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Increased expression of interleukin-22 in patients with giant cell arteritis

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Abstract

Objectives. GCA is characterized by arterial remodelling driven by inflammation. IL-22 is an attractive cytokine which acts at the crosstalk between immune and stromal cells. We hypothesized that IL-22 might be induced in GCA and might be involved in disease pathogenesis.

Methods. Patients subjected to temporal artery biopsies (TABs) naïve from therapy were enrolled: 27 biopsy-proven GCA, 8 biopsy-negative GCA, 21 biopsy-negative non-GCA patients. Expression of IL-22 was determined in TABs by immunohistochemistry, in plasma by ELISA, in peripheral blood mononuclear cells by real-time PCR and flow cytometry. Effects of IL-22 on viability and gene expression of primary cultures obtained from TABs were also evaluated.

Results. Inflamed TABs from GCA patients showed a higher expression of IL-22 and IL-22 specific receptor subunit (IL-22R1) than non-inflamed TABs. IL-22 was expressed in infiltrating immune cells and spindle shaped cells, IL-22R1 was expressed in endothelial cells. Patients with biopsy-proven GCA showed increased levels of IL-22 in plasma than patients with biopsy-negative GCA, without GCA and healthy subjects. Peripheral blood mononuclear cells from GCA patients expressed higher IL-22 transcript than healthy subjects. After stimulation *in vitro* with phorbol 12-myristate 13-acetate and ionomycin, the frequencies of Th22 and IL-22⁺ CD4⁺ lymphocytes were similar between patients with and without GCA. Treatment with IL-22 of primary cultures obtained from TABs increased cell viability under stress conditions and expression of B-cell activating factor.

Conclusion. IL-22 is increased in patients with GCA and affects viability and gene expression of arterial cells, supporting a potential role in disease pathogenesis.

Key words: giant cell arteritis, interleukin-22, arterial remodelling, inflammation, autoimmunity, pathogenesis

Rheumatology key messages

- IL-22 and IL-22R1 are expressed at higher levels in inflamed temporal artery biopsies from GCA patients.
- Patients with biopsy-proven GCA have increased levels of IL-22 in plasma.
- IL-22 might contribute to arterial remodelling and B cell responses in patients with GCA.

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Introduction

Giant cell arteritis (GCA) is the most common vasculitis in western countries in individuals older than 50 years of age. It is a chronic inflammatory disease affecting large and medium-sized arteries, especially the branches of the proximal aorta [1, 2]. What triggers GCA is actually unknown. What is known is that arteries from GCA patients show granulomatous infiltrate mainly consisting of CD4⁺ T lymphocytes of Th1 and Th17 subsets and macrophages with an increased production of cytokines (e.g. IFN γ , TNF α , IL-1 β , IL-2, IL-6, IL-9, IL-17 and IL-21), growth factors (e.g. vascular endothelial growth factor, fibroblast growth factor-2, platelet-derived growth factor, endothelin-1), elastolytic and proteolytic enzymes [1, 3, 4]. Inflammation leads to arterial remodelling and intimal hyperplasia with possible subsequent stenoses or occlusions resulting in ischaemic events [5].

IL-22 belongs to the IL-10 cytokine family. It signals through a heterodimeric receptor composed of a chain specific for IL-22 (IL-22R1) and a chain shared with IL-10 (IL-10R2). What makes IL-22 attractive is that it is produced by immune cells but acts only on non-haematopoietic cells because IL-22R1 has been detected only in stromal cells (e.g. epithelial and endothelial cells and fibroblasts). IL-22 has been reported to regulate the crosstalk between cells of the immune system and tissue-resident cells, particularly at barrier surfaces (e.g. respiratory, gastrointestinal systems and skin) [6, 7]. It has immunomodulator properties in infection, inflammation, autoimmunity, cancer, tissue regeneration and host defence. It is noteworthy that IL-22 increased expression can be either pathological or protective, depending on the duration and amount of IL-22, the overall cytokine milieu and tissue type [8]. We hypothesized that IL-22 might be expressed in GCA because IL-6, IL-21 and Notch pathways, which are activated in GCA [9–13], can induce the production of IL-22 [14]. Moreover, IL-22 might have a role in arterial remodelling, because it regulates the intercommunication between the immune system and stromal cells.

At the moment, no data are available on the IL-22 pathway in GCA. Our aim was to investigate if IL-22 is differentially expressed by patients with GCA compared with patients without GCA and determine the effects of IL-22 on primary cultures obtained from arteries.

Methods

Patients

Temporal artery biopsies (TABs) were performed for diagnostic purposes to confirm GCA histologically [2, 15, 16]. Two cohorts of patients subjected to TABs for a suspicion of GCA were included in the study. All patients were naïve for corticosteroid therapy at the time of TAB and blood withdrawal. One cohort was enrolled at the section of Rheumatology at the University of Palermo and was dedicated to immunohistochemistry analyses on TABs. It included 15 patients with GCA with inflamed TABs and 12 patients with non-inflamed TABs who received a

different diagnosis. All patients with GCA and none of the non-GCA patients satisfied the ACR criteria for GCA [17]. The other cohort was recruited at the section of Rheumatology at the Arcispedale Santa Maria Nuova-IRCCS (Reggio Emilia, Italy) from June 2012 to June 2016 and was dedicated to systemic analyses on plasma and peripheral blood mononuclear cells (PBMCs). It included the following: patients with GCA with positive TABs showing transmural inflammation as defined by Cavazza *et al.* [16] (n = 12); patients with negative, non-inflamed TABs diagnosed with GCA on the basis of clinical, imaging, laboratory findings and follow-up evaluation (n = 8; median age: 74 years, interquartile range (IQR): 67–81 years; median follow-up: 10 months, IQR: 7–25 months); and patients with negative TABs who received a different diagnosis (non-GCA patients; n = 9). All patients with TAB-positive GCA, 7/8 patients with TAB-negative GCA and none of the non-GCA patients satisfied the ACR criteria for GCA. The two cohorts of patients were similar regarding demographic and clinical features with the exception of TAB-positive GCA patients from Reggio Emilia who were older compared with TAB-positive GCA patients from Palermo (supplementary Table S1, available at *Rheumatology* Online).

