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BRIEF REPORT

Indication of Activated Senescence Pathways in the Temporal Arteries of Patients With Giant Cell Arteritis

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Objective. Giant cell arteritis (GCA) affects almost exclusively individuals above 50 years old, suggesting a role of aging-related changes such as cellular senescence in its pathobiology. The kinases p21(WAF1/CIP1) and p16/INK4A play key roles in 2 distinct pathways leading to senescence. The proinflammatory molecules interleukin-6 (IL-6) and granulocyte–macrophage colony-stimulating factor (GM-CSF), which are key components of the senescence-associated secretory phenotype (SASP), are effective targets of treatment in GCA. Here, we aimed to investigate the presence of p21+ and p16+ cells producing these SASP cytokines in temporal artery biopsies (TABs) of patients with GCA.

Methods. Eight patients with GCA and 14 age-matched, non-GCA individuals who underwent a TAB were included. Immunohistochemical staining of p21, p16, IL-6, and GM-CSF was performed. Multiplex immunofluorescent staining was performed to investigate the colocalization of p21 and p16 with IL-6, GM-CSF, and immune cell markers (CD68, CD3, CD20).

Results. We found that expression levels of p16, p21, IL-6, and GM-CSF were elevated in the TABs of patients with GCA. Both p16- and p21-expressing cells were mainly found near the internal lamina elastica, especially among giant cells and macrophages, although p21 and p16 expression could be found in all 3 layers of the vessels. Expression of p16 and p21 was occasionally found in T cells but not B cells. The p16+ and p21+ cells expressing GM-CSF/IL-6 were detected throughout the TABs.

Conclusion. Our data suggest the presence of activated senescence pathways at the site of vascular inflammation in GCA and support further research into the role of senescence in the pathophysiology of GCA.

INTRODUCTION

Giant cell arteritis (GCA) is a chronic autoinflammatory disease characterized by granulomatous inflammation of large- and medium-sized arteries, including the temporal arteries (1). Patients experiencing GCA are almost exclusively above 50 years old, suggesting a role of aging-related changes to the vascular bed and/or the immune system.

Cellular senescence is a cell fate occurring in response to molecular damage and is associated with stable proliferative arrest and resistance to cell death (2). Senescence is considered to be an essential anticancer mechanism, as it prevents proliferation in cells with DNA damage. Importantly, while senescent cells are rarely detectable in young tissues, their numbers increase exponentially with age (2). Induction of cellular senescence can be attributed to several factors, namely strong mitogenic signals leading to shortened telomers, activation of 2 pathways strongly associated with senescence (p12[WAF1/CIP1]- and p16/INK4A-associated pathways), and the development of a complex senescence-associated secretory phenotype (SASP) (3). SASP includes the secretion of several proinflammatory cytokines, chemokines, and growth factors such as interleukin-6 (IL-6), IL-1β, IL-8, chemokine (C-C motif) ligand 8 (CCL8), vascular endothelial growth factor (VEGF), and granulocyte–macrophage colony-stimulating factor (GM-CSF) (4). SASP has many biological functions and can promote paracrine senescence to surrounding

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healthy cells. Therefore, senescent cells are likely important in the development of autoimmune and autoinflammatory diseases in the elderly.

Even though age is the strongest risk factor for GCA, cellular senescence has not been properly assessed in GCA lesions. Furthermore, the SASP cytokines IL-6 and GM-CSF have been successfully targeted for the treatment of GCA (5,6). Here we sought clues of activated senescence pathways at the site of vascular inflammation in GCA by assessing the expression of p21 and p16, as well as IL-6 and GM-CSF.

PATIENTS AND METHODS

GCA patients and controls. We included 8 patients with GCA (median age 65.4 years [interquartile range (IQR) 63.6–79]) fulfilling the American College of Rheumatology (ACR) 1990 criteria for the classification of GCA (7) and who underwent a temporal artery biopsy (TAB) showing evidence of transmural inflammation. These biopsies were obtained prior to the initiation of treatment with glucocorticoids (n = 5) or within 10 days after initiation (n = 3). In addition, 14 non-GCA uninflamed TABs from age-matched individuals (median age 69.8 years [IQR 64.2–78.2]) were included as controls. These individuals ultimately received a different diagnosis than GCA or polymyalgia rheumatica. The study was approved by the institutional review board of the University Medical Center Groningen (METc2010/222). Written informed consent was obtained from all study participants. All procedures complied with the Declaration of Helsinki.

