RHEUMATOLOGY

Rheumatology 2011;50:2061–2070
doi:10.1093/rheumatology/ker163
Advance Access publication 25 August 2011

Original article

Tissue and serum markers of inflammation during the follow-up of patients with giant-cell arteritis—a prospective longitudinal study

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Abstract

Objective. To evaluate the association between inflammatory markers and relapse in GCA patients longitudinally assessed in a clinical trial of infliximab and glucocorticosteroids.

Methods. Forty-four newly diagnosed GCA patients in glucocorticosteroid-induced remission were randomized to receive infliximab 5 mg/kg or placebo plus daily glucocorticosteroids, tapered using a standardized schedule. Sera were analysed for inflammatory markers at multiple, pre-defined time points. Temporal artery biopsies were performed in four patients before and after treatment to analyse changes in inflammatory and vascular remodelling marker expression.

Results. Thirteen of 44 patients relapsed. Similar proportions of relapsed patients were present in both treatment arms. ESR, CRP, intercellular adhesion molecule (ICAM)-1, TNF-α, and IL-12p40 were significantly elevated near relapse. In post-treatment biopsies, mRNA expression of pro-inflammatory cytokines decreased, while vascular remodelling factors increased relative to baseline biopsies. Tissue IL-12p40 and IFN-γ mRNA remained elevated in relapsing *vs* remitting patients.

Conclusion. Despite prior findings of high concentrations of TNF-α in temporal artery biopsies of GCA patients, infliximab plus glucocorticosteroids did not result in improved clinical outcomes. Increased measures of this biomarker did not provide useful insight into the relative importance of TNF-α in the pathogenesis of GCA. Gene expression analysis in paired temporal artery biopsies pre- and post-treatment revealed decreased inflammatory activity and active vascular remodelling following treatment. In relapsing patients, increased expression of IFN-γ and IL-12p40 in post-treatment biopsies suggests a role in sustaining disease and setting the stage for relapse during treatment withdrawal.

Trial registration. ClinicalTrials.gov; http://www.clinicaltrials.gov; NCT00076726.

Key words: Serum markers, Giant cell arteritis, Relapse, Remission, Cytokines.

CLINICAL SCIENCE

Introduction

GCA is a granulomatous arteritis characterized by inflammation of large- and medium-sized arteries. Vascular inflammatory infiltrates are composed predominantly of macrophages, $CD4^+$ T lymphocytes and dendritic cells [1, 2]. Inflammatory infiltrates express a variety of pro-inflammatory cytokines (i.e. IL-1β, TNF-α, IL-6 and IFN-γ), chemokines (CCL-2), adhesion molecules [e.g. E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1] and growth factors (e.g. VEGF, TGF-β, PDGF) that are thought

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Submitted 13 December 2010; revised version accepted 21 March 2011.

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to contribute to vascular inflammation, injury, or tissue repair and remodelling (reviewed in [3–5]). Elevated serum concentrations of some of these molecules [e.g. IL-6, TNF-α, soluble intercellular adhesion molecule (sICAM)-1] can be detected in patients with active GCA [6–8]. Increased expression of TNF-α and CCL2 in lesions at diagnosis has been associated with persistent disease activity [9, 10].

Although treatment with glucocorticosteroids dramatically improves GCA symptoms [11, 12], glucocorticosteroid tapering leads to relapse in 40–50% of patients in the majority of series [11, 13, 14], and up to 84–91% in the context of clinical trials with rapid, pre-defined, glucocorticosteroid tapering [15, 16]. Relapses result in frequent retreatment and incremental glucocorticosteroid-related toxicity [11, 13, 14, 17]. In a recent clinical trial, the addition of infliximab to a standardized tapering schedule of glucocorticosteroids did not reduce relapse rates or cumulative glucocorticosteroid dosing in patients with newly diagnosed GCA [18]. In this study, we evaluated tissue and serum concentrations of IL-1β, TNF-α, IL-6, IFN-γ, IL-12, PDGF, MMP-9, ICAM-1 and TGF-β, each of which has been considered to play a role in GCA [3–10, 19–26]. We analysed the relationship of serum biomarker expression to GCA disease activity and expression of inflammatory and vascular remodelling factors in paired temporal artery biopsies before and after 1 year of treatment.

