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Expression of Interleukin-32 in the Inflamed Arteries of Patients With Giant Cell Arteritis

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Objective. **Giant cell (temporal) arteritis (GCA) is a vasculitis that mainly affects the large and medium arteries, especially the branches of the proximal aorta. Interleukin-32 (IL-32) is a recently described Th1 proinflammatory cytokine, and is mainly induced by interferon-γ (IFNγ), IL-1β, and tumor necrosis factor α (TNFα). This study was undertaken to investigate the expression and tissue distribution of IL-32 in artery biopsy specimens from patients with GCA.**

Methods. **Quantitative gene expression analysis of IL-32, IL-1β, TNFα, IFNγ, IL-6, and IL-27 was performed in artery biopsy specimens obtained from 18 patients with GCA and 15 controls. Immunohistochemistry analysis was performed to evaluate IL-32 tissue distribution and identify IL-32–producing cells. Circulating Th1 lymphocytes were evaluated by flow cytometry.**

Results. **We demonstrated a strong and significant up-regulation of IL-32 at both the messenger RNA and protein levels in the artery biopsy samples from patients with GCA. IL-32 was abundantly expressed by vascular smooth muscle cells of inflamed arteries and neovessels within inflammatory infiltrates. IL-32 expression strongly correlated with the intensity of the systemic inflammatory response. IL-32 overexpression was accompanied by strong overexpression of Th1 cytokines, such as IFNγ and IL-27p28, in inflamed arteries from GCA patients. The Th1 lymphocyte population was also expanded among peripheral blood mononuclear cells from GCA patients and produced higher amounts of IL-32 compared to controls.**

Conclusion. **Our findings indicate that overexpression of IL-32 together with a clear Th1 response immunologically characterizes the inflammatory response in GCA. In particular, IL-32 seems to be an important mediator of artery inflammation in GCA.**

In giant cell (temporal) arteritis (GCA), inflammation mainly affects the large and medium muscular arteries, especially the proximal aorta and its branches. One or more systemic manifestations are present in most patients and indicate the presence of a systemic inflammatory response (1).

The exact mechanisms that contribute to the pathogenesis of GCA are still largely unknown. It is well accepted, however, that inflammatory cellular infiltrates, mainly T lymphocytes and macrophages and their extensive production of proinflammatory mediators such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and IL-6, play a major role in the regulation of arterial immune responses (1). GCA has previously been ascribed to a Th1 response, based on a study demonstrating that interferon-γ (IFNγ) is a dominant cytokine in the inflamed arteries (2). In addition, it has been recently demonstrated that another proinflammatory cytokine, IL-17, is produced concomitantly with IFNγ in the inflamed arteries of GCA patients, contributing to the systemic and vascular manifestations of the disease (3).

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IL-32 is a recently described Th1-related proinflammatory cytokine, and has important functions in both innate and immune responses; its expression is induced by Th1 cytokines, such as IL-1β, TNFα, and IFNγ (4). IL-32 is also constitutively produced by human endothelial cells, indicating that it is a critical regulator of endothelial function through regulation of coagulation, endothelial cell activation, and atherosclerosis (5–7).

The role of IL-32 in human inflammatory diseases, such as Crohn's disease (8) and rheumatoid arthritis (RA) (9–12), has been extensively demonstrated, identifying IL-32 as a potential therapeutic target in chronic inflammation. Whether this cytokine could be implicated in the immune responses that are characteristic of GCA still remains to be investigated. In the present study, we investigated the expression of IL-32 in the context of Th1 response in temporal arterial biopsy specimens from patients with GCA. Since IL-27, a cytokine functionally and structurally related to IL-12, has been proposed as a critical factor for early Th1 differentiation (13), we also studied IL-27 expression.

PATIENTS AND METHODS

Patients. Eighteen consecutive patients (14 women, 4 men) with biopsy-proven GCA were studied. Their median age was 77 years (range 60–87 years), and their erythrocyte sedimentation rate (ESR) was 80 mm/hour (range 77–120). Headache and temporal artery tenderness were present in 15 of 18 patients. Decreased or absent temporal artery pulsation was present in 10 of 18 patients. Patients were diagnosed as having biopsy-proven GCA if histologic examination of the temporal artery biopsy specimen showed disruption of the internal elastic lamina, with infiltration of mononuclear cells into the arterial wall, with or without giant cells. All studied samples were obtained from untreated patients. At the time of diagnosis, 6 patients had experienced vision loss. Five patients had associated polymyalgia rheumatica (PMR).