Immunohistochemistry. We performed immunohistochemical staining of p21 and p16, as well as the SASP cytokines IL-6 and GM-CSF (see Supplementary Table 1 for details, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.42525). Formalin-fixed, paraffin-embedded TAB sections (3 μm) were deparaffinized and rehydrated, followed by 15 minutes of antigen retrieval in a microwave. Tissues were incubated with primary antibodies targeting p21, p16, IL-6, and GM-CSF (see Supplementary Table 1). Sections were then blocked for endogenous peroxidase activity and incubated with secondary antibodies tagged with horseradish peroxidase (HRP). Brown positive stains were developed by incubation with 3,3′-diaminobenzidine (DAB). Sections were counterstained with hematoxylin. All slides were scanned using a Nanozoomer Digital Pathology Scanner (NDP Scan U 10074-01, Hamamatsu Photonics). For each TAB, 3 layers of the vessel wall (including intima, media, and adventitia) were scored. The percentage of positive cells per layer was assessed with QuPath image analysis software version 0.3.2.

Multiplex immunofluorescence. Sequential opal immunofluorescence staining was performed to confirm the coexpression of IL-6, GM-CSF, CD68, CD3, and CD20 with the senescence markers p21 and p16 (n = 3 each, except n = 2 for CD20/p16 staining) (8). Formalin-fixed, paraffin-embedded TAB sections (3 μm) were deparaffinized and rehydrated, followed by 15 minutes of antigen retrieval with Tris-EDTA buffer (pH9) in a microwave. Tissues were incubated with primary antibodies targeting p21 or p16 (see Supplementary Table 1, http://onlinelibrary.wiley.com/doi/10.1002/art.42525) overnight at 4°C. Sections were then blocked for endogenous peroxidase activity and incubated with secondary antibodies tagged with HRP. Opal 650 or 620 fluorophore (Akoya Biosciences) was developed by incubation for 10 minutes. Bound antibodies and nonspecific fluorophores were stripped by 15 minutes of antigen retrieval with Tris-EDTA buffer (pH9) in a microwave. Tissues were incubated with primary antibodies targeting IL-6, GM-CSF, CD68, CD3, or CD20 (see Supplementary Table 1) for 1 hour at room temperature. Sections were then incubated with secondary antibodies tagged with HRP. Opal 620 or 650 fluorophore (Akoya Biosciences) was developed by incubation for 10 minutes. Bound antibodies and nonspecific fluorophore were stripped by 5 minutes of antigen retrieval with Tris-EDTA buffer (pH9) in a microwave. Sections were incubated with DAPI as a counterstain and sealed. Image cubes were captured at magnifications of 20× and 40× using Nuance Multispectral Imaging System 3.0.1 with NuanceFX 3.0.1 software (both from PerkinElmer). Spectral unmixing was performed with spectral libraries of each fluorophore assigned different colors, subtracting the background autofluorescence.

Statistics. To analyze the differences between GCA and non-GCA TABs, nonparametric Mann–Whitney U-tests (2-tailed) were used. Analysis was performed with GraphPad Prism 8.0.1 and *P* values less than 0.05 were considered significant.

RESULTS

Senescence markers p16 and p21 were expressed in inflamed GCA TABs (Figure 1A). Expression of p16 was mostly cytoplasmic and could most commonly be observed in cells surrounding the internal lamina elastica, such as the giant cells. Nuclear staining of p21 was also observed near the internal lamina elastica. In contrast, expression of p21 and p16 was scarce in control TABs. SASP cytokines IL-6 and GM-CSF were abundantly expressed in each layer of GCA TABs (Figure 1A). The percentage of positive cells expressing p16, p21, IL-6, and GM-CSF was significantly higher in inflamed TABs compared to uninflamed TABs (Figures 1B and C). This was true for the whole tissue section, as well as in each of the 3 artery layers.