Patients and methods

Patients and study design

The study design and clinical trial results have been previously described [18]. Briefly, 44 patients with newly diagnosed GCA in glucocorticosteroid-induced remission were randomly assigned to receive infliximab 5 mg/kg ($n = 28$) or placebo ($n = 16$) at Weeks 0, 2, 6 and every 8 weeks thereafter. Glucocorticosteroid dosing was tapered according to a pre-defined schedule. A consensus definition of relapse was created by the steering committee and investigators participating in this trial. GCA relapse was defined as an increase in ESR from normal to $\geqslant 40$ mm in the first hour, plus one protocol-specified symptom or sign of GCA [18]. The study was approved by the institutional review boards or ethics committees of the individual study sites. The study was conducted according to the current regulations of the US Food and Drug Administration, the International Conference on Harmonization guidelines, and the principles of the Declaration of Helsinki. All patients provided written informed consent before participating in any protocol-specific procedures. An independent safety monitoring committee reviewed safety information during the trial.

Biomarker selection

Biomarkers were selected by consensus by the investigators during the design of the clinical trial [18]. Selection was based on previously known expression in GCA lesions and association with disease phenotype [3, 9, 19, 23–26], histopathological characteristics [21, 22] or disease outcome [9, 10]. These included Th1-related cytokines (IL-12 and IFN-γ), pro-inflammatory cytokines (IL-1β, TNF-α, IL-6), adhesion molecules (ICAM-1), metalloproteinases (MMP-9) and vascular remodelling factors (TGF-β1, PDGF A and B). All these factors were investigated in tissue samples. With the exceptions of IL-1β and IFN-γ, which are barely detectable in human serum, other biomarker concentrations were also measured in patient's sera.

Serum biomarker assessments

Serum samples were collected at pre-defined time points during the trial (Weeks 0, 2, 6, 14, 22 and 30) and tested for TNF-α, IL-6, IL-12p40, PDGF, MMP-9, ICAM-1 and TGF-β concentrations (R&D Systems; Minneapolis, MN, USA). CRP concentrations were evaluated every 4 weeks using the Tina-quant kit (Roche; Indianapolis, IN, USA). ESR was measured in local laboratories.

Temporal artery biopsies

Four patients in whom temporal artery biopsies were obtained at the time of diagnosis before starting glucocorticosteroid treatment agreed to have second biopsies performed on the contra-lateral side after 46–52 weeks of treatment. One patient received placebo and three received infliximab in addition to glucocorticosteroids. After surgical removal, half of each of the four paired biopsies was embedded in RNAlater Ice and snap-frozen in liquid nitrogen. The rest of the specimen was embedded in optimum cutting temperature (OCT) compound and frozen in methylbutane pre-chilled in liquid nitrogen. Cryostat sections were haematoxylin–eosin stained for histopathological diagnosis and the remaining tissue was stored at -80°C until use for immunohistochemical studies.

RNA isolation and cDNA synthesis

Temporal artery biopsy samples were homogenized in TRIzol Reagent (Invitrogen; Carlsbad, CA, USA) and RNA was extracted according to manufacturer's instructions. Total RNA (1 μg) was reverse transcribed to cDNA using the Archive kit (Applied Biosystems; Foster City, CA, USA) in a final volume of 100 μl, using random hexamers as a priming method. Reaction conditions were adjusted to the recommendations of the manufacturer. Samples were stored at -20°C until use.

Real-time quantitative PCR

cDNA was quantified by real-time PCR using specific Pre-Developed TaqMan gene expression assays (Applied Biosystems). Fluorescence was detected with ABI PRISM 7900 Sequence Detection system and analysed with Sequence Detection Software v.2.3 (Applied Biosystems). All samples were normalized to the expression of endogenous control GUSb. The comparative C_T method was used to assess relative gene expression.

Gene signature array

To search for additional molecules associated with disease recurrence, 90 genes were explored in post-treatment biopsies using the TaqMan Human Immune gene signature array, a quantitative real-time PCR-based system (Applied Biosystems). This gene expression card includes 4 transcription factors, 10 signal transducer molecules, 32 cytokines/cytokine receptors, 11 chemokines/chemokine receptors, 5 cell cycle protein kinases, 17 cell surface receptors, 4 molecules related to stress response, 3 oxidoreductase products and 6 housekeeping genes to normalize results (www.appliedbiosystems.com) (see supplementary table 1, available as supplementary data at *Rheumatology* Online). Values obtained from a normal temporal artery biopsy from a patient not diagnosed with GCA were used as a standard reference for comparison of multiple genes (Applied Biosystems Sequence Detection Systems version 2.3). Based on the results obtained, two additional genes (IL-23p19 and IL-17), not included in the expression card, were explored in all biopsy samples by real-time PCR. IL-23p19 was also evaluated by immunohistochemistry.