Four parameters (score range 0–4), as described by Cid et al (14), were used to evaluate the baseline inflammatory response at diagnosis: fever, weight loss, an ESR of $\geq$85 mm/hour, and a hemoglobin level of $<$11.0 gm/dl. A strong clinical inflammatory response as well as a high inflammatory reaction demonstrated by laboratory results (14) were present in 6 of the 18 patients. After diagnosis, GCA patients were treated with prednisone at an initial dosage of 50 mg/day. Prednisone was tapered in all patients according to the same fixed schedule, starting after 1 month of therapy if symptoms had resolved.

For controls, we obtained 15 histologically normal temporal artery samples from 15 consecutive patients who had been suspected of having GCA but who had negative biopsy results. They were matched by sex and age with the biopsy-proven GCA group (12 women, 3 men, median age 75.8 years [range 66–85 years]). The ultimate diagnoses in these patients were fever of unknown origin (5 patients), isolated PMR (3 patients), large-vessel vasculitis involving the thoracic aorta and its branches but sparing the temporal artery (1 patient), and nonspecific headache in the presence of osteoarthritis (6 patients). From 11 patients with biopsy-proven GCA and 5 controls, total RNA could be extracted from frozen fragments of the temporal artery biopsy specimens that were excised before treatment was begun. This study was approved by the local ethics committee and all patients provided informed consent for the collection and storage of biologic material.

RNA extraction from temporal artery biopsy specimens and quantitative reverse transcriptase–polymerase chain reaction (PCR) for IL-32, IL-6, transforming growth factor β, IL-1β, TNFα, IFNγ, and IL-27p28. Soon after removal, temporal artery biopsy specimens were stored in RNAlater solution (Applied Biosystems). Each sample was lysed in a tissue homogenizer, and RNA was extracted using the commercially available illustra RNAspin Mini Isolation Kit, according to the instructions of the manufacturer (GE Healthcare). Total RNA was reverse-transcribed to complementary DNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Samples were stored at –20°C until use. For quantitative TaqMan real-time PCR, Master Mix and TaqMan gene expression assay kits for GAPDH control and for IL-6 (Hs00174131_m1), IL-32 (Hs00170403_m1), IL-1β (Hs00174097_m1), TNFα (Hs00174128_m1), IFNγ (Hs00989291_m1), IL-6 (Hs00174131_m1), and IL-27p28 (Hs00377366_m1) were obtained from Applied Biosystems. Experiments on each sample were run in triplicate using the Step-One Real-Time PCR system (Applied Biosystems). Relative changes in gene expression between control and arteritis samples were determined using the $\Delta\Delta C_t$ method. Levels of the target transcript were normalized to GAPDH endogenous control, expressed consistently in both groups (ΔC_t). For $\Delta\Delta C_t$ values, additional subtractions were performed between arteritis and control ΔC_t values. Final values were expressed as the fold induction.

Immunohistochemistry analysis of GCA biopsy specimens. Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. Immunohistochemistry analysis for IL-32 was performed on 5-μm–thick paraffin-embedded sections from arteries and from tonsils (used as positive controls). Following rehydration, antigen was unmasked for 45 minutes at 95°C using Dako Target retrieval solution (pH 6.0). Endogenous peroxidase was blocked for 10 minutes with Dako peroxidase blocking reagent, and nonspecific binding was blocked for 20 minutes with Dako protein block. The primary antibodies, mouse monoclonal anti-human IL-32 (IgG1, clone KU32-09; 1:200 dilution) and rabbit polyclonal anti-human IL-32 (1:250 dilution) (Sigma-Aldrich), were added and incubated for 1 hour at room temperature. Isotype-matched irrelevant antibodies were used as a negative control (mouse IgG1 monoclonal antibody [mAb] [ab27479] and rabbit IgG polyclonal antibody [ab27472]; AbCam). Following 3 washes with Tris buffered saline, slides were incubated for 30 minutes with peroxidase-conjugated Dako EnVision polymer. After 3 further washings, peroxidase activity was visualized using diaminobenzidine chromogen (Dako), and slides were lightly counterstained with hematoxylin before dehydration and mounting in DePex (VWR International).