For systemic analyses, 12 age-matched healthy subjects were recruited as unaffected controls. Their median age was 77 years (IQR: 72–78 years). They did not have any autoimmune diseases, cardiovascular diseases, infections and cancers.

This study was approved by the Local Ethics Committee (Reggio Emilia, Italy) in compliance with the Declaration of Helsinki and informed consent was obtained from all patients and healthy subjects.

Biologic samples

Peripheral blood was collected in EDTA-coated tubes. PBMCs were isolated by histopaque-1077 density gradient centrifugation (Sigma-Aldrich, St Louis, MO, USA) and stored frozen in liquid nitrogen in 90% heat-inactivated fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) 10% dimethyl sulfoxide (Sigma-Aldrich) until use.

Immunohistochemistry

TABs were immediately fixed with 4% formaldehyde and embedded in paraffin. Immunohistochemical analysis for IL-22, IL-22R1 and B cell activating factor (BAFF) was performed on 5- μ m-thick paraffin-embedded sections from arteries and from tonsils (used as positive controls) as previously described [18, 19]. Rabbit polyclonal anti-human IL-22 (1:200 dilution) (Novus Biologicals, Littleton, CO, USA), rabbit polyclonal anti-human IL-22R1 (1:100 dilution) (Novus Biologicals) and rabbit polyclonal anti-human BAFF (1:200 dilution) (Abcam, Cambridge, UK) were used as primary antibodies. The isotype-matched irrelevant rabbit IgG polyclonal antibody (ab27472, Abcam) was used as negative control. The number of IL-22, IL-22R1 and BAFF expressing cells was determined by counting immunoreactive cells on photomicrographs

obtained from three randomly obtained high-power microscopic fields under a DM2000 optical microscope, using a DFC320 digital camera (Leica, Wetzlar, Germany).

ELISA

Concentration of IL-22 in plasma was determined with the Human IL-22 ELISA kit from RayBiotech (Norcross, GA, USA) following the manufacturer's instructions. Plasma was diluted 2-fold with assay diluent A as recommended by the manufacturer. Standards and samples were incubated overnight at 4 °C with gentle shaking. The experimental minimum detectable dose of IL-22 was 32 pg/ml. The intra-assay coefficient of variation was <10% and the inter-assay coefficient of variation was <12%.

IL-22 treatment of primary cultures from TABs

Primary cultures were obtained from TABs following the method developed by Lozano *et al.* [20] and recently described [19]. Such cultures showed a myofibroblast phenotype expressing markers of smooth muscle cells (smooth muscle α -actin) and fibroblasts (fibroblast activating protein and fibroblast specific protein) but not of endothelial cells (von Willebrand factor), determined by real-time PCR. Forty thousand cells were seeded in each well of six-well plates in 2 ml DMEM with high glucose + 10% FBS (Gibco, Thermo Fisher Scientific), 2 mM glutamine and 50 μ g/ml gentamicin (Sigma-Aldrich) and cultured at 37 °C, 5% CO₂. After 24 h they were treated with IL-22 (50 ng/ml) (research grade from Miltenyi Biotec, Bergisch Gladbach, Germany) in 2 ml complete medium. Cells were collected after 6 and 24 h of treatment for gene expression analyses.

Cell viability

Cells obtained from TABs were seeded at 1500 cells per well in 96-well plates in 100 μ l DMEM with high glucose + 10% FBS, 2 mM glutamine and 50 μ g/ml gentamicin. The following day medium was replaced with medium containing IL-22 (10, 50, 100 ng/ml) in the presence and absence of FBS. Treatment was renewed every 72 h. To assess cell viability, the proliferation reagent WST-1 (Sigma-Aldrich) was used (1:10 final dilution) and optical density at 450 and 620 nm was measured after 4 h incubation at 37 °C, 5% CO₂.

Gene expression analysis

Total RNA was extracted from the primary cultures and PBMCs with the miRNeasy micro kit (Qiagen, Hilden, Germany). RNA (62.5 ng) was retro-transcribed with the Takara (Kusatsu, Japan) reverse transcription kit in a total volume of 20 μ l following the manufacturer's instruction with the elimination of genomic DNA. cDNA was diluted twice with RNase and DNase free water then expression of IL-22 (Hs01574154_m1), IL-22R1 (Hs00222035_m1), BAFF (Hs00198106_m1), a proliferation-inducing ligand (APRIL; Hs00601664_g1), chemokine (C-X-C motif) ligand 13 (CXCL13; Hs00757930_m1) and chemokine (C-C motif) ligand 21 (CCL21; Hs00989654_g1), was quantified by real-time PCR with TaqMan gene expression assays

and master mix (all from Thermo Fisher Scientific). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs02758991_g1, Thermo Fisher Scientific) was used to normalize data.