Immunohistochemistry

Among the markers detected by real-time PCR, IL-1β, TNF-α, IL-6, IFN-γ, IL-12p40, IL-12p35, IL-23p19, MMP-9 and TGF-β were also assessed by immunohistochemistry in pre- and post-treatment biopsies. Limited availability of OCT-embedded tissue prevented the extension of the study to additional markers. Cryostat (4–6 μm) sections were air-dried, fixed in cold acetone and permeabilized with 0.1% saponin. Endogenous peroxidase was blocked with H_2O_2 and the slides were incubated with the primary antibodies listed in Table 1. Optimal dilutions were tested on frozen sections of surgically excised human tonsils (positive control). Immunoglobulins obtained from the same species served as negative controls. Immunodetection was performed with an HRP-labelled polymer conjugated to secondary antibodies (EnVision Visualization method, Dako, Glostrup, Denmark) using diaminobenzidine as a chromogen. Slides were counterstained with haematoxylin. Immunostaining was scored by two investigators (J.H.-R. and M.C.C.) blinded to clinical data, as previously described [8, 10, 19]. Full agreement was achieved in the scores of 96% of the slides evaluated. After discussion, consensus was achieved for the remaining determinations.

Statistical analysis

Median serum marker concentrations were determined at Weeks 0, 2, 6, 14, 22 and 30. For patients who relapsed, summary statistics were provided for each serum marker over time and paired median marker concentrations were compared between baseline and time of relapse. Group-wise comparisons were made using analysis of variance on the van der Waerden scores. *P*-values unadjusted for multiplicity were calculated for exploratory analysis. Paired *t*-test (InStat program) was used for comparison of mRNA biomarker data between the first and second biopsies.

Results

Serum biomarker concentrations in relapsing *vs* remitting patients

At baseline (Week 0), all patients were in glucocorticosteroid-induced remission. Thirteen of 44 patients had protocol-specified relapses [18] during glucocorticosteroid tapering; four were receiving placebo and nine were receiving infliximab. Overall, higher ESR and median serum concentrations of TNF-α, IL-6, IL-12p40, TGF-β, MMP-9, ICAM-1, PDGF and CRP were observed in relapsing patients *vs* those who achieved sustained remission (Table 2). Differences were statistically significant for ESR, CRP, ICAM-1 and PDGF at particular time points during follow-up, especially at Week 2 after the study entry (Table 2). IL-1β and IFN-γ could not be measured because their concentrations were below or near the detection threshold.

In patients who relapsed, significantly ($P < 0.01$) elevated median values were observed at the protocol-specified visit closest to relapse *vs* baseline for ESR (77 *vs* 20 mm/h), CRP (1.90 *vs* 0.5 mg/dl), ICAM-1 (298.33 *vs* 235.70 ng/ml), TNF-α (10.95 *vs* 2.45 pg/ml) and IL-12p40 (60.06 *vs* 7.80 pg/ml).

Changes in serum markers according to treatment

Despite no observed differences between groups with regard to clinical outcomes, median concentrations of TNF-α, IL-12p40, ICAM-1 and CRP were significantly

Table 1 Antibodies used for immunohistochemistry

Target molecule	Species	Type	Source	Dilution
IL-1β	Mouse	Monoclonal (clone B1)	Genzyme	1:50
IL-6	Mouse	Monoclonal (clone 6708)	R&D Systems	1:40
TNF-α	Mouse	Monoclonal (clone 1825)	R&D Systems	1:40
IFN-γ	Mouse	Monoclonal (clone 25718.11)	R&D Systems	1:100
TGF-β	Rabbit	Polyclonal	R&D Systems	1:100
MMP-9	Mouse	Monoclonal (clone GE-213)	Chemicon	1:100
IL-12p40	Mouse	Monoclonal (clone I-1A4)	AbD Serotec	1:1000
IL-12p35	Rabbit	Polyclonal	ATLAS Antibodies	1:1000
IL-23p19	Rabbit	Polyclonal	Abcam	1:1500

Table 2 Median serum biomarker concentrations in patients who relapsed and non-relapsing patients over different time points

Biomarker	Week 0[a]	Week 2	Week 6	Week 14	Week 22
ESR, mm/h					
Relapse	20.00	47.50*	58.00*	67.00*	54.50
No relapse	10.00	10.00	23.00	23.00	31.00
CRP, mg/dl					
Relapse	0.50	2.05*	0.90	0.85	1.80
No relapse	0.40	0.40	0.65	0.60	1.00
TNF-α, pg/ml					
Relapse	2.45	4.90	12.85	12.17	10.18
No relapse	2.47	8.73	9.85	8.16	16.09
IL-6, pg/ml					
Relapse	6.57	16.76	3.27	8.05	17.46
No relapse	2.75	3.80	3.28	5.59	5.92
IL-12p40, pg/ml					
Relapse	7.80	32.16	57.85	105.35	189.78
No relapse	7.80	18.57	38.85	61.81	101.12
PDGF, pg/ml					
Relapse	22261.44	34160.05*	21564.19	17640.36	22216.81
No relapse	18201.11	19761.95	21323.37	21574.78	23713.13
MMP-9, ng/ml					
Relapse	1117.46	1957.49	1297.05	642.18	1138.22
No relapse	1585.64	1217.77	1329.28	931.03	1070.42
ICAM-1, ng/ml					
Relapse	235.70	436.71*	415.10	271.12	372.27*
No relapse	231.99	227.64	315.57	270.13	279.21
TGF-β, pg/ml					
Relapse	41.60	45.41	45.59	42.30	36.88
No relapse	38.72	38.29	38.39	39.61	38.62

