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Original article

Analysis of SF and plasma cytokines provides insights into the mechanisms of inflammatory arthritis and may predict response to therapy

Helen L. Wright[1], Roger C. Bucknall[2], Robert J. Moots[3] and Steven W. Edwards[1]

Abstract

Objectives. Biologic drugs have revolutionized the care of RA, but are expensive and not universally effective. To further understand the inflammatory mechanisms underlying RA and identify potential biomarkers predicting response to therapy, we measured multiple cytokine concentrations in SF of patients with inflammatory arthritides (IAs) and, in a subset of patients with RA, correlated this with response to TNF-α inhibition.

Methods. SF from 42 RA patients and 19 non-RA IA patients were analysed for 12 cytokines using a multiplex cytokine assay. Cytokines were also measured in the plasma of 16 RA patients before and following treatment with anti–TNF-α. Data were analysed using Mann–Whitney U-test, Spearman's rank correlation and cluster analysis with the Kruskal–Wallis test with Dunn's post-test analysis.

Results. RA SF contained significantly elevated levels of IL-1β, IL-1ra, IL-2, IL-4, IL-8, IL-10, IL-17, IFN-γ, G-CSF, GM-CSF and TNF-α compared with other IA SF. RA patients who did not respond to anti-TNF therapy had elevated IL-6 in their SF pre-therapy ($P < 0.05$), whereas responders had elevated IL-2 and G-CSF ($P < 0.05$). Plasma cytokine concentrations were not significantly modulated by TNF inhibitors, with the exception of IL-6, which decreased after 12 weeks ($P < 0.05$).

Conclusions. Cytokine profiles in RA SF vary with treatment and response to therapy. Cytokine concentrations are significantly lower in plasma than in SF and relatively unchanged by TNF inhibitor therapy. Concentrations of IL-6, IL-2 and G-CSF in SF may predict response to TNF inhibitors.

Key words: synovial fluid, cytokine, rheumatoid arthritis, DMARD, anti-TNF.

Introduction

Autoimmune diseases such as RA are caused, in part, by deregulation of cytokine pathways, preventing normal resolution of inflammation. Elevated levels of pro-inflammatory cytokines can be detected in blood serum of RA patients, sometimes many years before the onset of symptoms [1]. In the early stages of the disease, high local concentrations of cytokines in the joint cause synovial proliferation, hyperplasia and angiogenesis. Infiltrating immune cells such as macrophages, lymphocytes and neutrophils become activated, themselves secreting cytokines, proteases and ICs. This perpetuates inflammation and stimulates pannus formation, and ultimately results in damage of bone and cartilage in established disease [2]. Understanding the complex cytokine networks that contribute to the autoimmune processes associated with RA has become more relevant since the advent of biologic therapy. The discovery of TNF-α as a central cytokine in the pathogenesis of the disease, and the subsequent success of anti-TNF therapy, has triggered the search for other potential targets of anti-cytokine therapies. While anakinra (an IL-1 receptor agonist) is an effective treatment for juvenile arthritis, and has shown some success in clinical trials [3, 4], it has not proved useful in clinical practice for RA. Clinical trials of tocilizumab, an anti-IL-6 receptor mAb, have demonstrated good efficacy in RA [5], while rituximab (depleting CD20-positive B cells) and

[1]Institute of Integrative Biology, University of Liverpool, [2]Rheumatic Diseases Unit, Royal Liverpool University Hospital and [3]Institute of Ageing and Chronic Disease, University Hospital Aintree, Liverpool, UK.

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Correspondence to: Steven W. Edwards, Biosciences Building, Crown Street, University of Liverpool, Liverpool L69 7ZB, UK. E-mail: s.w.edwards@liv.ac.uk

abatacept (anti-CTLA-4) are also effective treatments for this disease [6, 7]. While these last two biologics do not act by directly blocking specific cytokines, they may mediate their effects by disrupting inflammatory processes through inhibition of cytokine production. Unfortunately, ~30% of RA patients who receive a TNF inhibitor fail to achieve an adequate response, but the reasons for this failure to respond are not known. Consequently, there are no clear biomarkers to determine which patients will, or will not, respond to such therapy [8]. Studying inflammatory SF therefore provides a unique opportunity to examine the microenvironment within the inflamed joint with a view to dissecting the complex cytokine networks perpetuating the inflammatory process, and also offers the potential for discovery of new targets for the development of RA therapies.