Flow cytometry

PBMCs were thawed and counted with a Fuchs-Rosenthal haemocytometer (Sigma-Aldrich). After overnight resting at 37 °C, 5% CO₂ in RPMI 1640 + 10% heat inactivated FBS, PBMCs were stimulated for 4 h with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μ g/ml ionomycin (Sigma-Aldrich) at 1×10^6 cells in 1 ml RPMI 1640 + 10% FBS. After 30 min stimulus, brefeldin A (Sigma-Aldrich) was added at 10 μ g/ml. Cells were collected and stained with 100 μ l Live/Dead Fixable Dead Cell Stain near-IR-fluorescent reactive dye at 0.1% in PBS (Thermo Fisher Scientific) for 10 min at room temperature and then with antibodies against the surface antigens CD4 and CD56 for 20 min at room temperature. PerCP-Cy5.5 mouse anti-human CD4 (clone RPA-T4, from BD) and PE anti-human CD56 (clone REA196 from Miltenyi Biotec) were diluted in 100 μ l PBS. After washing with PBS + 1% FBS, PBMCs were fixed and permeabilized with 250 μ l BD Cytofix/Cytoperm Fixation/Permeabilization solution (BD Biosciences, San Jose, CA, USA) for 20 min at 4 °C. After two washing with the BD Perm/Wash buffer, PBMCs were stained with antibodies against intracellular cytokines: anti-human IL-17A PE-Cyanine7, anti-human/mouse IL-22 APC, anti-human IFN- γ Alexa Fluor 488 (all from Thermo Fisher Scientific) in 100 μ l Perm/Wash buffer for 30 min at 4 °C. Antibodies were diluted as recommended by the manufacturers. After two washing with the BD Perm/Wash buffer, PBMCs were resuspended in PBS + 1% FBS and acquired with the FACSCanto II (BD Biosciences). Data were analysed with FACSDiva software (BD Biosciences). At least 60000 live lymphocytes were acquired.

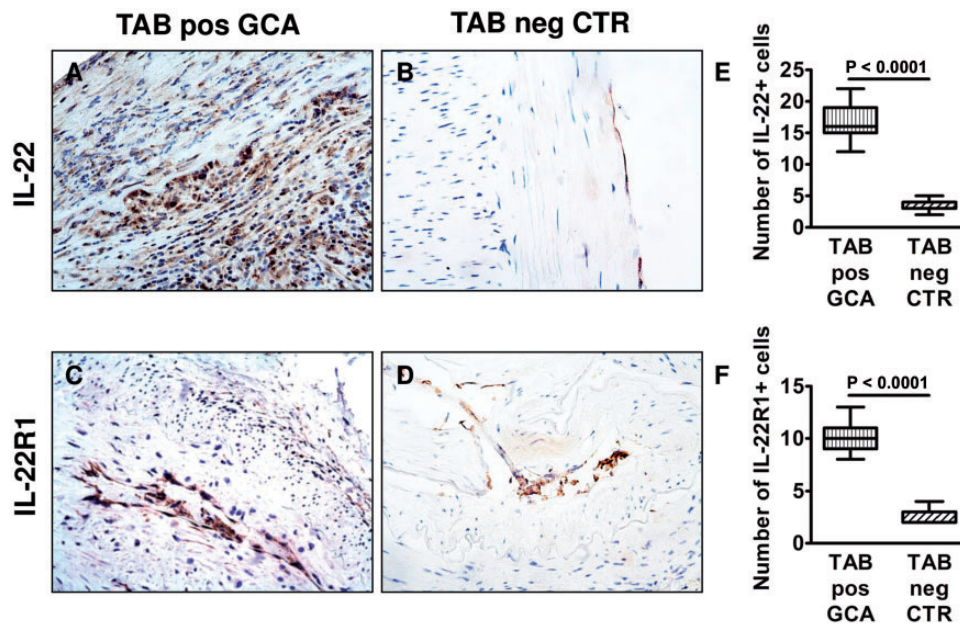
Statistical analysis

Statistical analyses were performed with Prism 6 software (GraphPad Software, La Jolla, CA, USA). The Mann-Whitney U test was used for comparisons between two groups, Spearman's test was used for correlations between two variables and a receiver operating characteristic curve was used to assess the performance of a binary classifier system. Fisher's exact test was used to analyse tables. $P < 0.05$ (two-tailed) was considered statistically significant.

Results

Increased expression of IL-22 and IL-22R1 in inflamed TABs

To evaluate whether IL-22 and its specific receptor (IL-22R1) were expressed in TABs and by which kinds of cells, immunohistochemistry was performed on inflamed TABs from GCA patients ($n = 15$) and normal TABs from non-GCA patients ($n = 12$). IL-22 was detected in infiltrating immune cells and spindle-shaped cells in inflamed

Fig. 1 Presence of IL-22 and IL-22R1 in temporal artery biopsies

(A–D) Representative images showing the expression of IL-22 and IL-22R1 in inflamed (A, C) and non-inflamed (B, D) TABs by immunohistochemistry. Diaminobenzidine was used as chromogen (Dako, Agilent, Santa Clara, CA, USA) and slides were slightly counterstained with haematoxylin before dehydration and mounting in DePex (VWR International, Milan, Italy). (E, F) Semi-quantitative analysis of IL-22 (E) and IL-22R1 (F) expressing cells in inflamed ($n = 15$) and non-inflamed ($n = 12$) TABs. Whiskers represent the 5th and the 95th percentile. Data were compared by the Mann–Whitney U test. IL-22R1: interleukin-22 receptor specific chain; TAB: temporal artery biopsy.