Sections were analyzed by 2 experienced pathologists

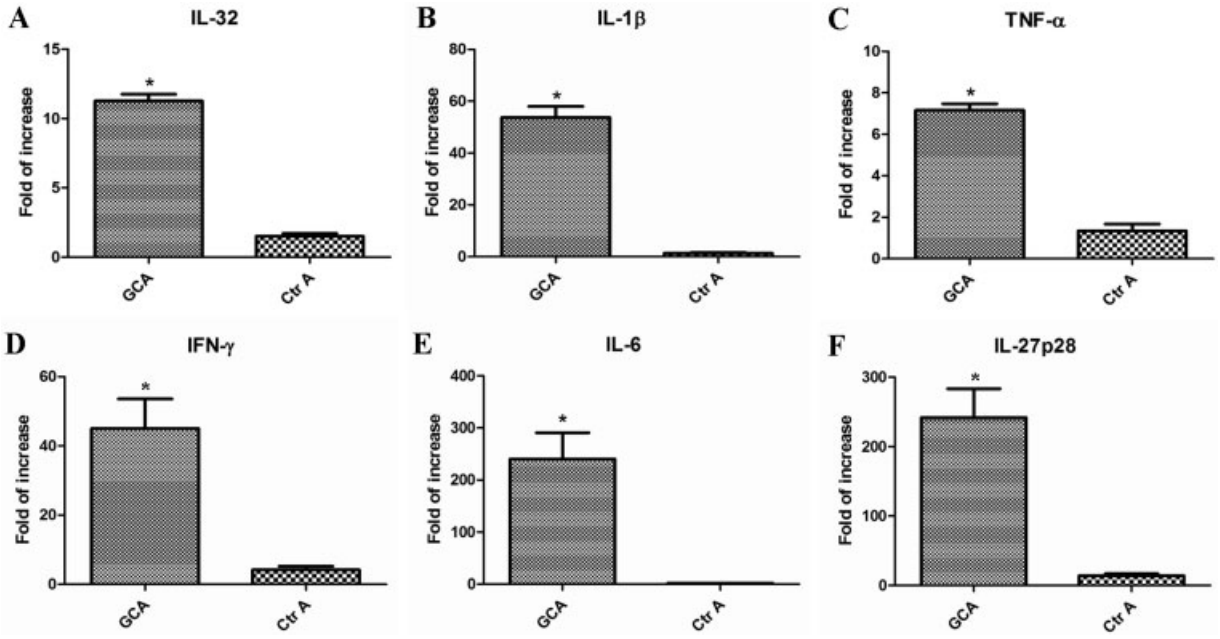

Figure 1. Interleukin-32 (IL-32), IL-1β, tumor necrosis factor α (TNFα), interferon-γ (IFNγ), IL-6, and IL-27p28 genes in inflamed arteries from patients with giant cell arteritis (GCA). Relative expression of mRNA for IL-32 (**A**), IL-1β (**B**), TNFα (**C**), IFNγ (**D**), IL-6 (**E**), and IL-27p28 (**F**) in arterial specimens obtained from 11 patients with GCA and 5 control arteries (Ctr A) was quantified by TaqMan real-time polymerase chain reaction. Bars show the mean ± SEM. $* = P < 0.0001$ versus control.

(AR and AC) who were blinded with regard to subject group. The number of IL-3–expressing cells was determined by counting IL-32–immunoreactive cells on photomicrographs obtained from 3 randomly obtained high-power microscopic fields (400× magnification) under a DM2000 optical microscope, using a DFC320 digital camera (Leica). Staining for IL-32 was scored semiquantitatively on a 4-point scale (range 0–3); a score of 0 represented no or minimal staining, a score of 1 indicated up to 40% positive cells, a score of 2 indicated up to 60% positive cells, and a score of 3 represented staining of >60% of the cells (evaluated using 200× magnification).