Immunofluorescence staining revealed that p16 (Figure 2 and Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.42525) and p21 (Figure 2 and Supplementary Figure 2) positive cells colocalized with IL-6 and GM-CSF (for isotype control, see Supplementary Figure 5). However, p16+ and p21+ cells that do not express GM-CSF/IL-6 were also detected,

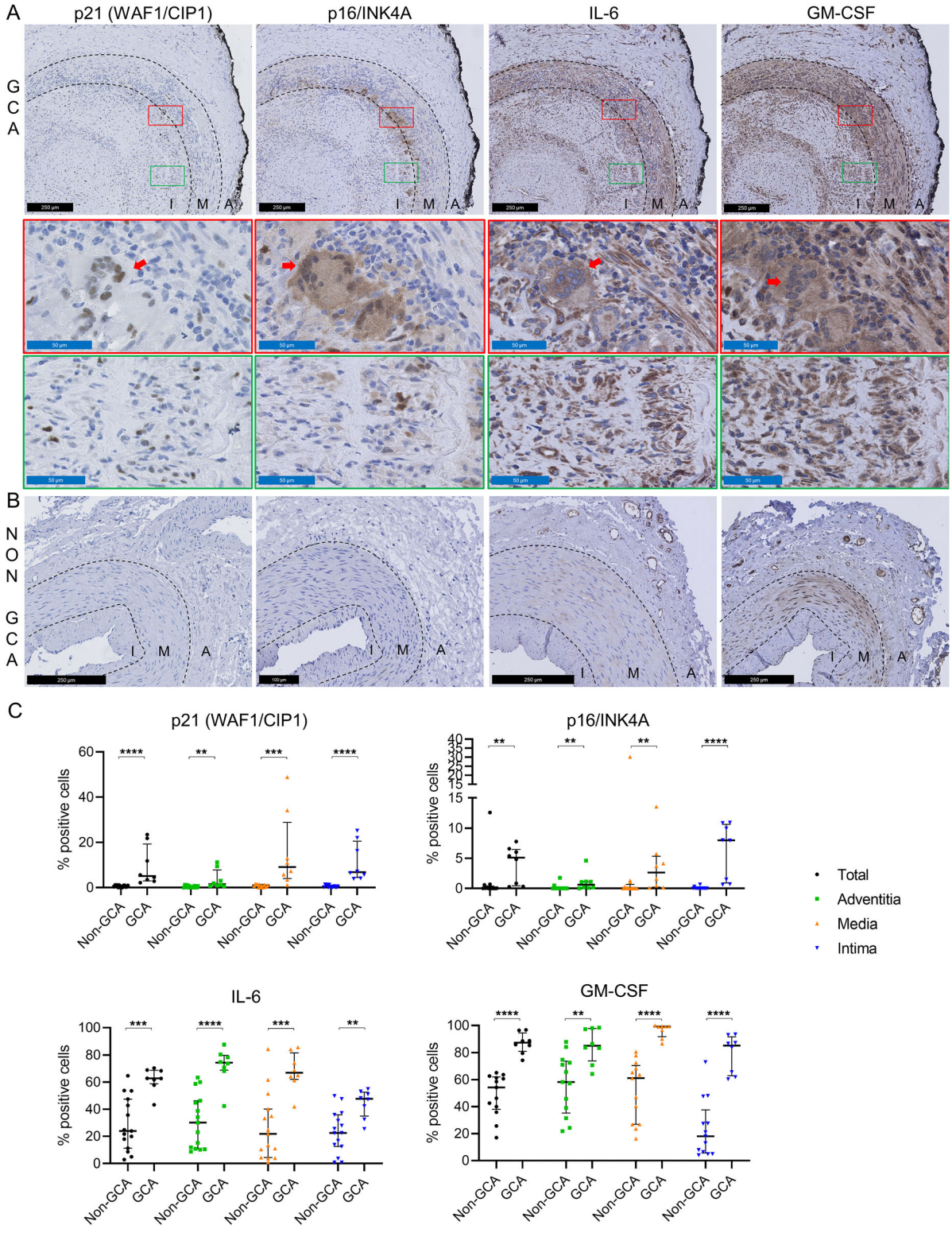

Figure 1. Expression of senescence markers p21 (WAF1/CIP1) and p16/INK4A and senescence-associated secretory phenotype (SASP) cytokines interleukin-6 (IL-6) and granulocyte–macrophage colony-stimulating factor (GM-CSF) in giant cell arteritis (GCA). **A**, Representative image of the expression of p21, p16, IL-6, and GM-CSF in the temporal artery (TAB) from a GCA patient. All 4 markers were abundantly expressed (**brown staining**) by giant cells (**red arrows**) surrounding the intimal elastic lamina (**red boxes**) and other infiltrating/vascular cells (**green boxes**). **B**, The expression of p21 and p16 was scarce in uninflamed control TABs. The expression of IL-6 (mostly by endothelial cells) and GM-CSF (mostly by vascular smooth muscle cells) was noted in control tissues, albeit at lower levels compared to inflamed GCA TABs. **C**, Quantitative scoring showed significant elevation of all 4 markers in all 3 layers of inflamed GCA TABs compared to non-GCA TABs. ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$, by Mann–Witney U test. I = intima; M = media; A = adventitia.