TABs (Fig. 1A), at a higher level in inflamed than normal TABs (comparison between Fig. 1A and B). Expression of IL-22R1 was detected in endothelial cells facing the arterial lumen and some adventitial cells, more in inflamed than normal TABs (comparison between Fig. 1C and D). The number of immunoreactive cells per field was significantly higher in inflamed compared with normal TABs for both IL-22 and IL-22R1 (Fig. 1E and F).

Systemic production of IL-22

To investigate whether IL-22 was deregulated systemically, levels of IL-22 were determined in plasma and PBMCs from patients with GCA (both TAB positive and TAB negative), patients subjected to TABs but with a final diagnosis different from GCA and a cohort of age matched healthy subjects. Concentration of IL-22 in plasma was significantly higher in patients with TAB positive GCA compared with patients with TAB negative GCA, non-GCA patients and healthy controls (Fig. 2A). Patients with TAB positive GCA clustered in two groups: with a concentration of IL-22 higher than 277 pg/ml and with a concentration of IL-22 equal to or lower than 277 pg/ml. This cut-off level classified patients with TAB positive GCA vs healthy subjects with 58% sensitivity and 92% specificity and vs TAB negative non-GCA patients with 58% sensitivity and 89% specificity by means of receiver operating

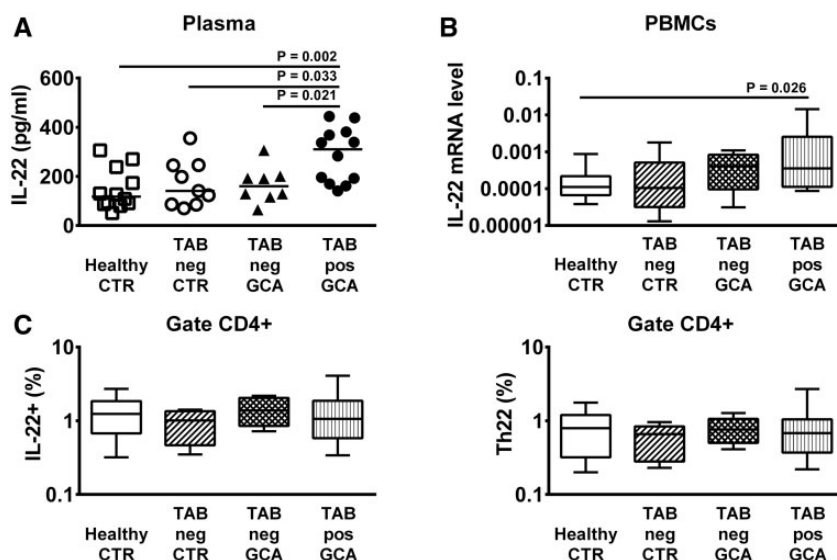
characteristic analysis (area = 0.8681, $P = 0.002$ and area = 0.7824, $P = 0.030$, respectively). Patients with a clinical diagnosis of GCA but with a negative TAB had levels of IL-22 equal to non-GCA patients and healthy subjects (Fig. 2A).

To determine whether circulating levels of IL-22 were associated with demographic and clinical parameters, patients subjected to TAB were divided according to the cut-off level of IL-22. There were no differences in the analysed parameters at diagnosis between patients with higher and lower IL-22 concentration in plasma (Table 1). Nevertheless, there was a positive correlation between IL-22 and CRP levels (Spearman's test, $P = 0.0310$, $r = 0.4012$, $n = 29$).

We then analysed levels of IL-22 in PBMCs *ex vivo* (by real-time PCR and flow cytometry) and after 4 h stimulation with PMA and ionomycin in the presence of brefeldin A (by flow cytometry). The known cellular sources of IL-22 are T helper cells (Th1, Th17, Th22), NK cells, $CD8^+$ T cells, $\gamma\delta$ T cells, innate lymphoid cells of group 3 and fibroblasts [7, 14]. We analysed $CD4^+$ T helper and $CD56^+$ NK lymphocytes by flow cytometry.

IL-22 transcript was higher in PBMCs from patients with TAB positive GCA compared with healthy subjects but no differences were found between patients with TAB positive GCA and those with TAB negative GCA and without GCA (Fig. 2B). There was a positive correlation between

Fig. 2 Systemic production of IL-22



(A) Concentration of IL-22 in plasma was determined by ELISA. Each symbol represents an individual subject. Horizontal lines show the median. (B) Box plot visualization of the levels of IL-22 mRNA in PBMCs *ex vivo* determined by real-time PCR. Expression as $2^{-\Delta C_t}$ (where ΔC_t is IL-22 C_t - GAPDH C_t) is shown on the y-axis. The median C_t of IL-22 amplification product in PBMCs from TAB positive GCA patients was 36.3; the median C_t of GAPDH was 24.4. (C) Box plot visualization of the frequencies of IL-22⁺ and Th22 (IL-22⁺ IL-17⁻ IFN γ ⁻) cells in the CD4⁺ lymphocyte gate determined by flow cytometry after 4 h stimulation of PBMCs *in vitro* with PMA + ionomycin in presence of brefeldin A. Dead cells were excluded from analysis. Whiskers represent the 5th and the 95th percentile. Analyses were performed on plasma samples and PBMCs from 12 biopsy-proven GCA patients (TAB pos GCA), 8 biopsy-negative GCA patients (TAB neg GCA), 9 biopsy-negative non-GCA patients (TAB neg CTR) and 12 healthy subjects (Healthy CTR). Data were analysed by Mann-Whitney U test. Ct: threshold cycle; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; PBMC: peripheral blood mononuclear cell; PMA: phorbol 12-myristate 13-acetate; TAB: temporal artery biopsy.