Flow cytometry for IL-32 on peripheral blood mononuclear cells (PBMCs) isolated from GCA patients. PBMCs, isolated from heparinized blood samples from 5 GCA patients and 5 controls, were stimulated with phorbol myristate acetate (1 μg/ml) and ionomycin (0.5 μg/ml) and incubated at 37°C in 5% CO_2. After 2 hours of incubation, 10 μg/ml brefeldin A (Sigma) was added and after 16 hours of incubation PBMCs were collected and stained with mAb. Reagents used for flow cytometric analysis were peridinin chlorophyll A protein–conjugated anti-CD4 (Becton Dickinson), mouse anti-human IL-32 purified antibody (BioLegend), and phycoerythrin-conjugated anti-mouse IgG (Sigma). Total cells were incubated with mAb on ice for 30 minutes and washed twice in phosphate buffered saline that contained 0.1% weight/volume NaN_3. After surface staining, the cells were fixed with 1% w/v paraformaldehyde (Sigma) for 30 minutes at 4°C and were then permeabilized with a permeabilization solution (Becton Dickinson) for 10 minutes at room temperature and stained with antibodies to intracellular antigens. Three-color flow cytometry was performed using a FACSCalibur (Becton Dickinson). At least 50,000 cells (events), gated on the lymphocyte region, were acquired for each sample. PBMCs were expressed as the percentage of cells within the lymphocyte gate. The acquired data were analyzed using CellQuest software (Becton Dickinson). IFNγ- and IL-32–producing T cells in PBMCs were determined by CD4, IFNγ, and IL-32 staining, and subsequent analysis on a FACSCalibur flow cytometer.

Statistical analysis. Statistical analysis of quantitative variables was performed using the Mann-Whitney rank sum test. Pearson's correlation coefficient was utilized to quantify the associations between the genes of interest. P values less than 0.05 were considered significant.

RESULTS

Clinical and histologic features. A strong clinical inflammatory response (fever and weight loss) was observed in 5 patients and a high inflammatory reaction demonstrated by laboratory results (ESR ≥85 mm/hour and hemoglobin <11.0 gm/dl) in 4 patients. Overall, 5 patients had an inflammatory reaction determined by clinical examination or laboratory analysis. All enrolled patients had a positive temporal artery biopsy result with intimal thickening due to proliferation and migration of vascular smooth muscle cells (VSMCs).

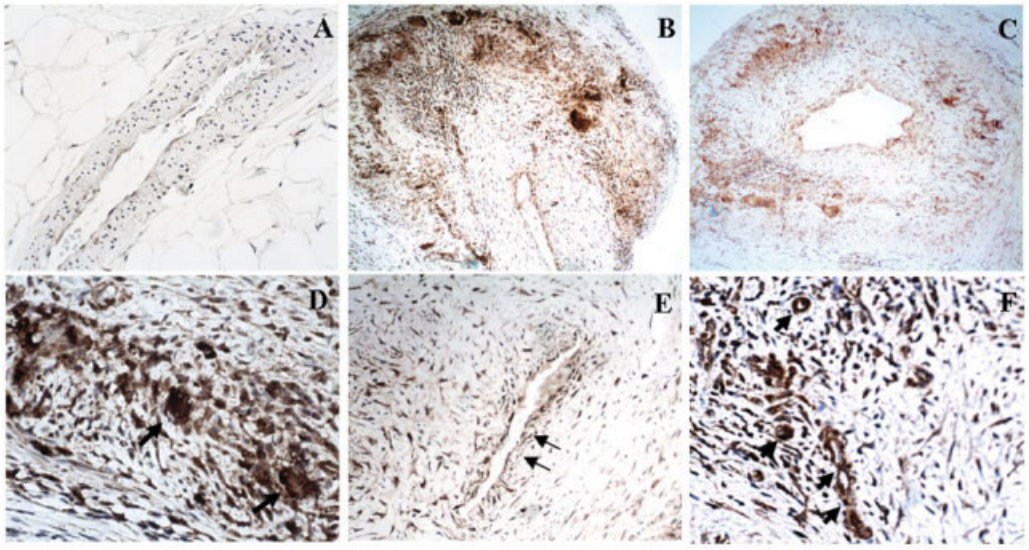

Figure 2. IL-32 expression in arterial wall. Temporal artery biopsy specimens were obtained from controls (**A**) and patients with GCA (**B–F**) and stained for IL-32. IL-32 expression was observed in the GCA patients, but not in control arteries. Abundant IL-32 expression was observed in a large number of giant cells present in the wall of inflamed arteries (**arrows** in **D**). Expression of IL-32 was more intense in microvessels scattered through the inflammatory infiltrates (**arrows** in **F**) than in the luminal endothelium (**arrows** in **E**). IL-32+ vascular smooth muscle cells were observed in all inflamed arteries (**B–F**). Representative photomicrographs of 5-μm–thick paraffin-embedded specimens are shown. Original magnification $\times$ 100 in **A–C**; $\times$ 250 in **D–F**. See Figure 1 for definitions.