[a]At week 0 all patients were in glucocorticoid-induced remission. *$P < 0.05$ for comparison between relapsed and non-relapsing patients.

different ($P < 0.05$) for patients receiving glucocorticosteroids plus infliximab *vs* those receiving glucocorticosteroids plus placebo at select time points post-treatment. Significantly higher median TNF-α concentrations were observed in patients receiving infliximab (12.17–19.12 pg/ml) *vs* placebo (2.60–2.92 pg/ml) across all time points. IL-12p40 concentrations were significantly higher at Weeks 6 and 14 in patients receiving glucocorticosteroids plus infliximab (56.58 and 72.06 pg/ml, respectively) *vs* glucocorticosteroids plus placebo (27.30 and 47.00 pg/ml, respectively). No differences in other markers were found between patients in each treatment arm.

mRNA expression in paired pre- and post-treatment temporal artery biopsies

The four patients with second biopsies had different clinical outcomes. Two of the four patients who experienced three and four relapses continued receiving glucocorticosteroids at 12.5 and 15 mg/day, and had received cumulative glucocorticosteroid doses of 6347 and 8247 mg, respectively. The other two patients were in remission; at second biopsy one had not relapsed and the other had experienced one relapse. They were receiving 0 and 8 mg prednisone/day, and had received cumulative prednisone doses of 4285 and 4987 mg, respectively.

With the exception of TNF-α, pro-inflammatory cytokine mRNA expression decreased post-treatment with glucocorticosteroid plus infliximab or placebo (Table 3). T-cell co-stimulatory and adhesion molecule ICAM-1 expression also decreased. Among Th1 cytokines, IFN-γ expression decreased whereas IL-12p40 tended to increase post-treatment.

A survey of 90 genes expressed in second biopsies revealed that patients with more frequent relapses had higher concentrations of IL-12p40 and IFN-γ mRNA in their post-treatment biopsies than patients who were more responsive to treatment (Fig. 1). IL-12p40 mRNA remained elevated in frequent relapsing patients but was undetectable in patients in remission. IL-12p35 mRNA was present in low concentrations in frequently relapsing patients. These levels suggested that persistence of IL-12p40 might be partly related to IL-23 (the IL-23p19 subunit is shared with the IL-12p40 subunit), which was not included in the gene-expression card. IL-23p19 mRNA and its related cytokine IL-17 were subsequently analysed by real-time PCR and found to be over-expressed in pre-treatment biopsies and decreased with treatment (Table 3). Although other mRNAs were elevated, no

Table 3 Changes in biomarker mRNA concentration [mean (SEM)] in paired temporal artery biopsies[a]

Biomarker	First biopsy	Second biopsy	*P*-value
IL-1β	63.19 (19.76)	17.23 (5.34)	0.144
TNF-α	10.18 (2.39)	19.94 (4.65)	0.246
IL-6	353.88 (90.72)	32.81 (18.46)	0.058
IFN-γ	232.62 (78.95)	16.80 (5.48)	0.064
IL-12p40	0.20 (0.20)	1.99 (0.71)	0.066
IL-12p35	7.22 (2.99)	13.34 (1.33)	0.051
IL-23p19	13.00 (6.47)	5.06 (1.94)	0.198
IL-17	2.57 (1.07)	0.35 (0.15)	0.250
ICAM-1	471.11 (67.82)	159.30 (24.94)	0.032
MMP-9	188.22 (46.22)	356.14 (124.78)	0.253
TGF-β	1080.78 (208.46)	1819.12 (55.16)	0.050
PDGF-A	598.58 (328.13)	1262.48 (311.36)	0.009
PDGF-B	354.17 (177.39)	532.76 (101.99)	0.253

[a]Number of paired biopsies = 4. Statistics are only indicative given the low number of samples studied.

additional genes clearly differentiated between frequently relapsing and remitting patients.