TABLE 1 Characteristics of patients stratified on the basis of circulating levels of IL-22

Demographic, clinical and laboratory characteristics	IL-22 > 277 pg/ml (n = 9)	IL-22 ≤ 277 pg/ml (n = 20)
Age at disease onset, median (IQR), years	80 (71–82)	74 (67–78)
Any cranial symptoms ^a , n (%)	7 (78)	16 (80)
Abnormalities of temporal arteries ^b , n (%)	6/7 (86)	8/19 (42)
Any visual symptoms, n (%)	2 (22)	2 (10)
Systemic signs/symptoms ^c , n (%)	6 (67)	13 (65)
PMR, n (%)	5 (56)	10 (50)
ESR, median (IQR), mm/h	82 (70–95)	62 (14–90)
CRP, median (IQR), mg/dl	3.6 (3.1–12.0)	3.2 (0.8–7.2)
Haemoglobin, median (IQR), g/dl	11.7 (10.5–13.6)	12.6 (11.4–13.0)
Platelets, median (IQR), × 10 ³ /μl	352 (276–406)	310 (233–405)
Leucocytes, median (IQR), × 10 ³ /μl	8.5 (6.8–10.0)	8.3 (7.4–10.0)
Lymphocytes, median (IQR), × 10 ³ /μl	1.6 (1.5–2.1)	1.8 (1.4–2.2)
Monocytes, median (IQR), × 10 ³ /μl	0.7 (0.5–0.8)	0.6 (0.4–0.7)
Neutrophils, median (IQR), × 10 ³ /μl	6.1 (4.0–7.6)	6.0 (4.1–7.5)

^aAt least one of the following: headache, scalp tenderness, jaw claudication. ^bTemporal artery tenderness to palpation or decreased pulsation. This information was not available for all patients. ^cAt least one of the following: fatigue, anorexia, weight loss of at least 4 kg, fever. IQR: interquartile range; TAB: temporal artery biopsy.

concentration of IL-22 in plasma and levels of IL-22 transcript in PBMCs (Spearman's test, $P = 0.0415$, $r = 0.3809$, $n = 29$).

Production of IL-22 was not detected by flow cytometry in unstimulated PBMCs but was detected after stimulation with PMA and ionomycin. The percentages of IL-22⁺ and Th22 cells in the CD4⁺ lymphocyte gate were similar in patients with TAB positive GCA, TAB negative GCA, without GCA and healthy controls (Fig. 2C). The Th22 subset was defined as IL-22⁺ IFN γ ⁻ IL-17⁻ CD4⁺ lymphocytes. There were also CD4⁺ lymphocytes expressing both IL-22 and IL-17 or IL-22 and IFN γ , with no differences between groups (data not shown). More than 94% of the IL-22 expressing lymphocytes were CD4⁺. CD56⁺ lymphocytes did not produce or negligibly produced IL-22.

IL-22 effects on primary cultures obtained from TABs

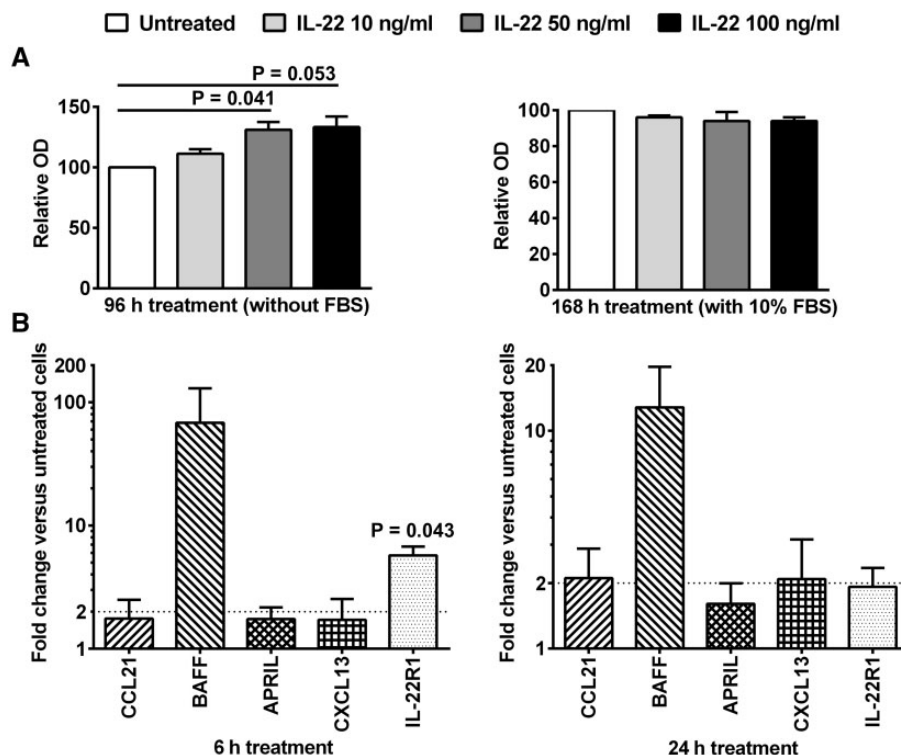
To determine possible effects of IL-22 on arterial cells, primary cultures obtained from normal TABs were treated *in vitro* with IL-22. Cells could be treated up to 96 h in the absence of FBS; afterwards they started to detach. In the