IL-32, IL-1β, TNFα, IFNγ, IL-6, and IL-27p28 messenger RNA (mRNA) expression in the temporal arteries of patients with GCA. We investigated whether arterial inflammation in GCA patients was characterized by up-regulation of IL-32 and proinflammatory Th1 cytokines involved in its regulation. As shown in Figure 1A, a marked and significant increase in arterial expression of IL-32 transcripts was observed in GCA patients compared with control artery biopsy specimens (mean $\pm$ SEM 11.16 $\pm$ 0.5 and 1.25 $\pm$ 0.13; $P < 0.0001$).

Consistent with IL-32 up-regulation, levels of IL-1β, TNFα, and IFNγ were significantly increased in

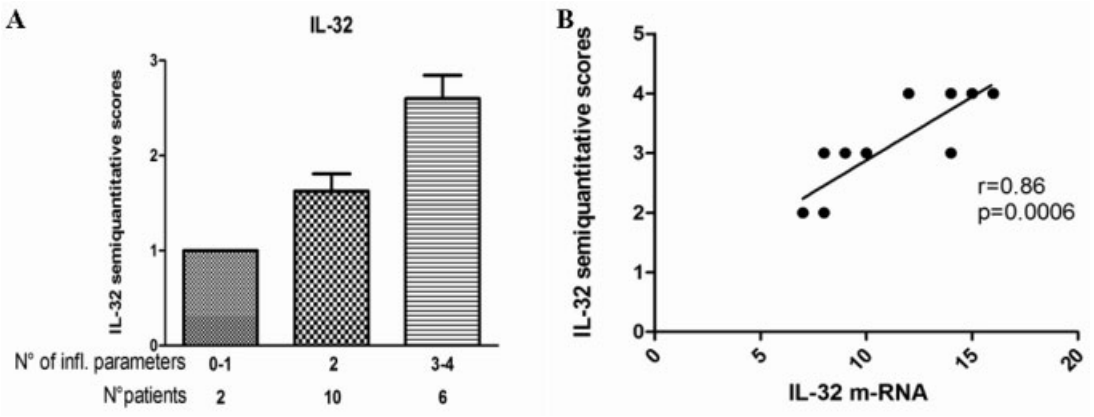

Figure 3. Correlation of immunohistochemical semiquantitative scores for interleukin-32 (IL-32) with number of inflammation (infl.) parameters and with expression levels of IL-32 mRNA. **A**, Patients with higher immunohistochemical scores for IL-32 exhibited a higher number of inflammation parameters. **B**, Semiquantitative IL-32 scores directly correlated with IL-32 mRNA levels. Values are the mean $\pm$ SEM.