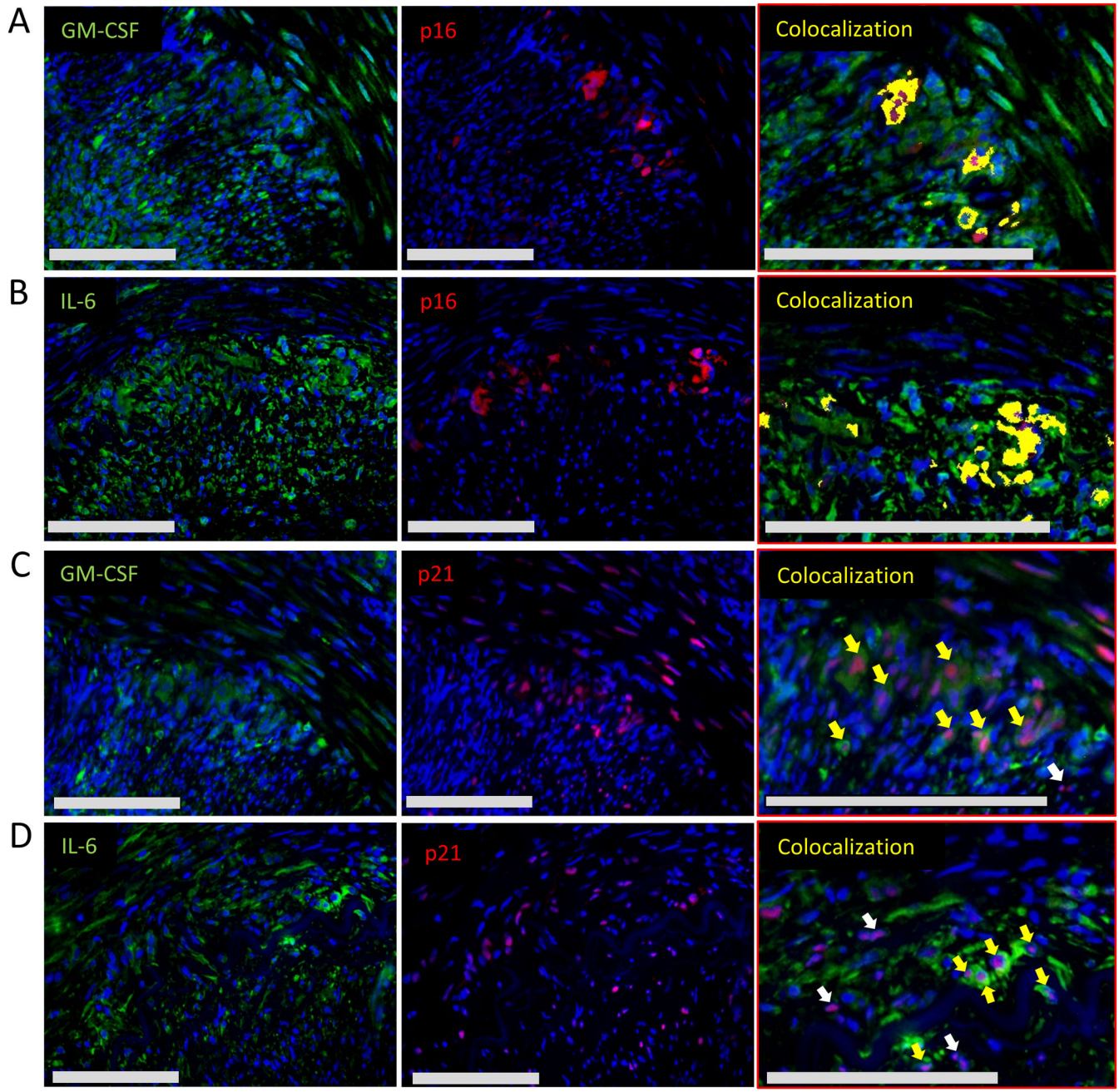

Figure 2. Expression of IL-6 and GM-CSF by p16/INK4A– and p21 (WAF1/CIP1)–positive cells in temporal artery biopsy of patients with GCA. **A** and **B**, Representative immunofluorescent staining showing coexpression of p16 (in red) and GM-CSF (**A**)/IL-6 (**B**) (in green). The color yellow represents colocalization of p16 and GM-CSF/IL-6 pixels. **C** and **D**, Representative immunofluorescent staining showing coexpression of p21 (in red) and GM-CSF (**C**)/IL-6 (**D**) (in green); **yellow arrows** point to p21+ cells coexpressing GM-CSF/IL-6, **white arrows** point to p21+ cells that lack expression of GM-CSF/IL-6, and **red boxes** show zoomed inset of the areas of colocalization. **Grey bar** represents a 100 μm ruler. See Figure 1 for definitions.

indicating that not all of the p16+ and p21+ cells were in the state of senescence.

Further classification of the immune-infiltrating cells revealed that macrophages are the main cell type expressing p16 and p21 (Figures 3A and D and Supplementary Figures 3 and 4, http://onlinelibrary.wiley.com/doi/10.1002/art.42525). Interestingly, both p16+ and p16– giant cells were detected in the same section (Figure 3A). T cells were also found to express p16 and p21 (Figures 3B and E and Supplementary Figures 3 and 4, http://onlinelibrary.wiley.com/doi/10.1002/art.42525), albeit to a lower extent compared to macrophages. However, the expression of both p16 and p21 was not detected in B cells