presence of FBS, cells were treated for a longer time (168 h) because they grow slowly with a duplication time of about 168 h. Treatment with IL-22 in the absence of FBS dose-dependently increased cell viability evaluated by the WST-1 metabolic assay (Fig. 3A, left) while treatment with IL-22 in the presence of 10% FBS did not affect cell viability (Fig. 3A, right). It has been shown that IL-22 can favour production of chemokines and assembly of tertiary lymphoid organs [21]. Treatment with IL-22 of primary cultures obtained from TABs increased gene expression of BAFF and IL-22R1 while it did not modify APRIL, CXCL13 or CCL21 gene expression (Fig. 3B). There was a change greater than 2-fold in BAFF gene expression between IL-22 treated and untreated cells in all the cultures after 6 h treatment and in 2/3 cultures after 24 h treatment. IL-22R1 gene expression was significantly increased only after 6 h treatment with IL-22 (Fig. 3B).

Correlation between IL-22 and BAFF levels in TABs

To verify whether IL-22 was biologically active in TABs, levels of BAFF were determined by

Fig. 3 Effects of IL-22 on primary cultures obtained from temporal artery biopsies



(A) Cultures obtained from TABs were treated with different doses of IL-22 in absence and presence of FBS. Cell viability was determined through the WST-1 colorimetric assay. OD is shown relative to that of untreated cells. (B) Cultures obtained from TABs were treated with 50 ng/ml IL-22 for 6 h and 24 h. Expression of CCL21, BAFF, APRIL, CXCL13, IL-22R1 was determined by real-time PCR and is shown relative to that of untreated cells by the formula: $2^{-\Delta\Delta C_t}$. Bars represent the mean with SEM of three independent experiments performed on three different cultures. Data were analysed by one sample *t*-test vs a theoretical mean of 100 (A) or 1 (B). APRIL: a proliferation-inducing ligand; BAFF, B cell activating factor; CCL21: chemokine (C-C motif) ligand 21; CXCL13: chemokine (C-X-C motif) ligand 13; FBS: fetal bovine serum; IL-22R1: interleukin-22 receptor specific chain; OD, optical density; TAB: temporal artery biopsy.

immunohistochemistry. BAFF expression was observed in neo-vessels and smooth muscle cells present in the medial layer, in infiltrating inflammatory mononuclear cells as well as in the context of granulomatous inflammation (Fig. 4A and B). The number of BAFF positive cells per field was significantly higher in inflamed than normal TABs (Fig. 4C) and directly correlated with the number of IL-22 positive cells per field in inflamed TABs (Fig. 4D).

Discussion

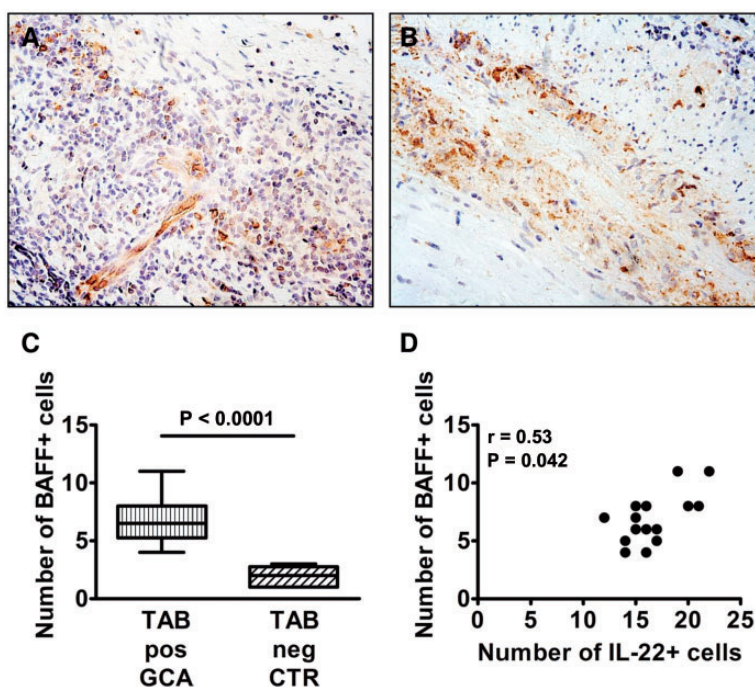
This is the first report about the IL-22 pathway in GCA. We found that IL-22 and IL-22R1 were expressed in TABs at higher levels in inflamed TABs from patients with GCA compared with normal, non-inflamed TABs. In addition, concentration of IL-22 was increased in plasma from biopsy-proven GCA patients compared with healthy subjects and biopsy-negative GCA and non-GCA patients. We might speculate that temporal artery inflammation is necessary but not sufficient to increase circulating levels of IL-22 and that pathways upstream of the systemic production of IL-22 are activated in a subset of biopsy-proven GCA patients (see below) but not in biopsy-negative GCA patients.

IL-22 transcript was detected in PBMCs from GCA patients *ex vivo*, but at a low level. Accordingly, IL-22 protein

was not detected in PBMCs unless they were stimulated with PMA plus ionomycin *in vitro*, meaning that IL-22 production mainly occurs at the tissue level where lymphocytes are activated. IL-22 expressing lymphocytes in PBMCs were mainly CD4⁺ (including Th1 and Th17 lymphocytes) and the CD4 negative ones were not CD56⁺, indicating that circulating cells of the NK lineage did not produce IL-22 in GCA.