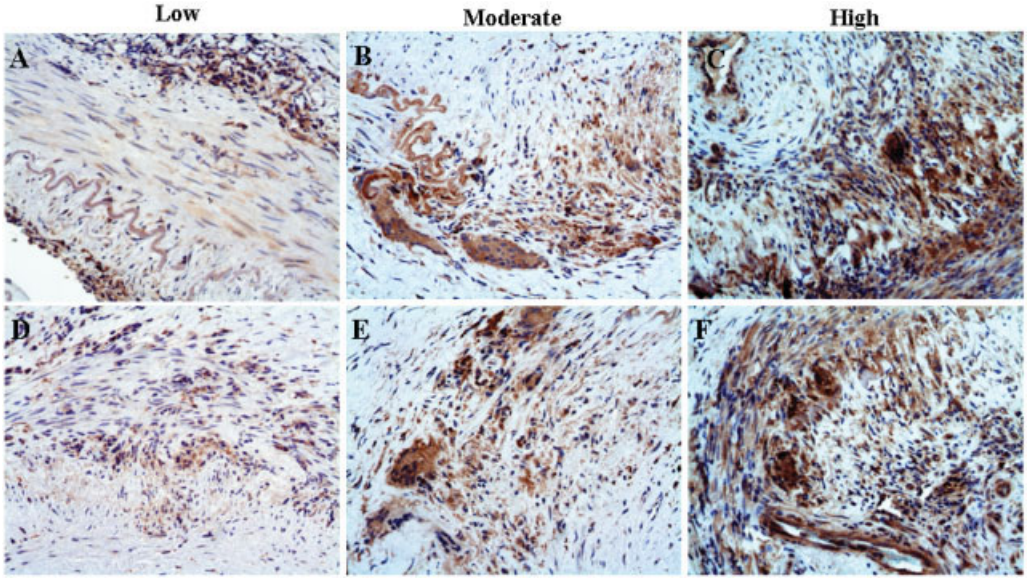

Figure 4. Evaluation of IL-32 immunostaining specificity. Representative photomicrographs of randomly selected 5-μm–thick paraffin-embedded sections of temporal artery biopsy specimens obtained from patients with GCA with low inflammation (**A** and **D**), moderate inflammation (**B** and **E**), or high inflammation (**C** and **F**), stained with 2 different anti–IL-32 antibodies (antibodies in **A–C** from BioLegend, in **D–F** from Sigma-Aldrich) are shown. The 2 antibodies yielded comparable results. Original magnification × 250. See Figure 1 for definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

GCA patients compared to controls (53.7 ± 4.24 versus 1.32 ± 0.15, 7.16 ± 0.27 versus 1.4 ± 0.33, and 45 ± 8.56 versus 4.2 ± 1.02, respectively; $P < 0.0001$) (Figures 1B–D), indicating the concomitant occurrence of a Th1 response in GCA. IL-32 up-regulation was also accompanied by strong IL-6 overexpression in GCA patients (240 ± 51.12 versus 1.8 ± 0.4; $P < 0.0001$) (Figure 1E). It is also relevant to note that IL-27p28 showed significantly higher transcript levels in GCA patients (241.66 ± 40.11 versus 14 ± 2.5) (Figure 1F).

IL-32 expression in tissue from patients with GCA. Given the strong up-regulation of IL-32 mRNA in biopsy specimens from patients with GCA, we next investigated its protein expression and distribution pat-

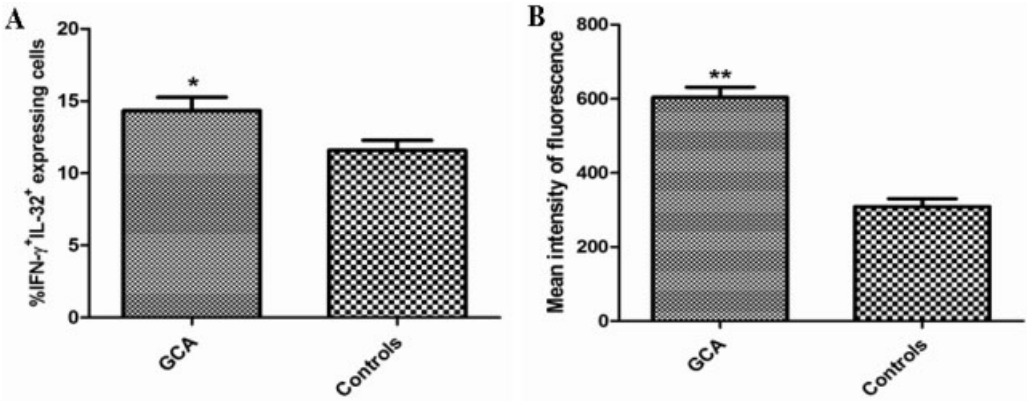

Figure 5. Th1 lymphocytes are expanded and produce high amounts of IL-32 in GCA. **A**, Higher percentage of CD4+IFNγ+IL-32+ lymphocytes in patients with biopsy-proven GCA than in controls. **B**, Twofold increase in IL-32 mean fluorescence intensity on CD4+IFNγ+ Th1 lymphocytes from GCA patients compared to controls. Bars show the mean ± SEM. * = $P = 0.0455$; ** = $P < 0.001$ versus control. See Figure 1 for definitions.