Methods

Patients

This study was approved by the Sefton Adults Ethics Committee. SF was obtained from 61 individuals, of whom 42 fulfilled the ACR criteria for RA [9]. Non-RA patients with joint effusions included adult patients with juvenile arthritis ($n = 4$), spondylitis ($n = 3$), reactive arthritis ($n = 3$), undifferentiated arthritis ($n = 3$), gout/pseudogout ($n = 4$), Behçet's disease ($n = 1$) and SLE ($n = 1$). Patient characteristics can be found in Table 1. There was no significant difference between the age of the patients in the RA and non-RA groups. However, non-RA patients had experienced a significantly shorter duration of disease than RA patients ($P < 0.01$). Twenty-eight patients were receiving (or about to receive) anti-TNF therapy, including 24 patients in the RA group and 4 patients in the non-RA group, all of whom were adult juvenile arthritis patients. All patients recruited onto the study gave written informed consent, and SF was aspirated into heparinized tubes and processed within 1 h. Aliquots of whole SF were centrifuged at 2000 *g* for 5 min and cell-free SF was decanted and frozen at −80°C. Blood plasma was obtained from 16 RA patients pre- and 12 weeks post-anti-TNF therapy, and 8 healthy controls. Patients about to commence anti-TNF therapy had a 28-joint DAS (DAS-28) of $\geqslant 5.1$, in line with EULAR guidelines [10].

Cytokine and chemokine multiplex assay

The concentrations of 12 cytokines in SF were quantified using a human cytokine Luminex multiplex assay (Biosource, Paisley, UK), according to the manufacturer's instructions. The cytokines/chemokines measured were IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, IFN-γ, G-CSF, GM-CSF and TNF-α. Detection ranges differed for each analyte, but were in the region of 3–54 000 pg/ml. Before the assay of plasma or SF, 50 μl of each sample was pre-incubated with 50 μl blocking buffer (40% mouse serum, 20% goat serum, 20% rabbit serum and 20% assay diluent) for 30 min at room temperature to prevent non-specific binding of antibody probes by RF, a method of efficient removal of RF that has been validated by Raza *et al*. [11]. Samples were centrifuged at 14 000 *g* for 10 min to remove ICs. The effectiveness of this method of removing autoantibodies was confirmed by testing the ability of the RF-depleted SF to activate the respiratory burst in GM-CSF-primed neutrophils. Untreated SF produced a large and sustained respiratory burst, which was completely absent when stimulating with RF-depleted SF (data not shown). The multiplex assay was carried out in a 96-well filter plate, using duplicate assays of protein standards, and wells were aspirated using a vacuum manifold. RF-blocked sample (50 μl) was incubated with 25 μl multiplex beads for 2 h on an orbital shaker, washed twice with wash buffer and incubated with 100 μl biotinylated detector antibody for 1 h. Wells were washed twice and incubated with 100 μl streptavidin-RPE for 30 min. Wells were washed three times and the bead conjugate re-suspended in 125 μl wash buffer. The plate was read in a Bio-Plex Suspension Array System, model Luminex 100 (Bio-Rad, Hemel Hempstead, UK). Cytokine concentrations were calculated by reference to the standard curve.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism v5.0 using the Mann–Whitney U-test and Spearman's rank correlation. Hierarchical cluster analysis based on average linkage was carried out using Genesis cluster analysis software version 1.7.1 [12] and analysis of variance between clusters was calculated using the Kruskal–Wallis test with Dunn's post-test analysis.

Table 1 Clinical characteristics of the patients with RA or non-RA with joint effusions

Characteristics	RA	non-RA	*P*-value
Number	42	19	
Female, *n*	34	6	
Age, median (IQR), years	54.5 (43–60)	47.1 (34–55)	0.09
Disease duration, median (IQR), years	12.5 (8–18)	0.7 (0–5)	<0.01
RF positive, *n*	30	0	
CRP, median (IQR), mg/l	26 (17–58)	32 (11–72)	0.73
ESR, median (IQR), mm/l	42 (23–66)	30.5 (13–63)	0.26
Anti-TNF, *n*	24	4	

Statistical analysis using Mann-Whitney U-test.

