients are mainly observed in close proximity with the inflammatory infiltrate. Second, it was demonstrated that cellular senescence in GCA arteries is limited to specific cell populations, most prominently in fibroblasts, macrophages and endothelial cells. In this way, cellular senescence can be conceptualised through specific cell types in terms of histological features, function and location within the layers of the vessel. Third, in line with previous studies suggesting that IL-6 and MMP-9 are profusely expressed in GCA TABs by inflammatory and resident cells,[30–35] we found that IL-6(+)/MMP-9(+) senescent cells are also present in GCA, confirming that SASP carries an inflammatory and impaired vascular remodelling potential. Interestingly, despite the fact that previous studies found MMP-9 expression by many cell types within GCA TABs, we detected MMP-9 only in senescent fibroblasts. Fourth, by quantifying IL-6(+) senescent cells, it was shown that the IL-6(+) senescent burden is proportional to the histological pattern/extension of the disease, underlining an important link among IL-6, degree of inflammation and cellular senescence in GCA. Finally, functional experiments demonstrated that the IL-6 rich microenvironment of TABs as opposed to sera from GCA patients has the capacity to induce IL-6 associated cellular senescence, implying a positive IL-6 dependent loop between the disease itself and cellular senescence. Given that resting fibroblasts do not express IL-6R,[36] the most likely scenario is that the complex of IL-6/sIL-6R is provided to fibroblasts via the TAB supernatant of GCA and not PMR patients and the signal is transduced through gp130 that is constitutively expressed by fibroblasts.

Our findings place cellular senescence as a potential contributor of the inflammatory process within the diseased arteries. Senescent cells probably pre-exist in temporal arteries of GCA patients, reflecting the 'normal' background ageing burden of cellular senescence. An excess of senescent cells was documented within the GCA arteries in our study that most likely is related to the inflammatory burden of the disease and represents inducible senescent cells. Whether GCA arteries display higher degree of pre-existing senescent cells or are more vulnerable to cellular senescence is difficult to be dissected, although recent studies have shown that environmental stimuli may provoke premature senescence state[37]; nevertheless our work clearly showed that soluble factors derived from the microenvironment of GCA and not PMR arteries, have the capacity to induce senescence and that IL-6 plays a significant role as attested by the inhibition experiments of IL-6R blockade. The fact that IL-6 receptor blockade resulted in significant but not total inhibition of cellular senescence, suggests that other factors may act synergistically to induce senescence. The excess of senescent cells in GCA arteries is of great clinical significance with important biological consequences. Despite the fact that senescent cells are characterised by cell cycle arrest with loss of proliferative capacity, they are resistant to apoptosis and cannot be easily removed and cleared from tissues.[9 15] The properties of senescent cells are largely defined by two factors: (1) the histological origin of senescent cells[38–40] and (2) the SASP components that contribute to their dynamic functional state and render them capable of interfering with the neighbouring structures. We found that senescent cells originated mainly by fibroblasts and macrophages in GCA arteries, expressing key molecules such as IL-6 and MMP-9. IL-6 derived from senescent cells may act in an autocrine and paracrine manner either to induce and spread cellular senescence to non-senescent neighbouring cells or to amplify the local inflammatory milieu.[9 30] Interestingly, it was recently shown that

persistent inflammatory stimulation of macrophages through TLR2 signalling may drive granuloma formation, a histological feature of GCA as well as DNA damage response, a well-known trait of cellular senescence.[41] Similarly, MMP-9 may contribute to the delay of the resolution of inflammation and deregulation of vascular remodelling which are closely related to aneurysm formation and refractoriness to treatment in GCA.[42] The role of endothelial senescent cells is less obvious. Although ICAM-1 has been found to be profusely expressed by inflammatory cells, endothelial cells and vascular smooth muscle cells,[43 44] endothelial senescent cells also display ICAM-1, reinforcing the inflammatory phenotype of endothelium.[45] Overall, it appears that the pathogenetic model of GCA could be enriched by integrating cellular senesce in a similar way to that observed in severe COVID-19 infection.[9 46] Pre-existing and inducible senescent cells in GCA arteries may play an active role in pathogenesis through perpetuation and maintenance of the inflammatory process as well as by interfering adversely with the tissue repair and remodelling of the inflamed vessel (figure 6). Significant comorbidities of GCA and mainly atherosclerosis can be further interpreted by the interference of foamy senescent macrophages which have been proposed to display atherogenic functions.[38 40] This model is further supported by the fact that the proportion of senescent cells correlates with the extent of inflammation within the affected arteries. In this line, the RNAseq analysis, revealed an enrichment of genes and molecular pathways related to cellular senescence. Furthermore, genome-wide association studies (GWAS) in GCA, pointed out specific SNP of important SASP components such as IL-6 or other cytokines, that may confer genetic predisposition to GCA through cellular senescence.[47] Finally, the epigenetic regulation of cellular senescence has been recognised to be involved in pathogenesis of cardiovascular diseases[48] and especially in GCA specific microRNAs linked to senescence were found upregulated in GCA arteries.[20] Despite the implication of cellular senescence in perpetuating inflammation, the exact role of senescent cells in the onset of GCA remains unclear. Temporal artery biopsies are obtained from patients who were already suffering from the disease at the time of diagnosis and carry a high inflammatory burden, connoting an advanced disease state. To address such questions research efforts should be focused on animal models.

Undoubtably, the application of the multimarker algorithm is one of the major strengths of this study. This approach was crucial to 'quantify' cellular senescence, opening a new avenue to study the role of senescence in GCA. Another strength is the large number of TABs that were studied and analysed by the same scientific team to ensure statistical power, accuracy and reproducibility of the research findings. One important weakness is the limited investigation of SASP, including only IL-6 and MMP-9 which as a route of pathogenicity, is characterised by significant diversity that incorporates among others IL-17A and GM-CSF, which will be extensively investigated in future studies. Another limitation is the use of steroids in a significant number of patients prior to TAB. Although the histopathology in GCA TABs has been proven not to be affected by short-term course of glucocorticosteroids (GCs),[49] functional consequences may occur[50] and, therefore, the impact of steroid treatment on induction of cellular senescence remains unknown. However, in our study, the proportion of senescent cells among GCA patients who have received GCs for different duration was not statistically different compared with untreated GCA patients. The lack of TABs from GCA patients in remission is also a weakness of our study. A second TAB after treatment administration would offer the opportunity to study the biological behaviour and features of senescent cells in the inactive state of the disease. Finally, additional functional experiments are required to identify molecules other than IL-6 that could mediate cellular senescence and have a role in GCA pathogenesis. Finally, all our experiments were focused only at the protein level (IHC, IF) without performing any investigations at the RNA levels (eg, *in situ* hibridisation, q-PCR).

In conclusion, senescent cells are present in GCA arteries and display an IL-6/MMP-9 SASP that potentially mediates inflammation and impaired vascular remodelling. Their detection in tissue biopsy may serve as a candidate tissue biomarker for disease severity, response to treatment and prognosis of serious complications. Further, these data set reasonable questions on the potential of senolytic treatment as a complementary strategy in the management of GCA.

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