tern. Histologically normal arteries did not exhibit significant immunostaining for IL-32 (Figure 2A). IL-32+ infiltrating cells were detectable only in inflamed arterial samples from patients with GCA (Figures 2B–F). Semiquantitative evaluation of the staining for IL-32 demonstrated that 6 of the 18 patients with biopsy-proven GCA had a score of 3, 10 had a score of 2, and 2 had a score of 1. Patients with higher scores for IL-32 expression also exhibited a higher number of inflammation parameters, as described in Patients and Methods (2 patients with 0–1 parameters of inflammation each had a score of 1, 10 patients with 2 parameters of inflammation had a mean ± SEM score of 1.62 ± 0.18, and 6 patients with >2 parameters of inflammation had a score of 2.6 ± 0.24) (Figure 3A). Interestingly, the highest levels of IL-32 protein expression were observed in those patients who displayed the highest IL-32 mRNA levels.

The morphologic appearance of IL-32–infiltrating cells was mainly characterized by a large cytoplasm and often dendritic morphology, consistent with a monocyte-derived cell lineage (i.e., macrophages/dendritic cells). IL-32+ giant cells scattered through the inflammatory infiltrates were also observed in those patients exhibiting prevalent granulomatous inflammation (Figure 2D).

Proliferation and migration of VSMCs are the major determinants of intimal hyperplasia observed in inflamed arteries (15,16). VSMCs, which have been demonstrated to play a pivotal role in arterial wall inflammation through the production of proinflammatory cytokines, exhibited strong positivity for IL-32 in GCA patients (Figures 2B–F).

Finally, intense staining for IL-32 was observed in microvessels distributed within the vessel wall of inflamed arteries (Figures 2E and F). Since control specimens did not show vessels scattered through arterial wall, the majority of microvessels identified in the inflamed arteries were considered to be neovessels resulting from inflammation-dependent angiogenesis.

In order to confirm the specificity of anti–IL-32 immunostaining, we performed a new series of experiments using 2 different antibodies against IL-32 (a previously used mouse monoclonal and a rabbit polyclonal anti-human IL-32) on 3 tissue samples randomly selected, one with low expression of IL-32, one with moderate expression, and one with high expression. As shown in Figure 4, the 2 antibodies worked similarly.

IL-32 production by Th1 lymphocytes among GCA patient PBMCs. In order to assess whether peripheral Th1 cells from GCA patients could produce IL-32, the frequency of IL-32+ IFNγ-producing Th1 CD4+ T cells was evaluated in 5 patients with untreated GCA and in 5 age-matched controls. In GCA patients, the frequencies of circulating IFNγ+IL-32+ Th1 cells among CD4+ T cells were slightly but significantly expanded compared to controls (mean ± SEM 14.33 ± 0.92% versus 11.58 ± 0.7%; $P = 0.0455$) (Figure 5A). A 2-fold increase in IL-32 mean fluorescence intensity on CD4+IFNγ+ Th1 lymphocytes was observed in GCA patients compared to controls (603.5 ± 27.86 versus 308.8 ± 20; $P < 0.001$) (Figure 5B).

DISCUSSION

IL-32 is a recently described Th1 cytokine that is mainly produced by natural killer cells, T cells, epithelial cells, and blood monocytes (4). It is prominently induced by IFNγ (4), and it has been demonstrated to be an important mediator of both innate and adaptive immune response. Additionally, IL-32 exhibits several properties typical of proinflammatory cytokines, inducing the release of cytokines and chemokines (17).

IL-32 overexpression has been demonstrated in human inflammatory disorders such as RA and Crohn's disease. In RA synovial tissue, IL-32 is highly expressed and induces active osteoclast generation, and its levels correlate with the severity of inflammation and TNFα expression (9–12). IL-32 expression is also markedly increased in the inflamed mucosa of patients with inflammatory bowel disease compared to unaffected mucosa (8).