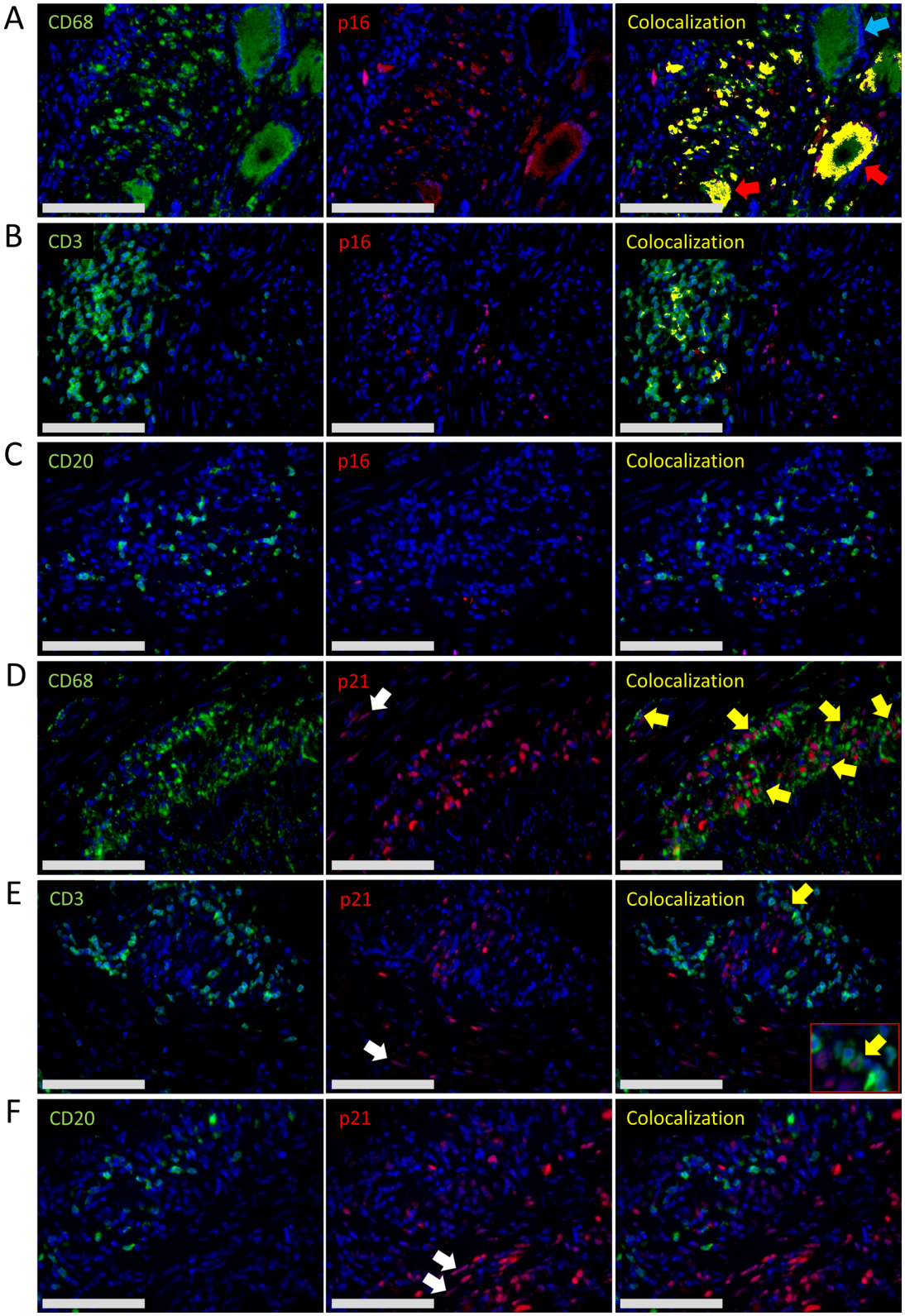

Figure 3. Macrophages and T cells but not B cells express p16/INK4A and p21(WAF1/CIP1), in the temporal artery biopsy of patients with giant cell arteritis. **A**–**B**, Representative staining showing (**A**) extensive presence of p16+ macrophages and (**B**) occasional p16+ T cells; no p16+ B cells were detected (**C**). **D**–**F**, Representative staining showing (**D**) p21+ macrophages and (**E**) p21+ T cells; no p21+ B cells were detected (**F**). **Red arrows** in **A** show p16+ giant cells; **blue arrow** in **A** shows a p16– giant cell; **yellow arrows** in **D** and **E** show p21+ macrophages and T cells; **white arrows** in **D**–**F** show p21+ vascular smooth muscle cells in the media (elongated nucleus). **Red box** shows the zoomed in inset of the colocalization; **grey bar** represents a 100 μm ruler. See Figure 1 for definitions.

(Figures 3C and F and Supplementary Figure 5, http://onlinelibrary.wiley.com/doi/10.1002/art.42525). Positivity of p16 and p21 was also found in cells morphologically resembling vascular smooth muscle cells in the media and (myo)fibroblasts in the intima (Figure 3 and Supplementary Figure 3, http://onlinelibrary.wiley.com/doi/10.1002/art.42525).

DISCUSSION

Our data suggest the presence of activated senescence pathways at the site of vascular inflammation in GCA and support further research into the role of senescence in the development of GCA. The expression of p16 and p21 in inflamed TABs is an important clue for activation of senescence pathways at the site of vascular inflammation in GCA (3). Both p21 and p16 target cyclin-dependent kinases that regulate proliferation, which can induce SASP via transcription factors such as GATA4 (3,9). Cellular senescence, indicated by prolonged p21 expression, has been postulated as a triggering event that attracts macrophages and cytotoxic T cells by a wide range of SASP factors (10). However, even though the expression of p21 and p16 is an important