Two subsets of biopsy-proven GCA patients, who did not differ clinically and demographically at diagnosis, emerged on the basis of plasmatic concentration of IL-22: high and low producers. This might reflect an enhanced activation of pathways upstream of IL-22 production (e.g. IL-6, IL-21, IL-23, Notch and aryl hydrocarbon receptor) [14] in a subset of patients. In particular, IL-23 is the main factor driving IL-22 expression *in vivo*, which seems indispensable for IL-22 induction during inflammation. Data on IL-23 in GCA are scarce but indicate a likely activation of the IL-23 pathway. Indeed, PBMCs from GCA patients showed a higher production of IL-23 than those from healthy subjects after stimulation with PMA plus ionomycin [12, 22]. Moreover, IL-23p19 peptide, one of the subunits of IL-23, has been recently detected in inflamed TABs, likely inducing expression of adhesion molecules in endothelial cells and favouring leucocyte attachment and transendothelial migration [23].

Fig. 4 Correlation between IL-22 and B cell activating factor levels in inflamed temporal artery biopsies



(A, B) Representative images showing BAFF immunostaining in inflamed TABs from GCA patients. (C) Semi-quantitative analysis of BAFF-positive cells in inflamed ($n = 15$) and non-inflamed ($n = 12$) TABs. Whiskers represent the 5th and the 95th percentile. Data were compared by the Mann-Whitney U test. (D) Correlation between the number of BAFF-positive cells and IL-22-positive cells in inflamed TABs (Spearman's correlation, $n = 15$). BAFF: B cell activating factor; TAB: temporal artery biopsy.

IL-22 and Th22 lymphocytes have been found deregulated in other inflammatory and autoimmune diseases. Therefore the IL-22–IL-22R1 axis is emerging as an attractive target for therapeutic interventions [24]. In particular, there is good evidence that inhibiting the IL-22–IL-22R1 axis could be beneficial in psoriasis, while its enhancement could be beneficial in inflammatory bowel diseases [24], which underlies that this axis is two-faced. Increased production of IL-22 and/or frequency of Th22 have been reported also in patients with RA, spondyloarthritis, SLE, multiple sclerosis, Behçet's disease, myositis and SS, but their role in these disease is still controversial and needs further investigations [18, 25–27].

IL-22 and Th22 lymphocytes have also been found deregulated in cardiovascular diseases, which can have some pathogenic overlaps with GCA. Levels of IL-22 in plasma and Th22 lymphocytes are increased in patients with acute myocardial infarction and unstable angina compared with stable angina and healthy controls [28]. Moreover, in a mouse model of atherosclerosis, IL-22 promoted plaque formation and vascular repair by stimulating dedifferentiation of medial smooth muscle cells into a synthetic phenotype and migration into the intimal layer. Mice deficient for IL-22 showed reduced plaque size compared with mice expressing IL-22 [29]. These data suggest a pathogenic role for IL-22 in cardiovascular diseases that might also occur in GCA.

Our study has some limitations but also some strong points. The number of patients was low and therefore it is necessary to confirm our findings on larger cohorts. However, we analysed only patients naïve from therapy. Having patients naïve from therapy is difficult because as soon as GCA is suspected, corticosteroid therapy must be started to prevent ischaemic cranial events. Because of this, only 29 patients with a suspicion of GCA reached our hospital naïve from therapy from June 2012 to June 2016. Another limitation of the present study is that although we detected IL-22 as expressed at different levels in GCA compared with non-GCA patients, the role of IL-22 (pathogenic or protective) in GCA needs to be further defined. However, in an attempt to identify possible effects of IL-22, we treated primary cultures obtained from TABs with IL-22 *in vitro*. IL-22 increased cell viability but only in the presence of stressed conditions (growth in absence of FBS). Moreover, primary cultures from TABs showed an increased expression of BAFF after treatment with IL-22. There was a positive correlation between the number of IL-22 and BAFF immunoreactive cells per field in inflamed TABs indicating that IL-22 can signal in TABs. BAFF regulates the proliferation, differentiation and maintenance of B cells. It has been shown to be increased in TABs (data here and reference [19]) as well as in plasma from GCA and PMR patients and associated with disease activity [30]. We can thus speculate that IL-22 might be involved in arterial remodelling and B cell responses in patients with GCA.

In conclusion, we documented for the first time an increased expression of IL-22 in temporal arteries and plasma from patients with GCA and effects of IL-22 on

viability and gene expression of primary cultures established from TABs, suggesting that IL-22 might be involved in disease pathogenesis and unveiling a new potential therapeutic target in GCA.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

References

- Salvarani C, Pipitone N, Versari A, Hunder GG. Clinical features of polymyalgia rheumatica and giant cell arteritis. *Nat Rev Rheumatol* 2012;8:509–21.
- Salvarani C, Cantini F, Hunder GG. Polymyalgia rheumatica and giant-cell arteritis. *Lancet* 2008;372:234–45.
- Weyand CM, Goronzy JJ. Giant-cell arteritis and polymyalgia rheumatica. *N Engl J Med* 2014;371:50–7.
- Watanabe R, Hosgur E, Zhang H *et al.* Pro-inflammatory and anti-inflammatory T cells in giant cell arteritis. *Joint Bone Spine* 2016;84:421–6.
- Weyand CM, Goronzy JJ. Immune mechanisms in medium and large-vessel vasculitis. *Nat Rev Rheumatol* 2013;9:731–40.
- Sonnenberg GF, Fouser LA, Artis D. Functional biology of the IL-22–IL-22R pathway in regulating immunity and inflammation at barrier surfaces. *Adv Immunol* 2010;107:1–29.
- Witte E, Witte K, Warszawska K, Sabat R, Wolk K. Interleukin-22: a cytokine produced by T, NK and NKT cell subsets, with importance in the innate immune defense and tissue protection. *Cytokine Growth Factor Rev* 2010;21:365–79.
- Perusina LM, Lin Y, Fang J, Zou W, Frankel T. Biological and pathological activities of interleukin-22. *J Mol Med* 2016;94:523–34.
- Hernandez-Rodriguez J, Segarra M, Vilardell C *et al.* Tissue production of pro-inflammatory cytokines (IL-1beta, TNFalpha and IL-6) correlates with the intensity of