Results

Cytokine and chemokine levels

SF from the knee joints of 61 patients were analysed for cytokines. Forty-two samples were from patients with RA. This was a heterogeneous group of patients in terms of both age and disease duration (Table 1). Patients were receiving one or more DMARDs and/or anti-TNF therapy, and included SF from both chronic non-responders to therapy and normally well-managed patients experiencing a disease flare. SF from 19 non-RA patients was measured as an inflammatory control group. SF from RA patients contained significantly higher concentrations of IL-1β ($P < 0.01$), IL-1ra ($P = 0.01$), IL-2 ($P < 0.05$), IL-4 ($P < 0.01$), IL-8 ($P < 0.05$), IL-10 ($P < 0.01$), IL-17 ($P < 0.01$), IFN-γ ($P < 0.01$), G-CSF ($P < 0.01$), GM-CSF ($P < 0.01$) and TNF-α ($P < 0.01$) (Fig. 1). IL-6 was detected in all but two samples, but was not significantly elevated in either group ($P > 0.05$). This confirms the more general role of IL-6 in inflammation rather than as a disease-specific cytokine. Blood CRP levels significantly correlated with SF IL-6 ($P < 0.01$), IL-8 ($P < 0.05$), IL-10 ($P < 0.01$) and GM-CSF ($P < 0.01$) and ESR titres correlated with IL-1ra ($P < 0.01$) and IL-8 ($P < 0.01$) across the entire patient group. While a statistical analysis of the differences in cytokine levels in fluids within each disease classification in the non-RA group was not possible due to the small numbers of patients with each condition, TNF-α was present only in the SF from juvenile arthritis patients.

Effect of drug therapy and response to therapy on cytokine levels in RA

The introduction of biologic therapy has enabled the direct targeting of specific inflammatory mediators in the pathogenesis of RA. Anti-TNF drugs, for example, act by directly blocking TNF-α and/or removing TNF-producing cells [13], but other downstream events may be affected following the blocking of this cytokine, as TNF-α regulates multiple inflammatory processes. In order to determine the *in vivo* effect of TNF-α blockade on the cytokine networks, levels of the cytokines in the SF of RA patients were analysed. Drug therapy at the time of sampling was noted and fluids were classified according to treatment as DMARD or anti-TNF. SFs classified as DMARD were from patients receiving MTX, AZA and/or LEF, and fluids classified as anti-TNF were from patients receiving infliximab, adalimumab or etanercept with or without MTX as a combination therapy. IL-1β, IL-2, IL-10, IL-17 and GM-CSF were significantly elevated in fluids from patients receiving DMARDs compared with the anti-TNF group ($P < 0.05$, Fig. 2A–E).

There are currently no reliable biomarkers to predict which patients are likely to respond to anti-TNF therapy, and identifying such a biomarker could accelerate the rate at which patients receive effective therapy [14]. SF from RA patients receiving or about to commence anti-TNF therapy was analysed and grouped based on whether they subsequently achieved a response to anti-TNF 12 months after initiating therapy, based on EULAR criteria of a decrease in DAS-28 of >1.2 [10]. The data revealed that IL-6 was significantly elevated in SF from those patients who did not go on to adequately respond to anti-TNF therapy ($P < 0.05$, Fig. 2F). In addition, IL-2 and G-CSF were significantly elevated in fluids from patients who ultimately achieved a response to anti-TNF therapy ($P < 0.05$, Fig. 2G and H), possibly indicating a different underlying disease pathology in patients who will respond effectively to anti-TNF therapy compared with those who will not.