clue for senescence, the presence of these factors cannot be seen as absolute proof of senescence, as nonsenescent cells may also express these markers (e.g., activated cells in inflammatory conditions may gain expression of p21 and/or p16) (11). The key SASP cytokines IL-6 and GM-CSF are successful therapeutic targets in GCA. While the IL-6 receptor blocker tocilizumab has been implemented in daily clinical practice after showing efficacy in GCA patients (6), a phase II trial has also recently shown promising effects of the GM-CSF receptor blocker mavrilimumab in this disease (5).

As patients experiencing GCA are almost exclusively over 50 years of age, an accumulation of senescent cells may be expected to some extent. Therefore, the question remains if senescent cells may contribute to the pathogenesis of this disease. Our data show that myeloid-derived cells (macrophages and giant cells) are the major inflammatory infiltrating cells expressing p16 and p21. Indeed, giant cell formation has been linked to senescence and has been suggested as a potential target in treatments promoting longevity (12). However, not all giant cells expressed p16 or p21, suggesting that not all of these cells were senescent. In the lymphoid compartments, our data showed that T cells were also capable of expressing p16 and p21, albeit in lower numbers compared to the macrophages. In line with our data, the expression of senescence markers p53 and p21, as well as the infiltration of NKG2D+CD28– senescent-like T cells in the TABs of GCA patients, has been previously documented (13,14). Apart from the immune cells, our data pointed at the expression of p16 and p21 in stromal cells such as vascular smooth muscle cells and (myo)fibroblasts. Previously, several microRNAs associated with cellular senescence (miR-146a, miR-146b-5p, miR-21, and miR-155) were found to be up-regulated in GCA TABs (15). All in all, our data complements these studies, suggesting that senescence is relevant to the pathobiology of GCA.