Immunohistochemical expression in paired temporal artery biopsies obtained pre- and post-treatment

A substantial reduction in the intensity of inflammatory infiltrates was observed in biopsies collected post-treatment with glucocorticosteroids plus infliximab or placebo compared with those obtained at the time of diagnosis. Only scattered foci of inflammatory cells persisted in second biopsies. Immunohistochemical scores for all of the markers tested were reduced in proportion to the decrease in inflammatory cells (Fig. 2). Figure 3 shows the topographic distribution of the products associated with relapse in pre- and post-treatment biopsies. As with other markers, IFN-γ, IL-12p35, IL-23p19 and IL-12p40 immunohistochemical expression decreased. Intense expression of these markers was observed in a cluster of infiltrating cells located in the adventitia in post-treatment biopsies. Smooth muscle cells immunostained slightly

Fig. 1 mRNA concentrations (Th1 cytokines) in post-treatment temporal artery biopsies from patients who relapsed and those who did not relapse. Values are relative to those found in a normal temporal artery. In addition to glucocorticosteroids, Patient 1 received placebo and Patients 2–4 received infliximab.

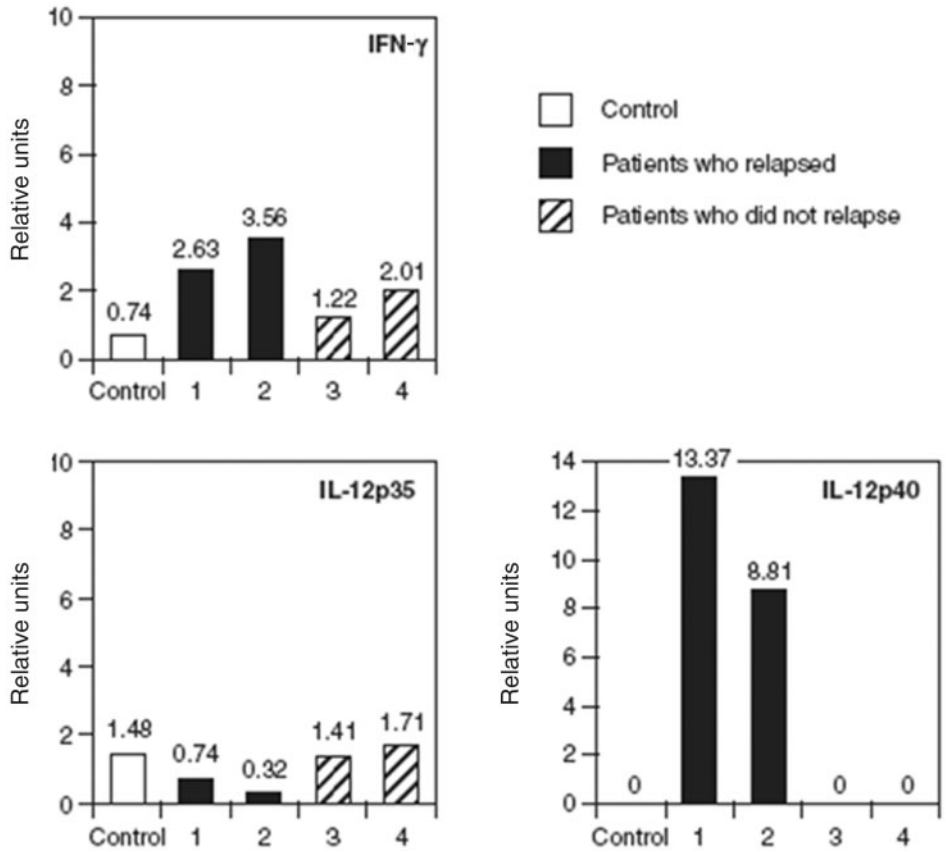
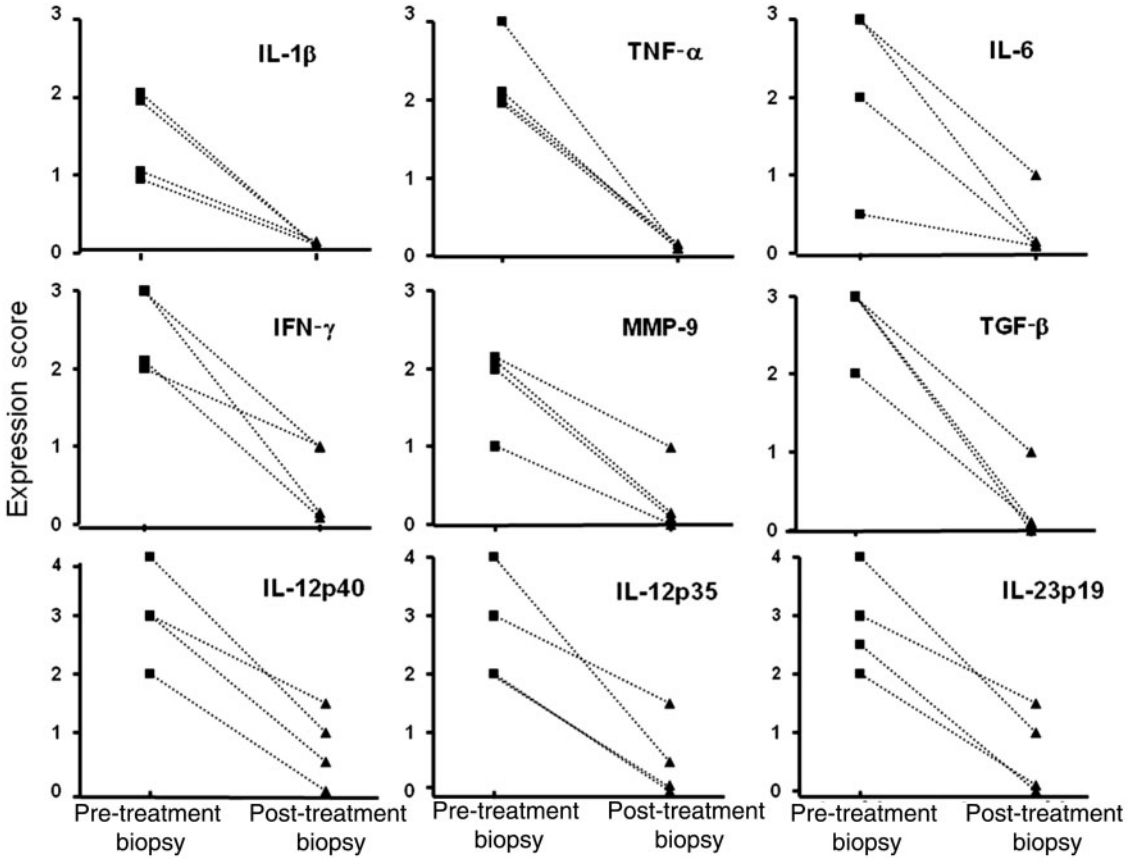