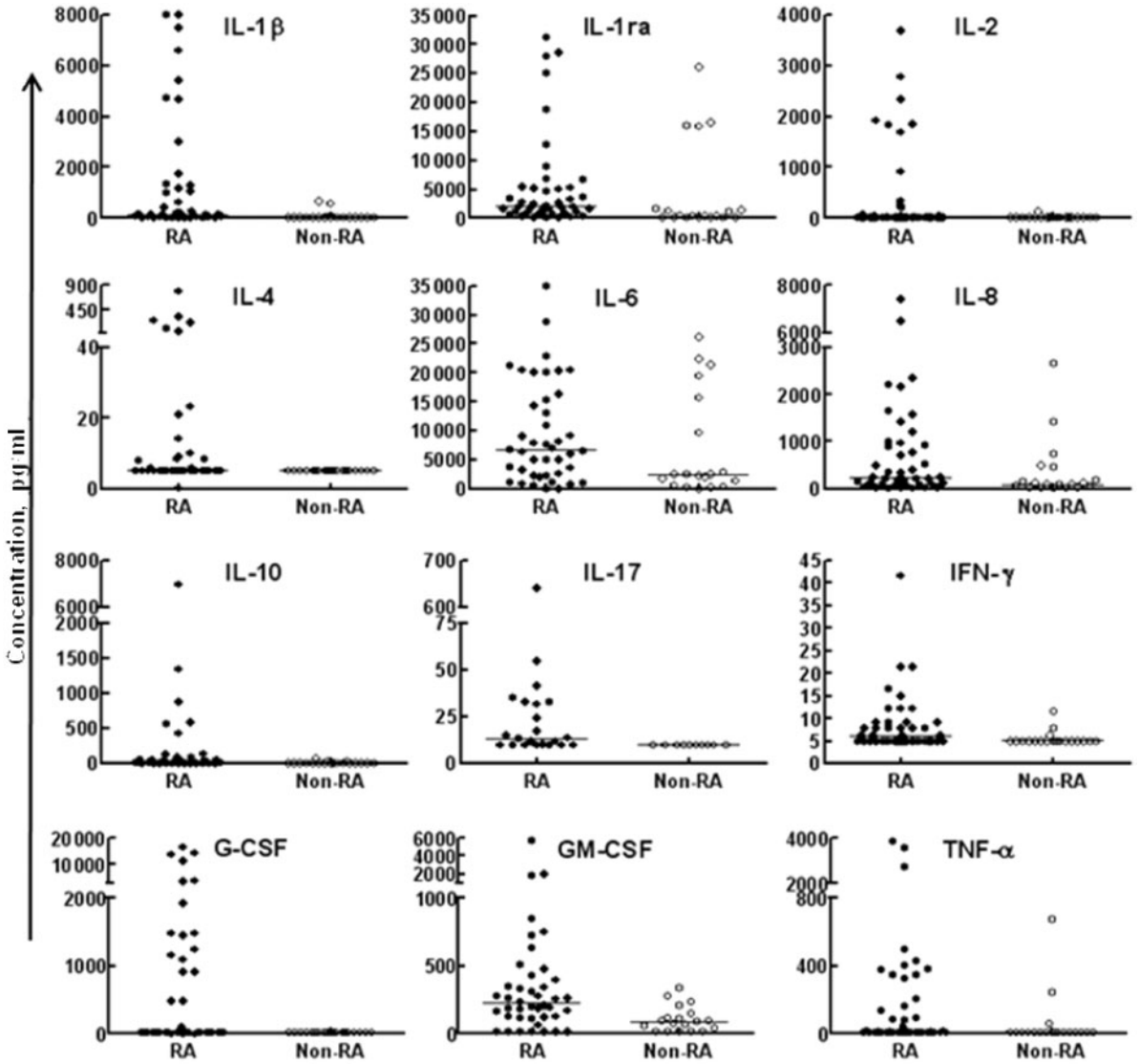
Cluster analysis of RA SFs

RA is a heterogeneous disease, and therefore SFs were analysed by hierarchical cluster analysis to identify any subgroups of RA patients with similar SF cytokine profiles. SFs clustered into six groups (Fig. 3), and patient characteristics including age, disease duration, RF titre, CRP and ESR were not significantly different across the clusters ($P > 0.05$). Comparison of the SF cytokine concentrations in each cluster was carried out using the Kruskal–Wallis test with Dunn's post-test analysis. Cluster 1 was significantly higher in IL-1β ($P < 0.01$), IL-2 ($P < 0.01$), IL-4 ($P < 0.05$), G-CSF ($P < 0.01$), GM-CSF ($P < 0.05$) and TNF-α ($P < 0.01$), suggesting that the patients falling within this cluster had very high levels of inflammation, with many cell types contributing to cytokine production. Cluster 2 contains only two SFs, which are characterized by the absence of IL-6, suggesting low-grade inflammation and therefore possibly indicating underlying OA in these two individuals. Cluster 3 SFs contain only low concentrations of cytokines and have significantly lower levels of IL-1ra ($P < 0.01$) and IL-8 ($P < 0.05$). Interestingly, none of the patients in this cluster was receiving anti-TNF therapy, with the lower levels of SF cytokines corresponding to lower levels of disease activity in these patients. Cluster 4 SFs contain significantly higher concentrations of IL-17 ($P < 0.05$) and TNF ($P < 0.01$), and also have the highest median IL-6 levels. Analysis showed that the patients in this group were all either unresponsive to their current biologic or were normally well-managed patients experiencing a flare of arthritis. Clusters 5 and 6 have very similar cytokine profiles and are differentiated by significantly lower G-CSF concentrations in Cluster 5 ($P < 0.05$). These latter two clusters of patients are distinguished from Cluster 4 by the absence of TNF-α.