- the systemic inflammatory response and with corticosteroid requirements in giant-cell arteritis. *Rheumatology* 2004;43:294–301.
- 10 Garcia-Martinez A, Hernandez-Rodriguez J, Espigol-Frigole G *et al.* Clinical relevance of persistently elevated circulating cytokines (tumor necrosis factor alpha and interleukin-6) in the long-term followup of patients with giant cell arteritis. *Arthritis Care Res* 2010;62:835–41.
 - 11 Roche NE, Fulbright JW, Wagner AD *et al.* Correlation of interleukin-6 production and disease activity in polymyalgia rheumatica and giant cell arteritis. *Arthritis Rheum* 1993;36:1286–94.
 - 12 Terrier B, Geri G, Chahar W *et al.* Interleukin-21 modulates Th1 and Th17 responses in giant cell arteritis. *Arthritis Rheum* 2012;64:2001–11.
 - 13 Piggott K, Deng J, Warrington K *et al.* Blocking the NOTCH pathway inhibits vascular inflammation in large-vessel vasculitis. *Circulation* 2011;123:309–18.
 - 14 Rutz S, Eidenschenk C, Ouyang W. IL-22, not simply a Th17 cytokine. *Immunol Rev* 2013;252:116–32.
 - 15 Salvarani C, Cantini F, Boiardi L, Hunder GG. Polymyalgia rheumatica and giant-cell arteritis. *N Engl J Med* 2002;347:261–71.
 - 16 Cavazza A, Muratore F, Boiardi L *et al.* Inflamed temporal artery: histologic findings in 354 biopsies, with clinical correlations. *Am J Surg Pathol* 2014;38:1360–70.
 - 17 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.
 - 18 Ciccina F, Rizzo A, Alessandro R *et al.* Activated IL-22 pathway occurs in the muscle tissues of patients with polymyositis or dermatomyositis and is correlated with disease activity. *Rheumatology* 2014;53:1307–12.
 - 19 Ciccina F, Rizzo A, Maugeri R *et al.* Ectopic expression of CXCL13, BAFF, APRIL and LT-beta is associated with artery tertiary lymphoid organs in giant cell arteritis. *Ann Rheum Dis* 2017;76:235–43.
 - 20 Lozano E, Segarra M, Garcia-Martinez A, Hernandez-Rodriguez J, Cid MC. Imatinib mesylate inhibits in vitro and ex vivo biological responses related to vascular occlusion in giant cell arteritis. *Ann Rheum Dis* 2008;67:1581–8.
 - 21 Barone F, Nayar S, Campos J *et al.* IL-22 regulates lymphoid chemokine production and assembly of tertiary lymphoid organs. *Proc Natl Acad Sci U S A* 2015;112:11024–9.
 - 22 Saadoun D, Garrido M, Comarmond C *et al.* Th1 and Th17 cytokines drive inflammation in Takayasu arteritis. *Arthritis Rheumatol* 2015;67:1353–60.
 - 23 Espigol-Frigole G, Planas-Rigol E, Ohnuki H *et al.* Identification of IL-23p19 as an endothelial proinflammatory peptide that promotes gp130-STAT3 signaling. *Sci Signal* 2016;9:ra28.
 - 24 Sabat R, Ouyang W, Wolk K. Therapeutic opportunities of the IL-22–IL-22R1 system. *Nat Rev Drug Discov* 2014;13:21–38.
 - 25 Nikoopour E, Bellemore SM, Singh B. IL-22, cell regeneration and autoimmunity. *Cytokine* 2015;74:35–42.
 - 26 Azizi G, Simhag A, El Roubi NM, Mirshafiey A. Th22 cells contribution in immunopathogenesis of rheumatic diseases. *Iran J Allergy Asthma Immunol* 2015;14:246–54.
 - 27 Ciccina F, Guggino G, Rizzo A *et al.* Potential involvement of IL-22 and IL-22-producing cells in the inflamed salivary glands of patients with Sjogren's syndrome. *Ann Rheum Dis* 2012;71:295–301.
 - 28 Zhang L, Wang T, Wang XQ *et al.* Elevated frequencies of circulating Th22 cell in addition to Th17 cell and Th17/Th1 cell in patients with acute coronary syndrome. *PLoS One* 2013;8:e71466.
 - 29 Rattik S, Hultman K, Rauch U *et al.* IL-22 affects smooth muscle cell phenotype and plaque formation in apolipoprotein E knockout mice. *Atherosclerosis* 2015;242:506–14.
 - 30 van der Geest KS, Abdulahad WH, Rutgers A *et al.* Serum markers associated with disease activity in giant cell arteritis and polymyalgia rheumatica. *Rheumatology* 2015;54:1397–402.