FIG. 2 Immunohistochemical expression scores for the cytokines studied in pre- and post-treatment temporal artery biopsies.



positive for IL-12p35 (Fig. 3), reflecting the constitutive expression of this IL-12 subunit in normal arteries (Fig. 1).

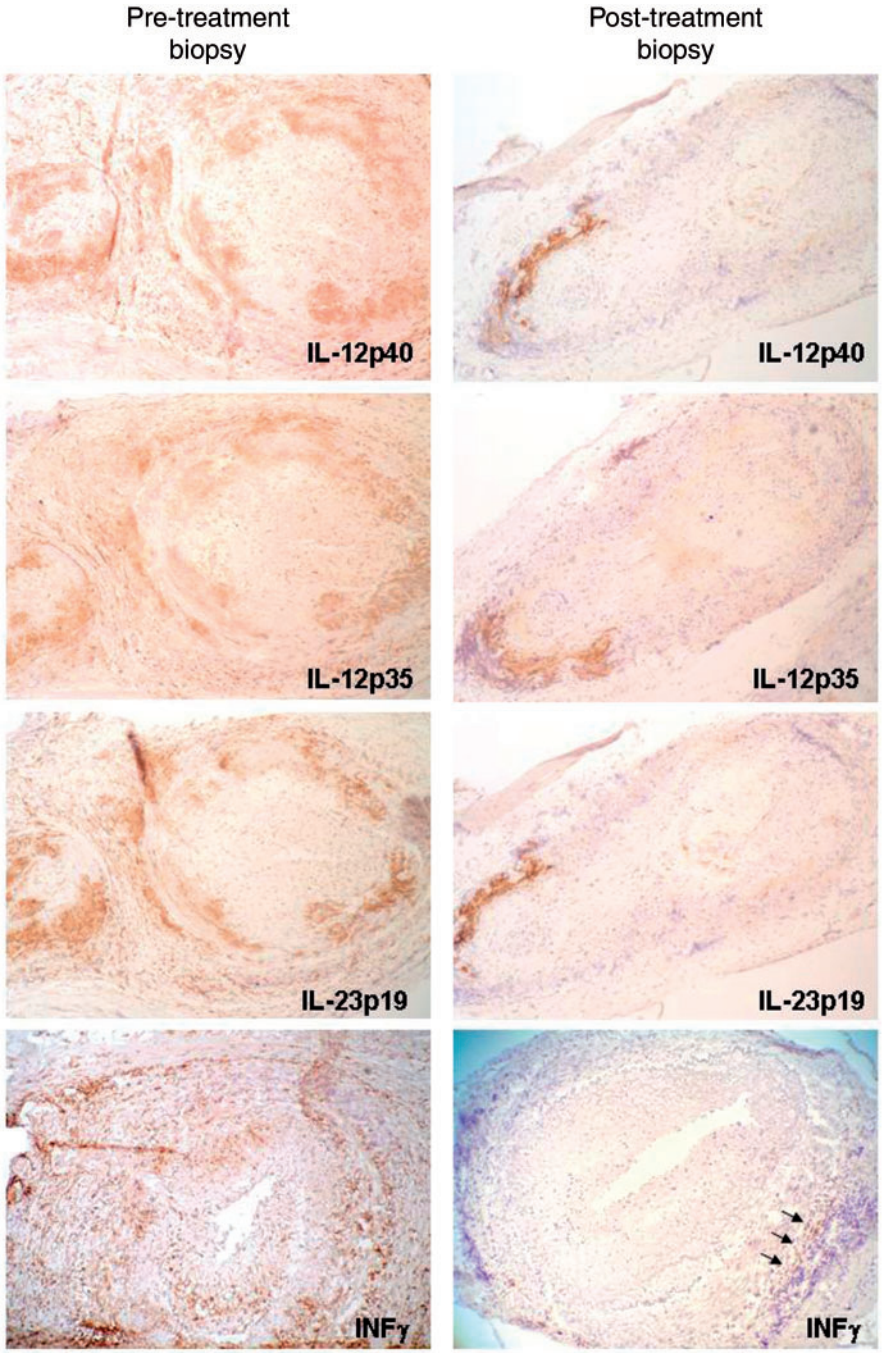
Discussion

This is the first prospectively designed study of GCA [18] to examine biomarkers and a large number of genes and proteins in paired sets of pre-treatment *vs* post-treatment biopsies [27, 28]. Patients with relapsing GCA had higher overall concentrations of acute-phase reactants, adhesion molecules and inflammatory cytokines than patients who did not relapse. Significantly increased values of CRP, ESR, ICAM-1, TNF-α and IL-12p40 were observed at relapse relative to baseline.

This study confirmed previously published data showing associations between elevated tissue TNF-α concentrations and active disease [8, 9, 29]. Nonetheless, our trial evaluating the addition of infliximab to glucocorticosteroids did not result in improved clinical outcomes [17]. In the context of GCA and TNF, we conclude that increased measures of this biomarker did not provide useful insight into just how critical TNF is in GCA. Attempting to achieve therapeutic benefit by blocking TNF was unsuccessful. One could argue that the dose and treatment schedule employed was insufficient to see an effect. However, the lack of any clinical signal of benefit, with doses that are usually effective in other diseases, makes this unlikely. It is more likely that our results reflect an incomplete understanding of cytokine-cell cross-talk and immunoregulation in GCA.