Although the extremely small number of patients enrolled limits the strength of the conclusions, this is the first reported study to show that IL-32 expression is markedly up-regulated in the inflamed arteries of patients with GCA. IL-32 up-regulation was accompanied by strong overexpression of IL-27p28, which is a potent and earlier inducer of Th1 polarization (13). IL-27, in fact, commits naive CD4+ T cells to differentiate into Th1 cells by inducing IL-12 receptor β2 and T-bet (12), as well as inducing production of Th1 cytokines such as IL-1β and TNFα in monocytes (18,19) and overexpression of IL-6 in RA fibroblast-like synoviocytes (20). In this regard, IL-6, the expression of which depends on IL-27 and IL-32 signaling (21,22), was also up-regulated in our GCA patients. These findings prompted us to hypothesize that IL-32 and IL-27p28 could participate in a positive-feedback mechanism, leading to the induction and perpetuation of arterial inflammatory immune responses.

GCA is a chronic vasculitis in which both the innate and the adaptive immune systems play a patho-

genetic role (1). Historically, GCA has been considered as a Th1-mediated disease (2). Recently, Deng et al (3) have reported a concomitant Th1 and Th17 response in tissue samples from GCA patients. In our study, IL-32 expression was associated with a strong Th1 inflammatory response, as demonstrated by the increased levels of IFNγ and IL-27p28.

IL-32 expression has been demonstrated in natural killer cells, epithelial and endothelial cells, lymphocytes, and blood monocytes (17). Endothelial cells are key elements in the development of inflammatory infiltrates through the cytokine-dependent overexpression of adhesion molecules. Neoangiogenesis is noted in most GCA specimens (23), and neovessels are mainly where leukocyte–endothelial cell interactions take place. IL-32 has also been demonstrated to contribute directly to endothelial cell activation by regulating adhesion molecule expression; our observation of intense IL-32 expression occurring prevalently in neovessels of inflamed arteries provides strong evidence for a pivotal role of IL-32 in the organization of the vascular inflammatory response.

In GCA, all injury mechanisms have been related to effector macrophages (16). IL-32 has been demonstrated to induce giant cell formation from CD14+ monocytes (12) and in the present study, macrophages and giant cells showed intense positivity for IL-32, emphasizing the central role of these cells in the pathogenesis of GCA. Macrophages and giant cells produce platelet-derived growth factor and vascular endothelial growth factor, which actively induce proliferation of VSMCs and intimal hyperplasia (15). VSMCs from inflamed arteries of GCA patients showed a strong positivity for IL-32 in the present study, suggesting a direct immunologic participation in arterial inflammation. The exact role of IL-32 on muscular cell activation and proliferation, however, needs to be better elucidated.

Sensing of microbial pathogens is primarily mediated by the recognition of pathogen-associated molecular patterns, which are conserved structures expressed by microbes of the same class. The most important of these recognition systems are the Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) families of proteins (25). IL-32 and IL-27 overexpression observed in GCA patients could be the result of innate immune system activation, since IL-32 has been demonstrated to specifically synergize with NOD1 and NOD2 ligands for the synthesis of IL-1β and IL-6 (21), and IL-27p28 is induced through stimulation of TLR-3, TLR-4, TLR-7, and TLR-8 (26). In inflamed arteries, IL-27p28 could induce a cytokine milieu that is favorable for IL-32 expression through the induction of IL-32–inducing cytokines, such as IL-1β and TNFα; both IL-32 and IL-27p28 may in turn stimulate Th1 lymphocyte functions, thereby transforming the innate immunity signals into adaptive immunity responses.

In conclusion, the demonstration of IL-32 overexpression in inflamed arteries of GCA patients implicates IL-32 as a pathogenetic mediator participating in the arterial wall inflammation occurring in this disease. The correlation of IL-32 expression with clinical markers of disease activity could support a potential use of therapeutic strategies directed at reducing IL-32 activity in patients with GCA.

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. Triolo 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. Ciccia, Alessandro, Rizzo, Principe, Raiata, Cavazza, Guggino, Accardo-Palumbo, Boiardi, Ferrante, Principato, Giardina, De Leo, Salvarani, Triolo.

Acquisition of data. Ciccia, Alessandro, Rizzo, Principe, Raiata, Cavazza, Guggino, Accardo-Palumbo, Boiardi, Ferrante, Principato, Giardina, De Leo, Salvarani, Triolo.

Analysis and interpretation of data. Ciccia, Alessandro, Guggino, Accardo-Palumbo, De Leo, Triolo.

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