Further studies are required to definitively prove that senescent cells are present in GCA lesions. As SASP cytokines can also reflect activation of cells through inflammatory signalling cascades, one important step will be the identification of true senescent cells. Future research should leverage the use of high-plex imaging techniques involving comprehensive sets of cell-type and senescence-specific markers (such as the positivity for SASP cytokines, DNA damage markers, transcription factors regulating SASP, and the negativity of cellular proliferation markers) to further confirm our findings. If proven important in GCA, senescence pathways may constitute new targets of intervention for either senolytics (i.e., agents that selectively eliminate senescent cells) or senomorphics (agents that can suppress SASP) (16).

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sandovici had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Jiemy, van Sleen, Graver, Boots, Sandovici.

Acquisition of data. Jiemy, Graver.

Analysis and interpretation of data. Jiemy, van Sleen, Pringle, Brouwer, van der Geest, Cornec, Boots, Sandovici.

REFERENCES

1. Dejaco C, Duftner C, Buttgereit F, et al. The spectrum of giant cell arteritis and polymyalgia rheumatica: revisiting the concept of the disease. Rheumatology 56:506–15.
2. Hernandez-Segura A, Nehme J, Demaria M. Hallmarks of cellular senescence. Trends Cell Biol 2018;28:436–53.
3. Cohn RL, Gasek NS, Kuchel GA, et al. The heterogeneity of cellular senescence: insights at the single-cell level. Trends Cell Biol 2023;33:9–17.
4. Ovadya Y, Krizhanovsky V. Senescent cells: SASPected drivers of age-related pathologies. Biogerontology 2014;15:627–42.
5. Cid MC, Unizony SH, Blockmans D, et al. Efficacy and safety of mavrilimumab in giant cell arteritis: a phase 2, randomised, double-blind, placebo-controlled trial. Ann Rheum Dis 2022;81:653–61.
6. Stone JH, Tuckwell K, Dimonaco S, et al. Trial of tocilizumab in giant-cell arteritis. N Engl J Med 2017;377:317–28.
7. Hunder GG, Bloch DA, Michel BA, et al. The American College of Rheumatology 1990 criteria for the classification of giant cell arteritis. Arthritis Rheum 1990;33:1122–8.
8. Kwon OC, Lee E, Chang E, et al. IL-17A GM-CSF neutrophils are the major infiltrating cells in interstitial lung disease in an autoimmune arthritis model. Front Immunol 2018;9:1544.
9. Kang C, Xu Q, Martin TD, et al. The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4. Science 2015;349:aaa5612.
10. Sturmlechner I, Zhang C, Sine CC, et al. p21 produces a bioactive secretome that places stressed cells under immunosurveillance. Science 2021;374:eabb3420.

11. Hall BM, Balan V, Gleiberman AS, et al. p16(Ink4a) and senescence-associated beta-galactosidase can be induced in macrophages as part of a reversible response to physiological stimuli. Aging (Albany) 2017;9:1867–84.
12. Kloc M, Uosef A, Subuddhi A, et al. Giant multinucleated cells in aging and senescence—an abridgement. Biology 2022;11:1121.
13. Nordborg C, Åman P, Persson M, et al. Cell-type-specific expression of p53 and p21 in giant cell arteritis. APMIS 2005;113:594–9.
14. Dejaco C, Duftner C, Al-Massad J, et al. NKG2D stimulated T-cell autoreactivity in giant cell arteritis and polymyalgia rheumatica. Ann Rheum Dis 2013;72:1852–9.
15. Croci S, Zerbini A, Boiardi L, et al. MicroRNA markers of inflammation and remodelling in temporal arteries from patients with giant cell arteritis. Ann Rheum Dis 2015;75:1527–33.
16. Childs BG, Gluscevic M, Baker DJ, et al. Senescent cells: an emerging target for diseases of ageing. Nat Rev Drug Discov 2017;16:718–35.