We did find a sharp increase in median serum concentrations of other select inflammatory and vascular remodelling markers at Week 2 after enrolment showing a clear difference in marker levels between patients who would experience a subsequent relapse and patients who did not relapse. Differences were statistically significant for CRP, ESR, ICAM-1 and PDGF. Trends were observed for IL-6, IL-12p40 and PDGF, although these did not reach a level of statistical significance. This rebound effect coincided with the beginning of glucocorticosteroid tapering, suggesting that relapsing patients may be identified early during the course of the disease and that monitoring early changes in select serum markers may be warranted after treatment initiation. Unfortunately, none of the molecules showed consistent or dramatic differences between relapsing and remitting patients across all the time points tested. Patients treated with infliximab had paradoxically higher serum concentrations of TNF-α and IL-12p40. While

FIG. 3 Distribution of IL-12p40, IL-12p35, IL-23p19 and IFN-γ expression in pre- and post-treatment temporal artery biopsies from two relapsing patients. The first three rows correspond to one patient and the fourth row to the other.



the reasons for this are uncertain, one might speculate that it may result from interference with negative feed-back loops, as observed in other settings [30, 31].

In paired biopsies obtained from four patients with GCA, mRNA concentrations of select inflammatory markers (IL-1β, IL-6, IFN-γ, ICAM-1) decreased and concentrations of factors participating in vascular remodelling (MMP-9, TGF-β, PDGF-A and PDGF-B) were increased after 1 year of treatment, suggesting progression towards tissue repair.

Immunohistochemical scores for inflammatory markers decreased post-treatment and correlated with the reduction in inflammatory cells in biopsies.

Simultaneous screening for 90 genes revealed elevated mRNA concentrations of IFN-γ and IL-12p40 in the second biopsies of two patients with multiple relapses.

Given that the IL-12p70 heterodimer (p40/p35) is a stimulator of Th1 differentiation and IFN-γ production, our results support a relationship between Th1-mediated response and relapsing disease. However, the IL-12p35 subunit of IL-12 was decreased in the second biopsies of relapsing patients, suggesting that increased IL-12p40 could also be part of the IL-23 (p40/p19) heterodimer. Indeed, IL-23p19 and its related cytokine IL-17A were up-regulated in our patients, as recently reported in larger series of active patients [32–34]. Immunostaining of post-treatment biopsies disclosed clusters of cells expressing these cytokines in the adventitia, where arterial inflammation is initiated in GCA [22, 35, 36].

Our study has several important limitations. The time points of serum sampling were pre-defined and thus were not precisely coincident with the occurrence of relapse. The small number of arterial samples analysed prevents rigorous assessment of the effects of glucocorticoids plus infliximab *vs* placebo on tissue expression of biomarkers. In addition, second biopsies may not have completely reflected treatment-induced changes in the first biopsy. The incomplete reflection of changes is an inherent limitation to any study performed on serial biopsies obtained before and after intervention. Nonetheless, our results suggest that both IFN-γ and IL-23 may be related to disease persistence. This hypothesis on the relationship of IFN-γ and IL-23 to disease persistence is consistent with experimental models showing that IL-12p40 knockout mice are resistant to developing various chronic inflammatory diseases and that IL-12p35 knockout mice develop more severe disease phenotypes [37, 38]. Further studies are needed to explore the relative functional contribution of IL-12/IL-23p40 and IFN-γ to disease persistence and the potential to exploit them as therapeutic targets in GCA.

Rheumatology key messages

- Patients with relapsing GCA have moderately elevated acute-phase reactants and other inflammatory molecules.
- Serial temporal artery biopsies reveal decreased pro-inflammatory cytokines and increased expression of vascular remodelling factors.
- Persistent tissue expression of IFN-γ and IL-12p40 in relapsing patients suggests involvement in disease chronicity.

Acknowledgements

Results presented at the 2008 EULAR Meeting (Paris, France, 11–14 June 2008) and at the 72nd Annual Scientific meeting of the American College of Rheumatology (San Francisco, CA, 24–29 October 2008).

The authors acknowledge Sunita Bhagat, Jhilik De and Derick Siegel from Centocor Ortho Biotech, Inc. for their laboratory expertise; Montse Sánchez from Pathology Department, Hospital Clínic, Barcelona for her immunostaining expertise; and Mary Whitman, PhD, Jennifer Han and Kirsten Schuck from Centocor Ortho Biotech Services, LLC, for their editorial expertise.

Investigators who enrolled patients into the study (listed by country) were as follows. USA: Steven Carsons, Mineola, NY; David Curtis, San Francisco, CA; Peter A. Merkel, Robert W. Simms, Aileen Schiller and Hyon K. Choi, Boston, MA; Eric L. Matteson, Rochester, MN; Kenneth Nies, Torrance, CA; Eric Peters, Paradise Valley, AZ; Gary Hoffman, Tiffany Clark, William Wilke and Karen Rendt-Zagar, Cleveland, OH; Robert Sergott, Philadelphia, PA; Sheldon Solomon, Voorhees, NJ; Joseph Forstot, Boca Raton, FL; Kenneth Calamia, Jacksonville, FL; Fredrick Murphy, Duncansville, PA. Belgium: Jean-Pierre Devogalr and Serge Steinfeld, Brussels; Rene Westhovens, Leuven; Lucas Williame and Frank Raeman, Antwerp; Beatrix Maeyaert, Assebroek. United Kingdom: Kuntal Chakavarty, Romford; Bhaskar Dasgupta, Essex; Chandra Chattopadhyay, Wigan. Italy: Carlo Salvarani, Reggio Emilia. Spain: Maria C. Cid, Ana García-Martínez, José Hernández-Rodríguez and Fátima Mota, Barcelona.

Funding: The Infliximab-GCA study was sponsored by Centocor Research and Development, Inc. and Schering-Plough Corp. and conducted by a Steering Committee consisting of: G.S.H., Cornelia Weyand, M.C.C., John Stone, Karen Rendt-Zagar and M.U.R.

Disclosure statement: M.C.C. and colleagues received support from Ministerio de Ciencia e Innovación (SAF 08/04328) and Marató TV3 (06/0710). S.V. currently works for Hoffman La Roche and owns stock in Johnson & Johnson, Inc. S.X. works for Centocor and owns stock in Johnson and Johnson. M.U.R. was employed by Centocor R&D, a fully owned subsidiary of Johnson & Johnson, Inc. at the time the study was conducted and owns Johnson & Johnson stocks. All other authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

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