Plasma cytokine concentrations in RA patients about to commence anti-TNF therapy

Blood plasma was collected from 16 RA patients about to commence anti-TNF therapy and at 12-week post-therapy. Before therapy, all patients had a DAS-28 $\geqslant 5.1$ and fulfilled EULAR criteria for anti-TNF therapy [10]. In addition, blood plasma was collected from eight healthy control individuals. Pre-therapy cytokine concentrations of IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-17, G-CSF and IFN-γ were significantly higher in patient plasma than in control plasma ($P < 0.05$), and IL-6 was the only cytokine that

FIG. 1 Cytokine concentrations in inflammatory SFs. Cytokines were measured in SF samples from RA (filled circle, $n = 42$) and non-RA (open circle, $n = 19$) patients. The median concentration of each cytokine is represented by a horizontal bar.

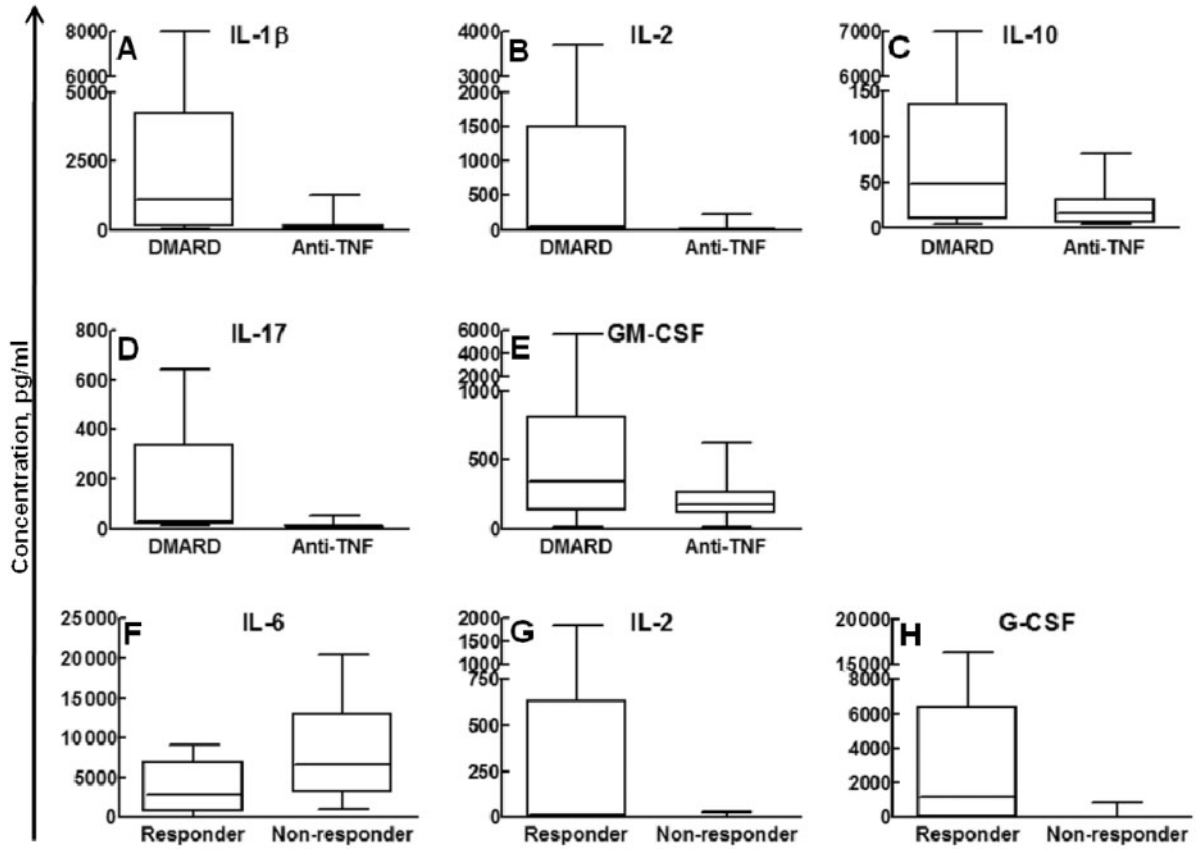


decreased significantly in patient plasma after therapy ($P < 0.05$) (Fig. 4). TNF-α was undetectable in control plasma, and was only detectable in plasma from 4 of 16 patients before commencement of anti-TNF therapy (range 35.3–419.6 pg/ml). The median concentration of TNF-α in patient plasma increased following therapy, and 12 weeks after therapy it was detected in 8 of 16 samples (range 22.7–436.4 pg/ml). The low pre-therapy concentrations of TNF-α and the subsequent increase following therapy may seem surprising. However, these data are consistent with other published research [15, 16], and may be due, in part, to the process of clearance of TNF-α by the anti-TNF drugs, suggesting that the TNF-α measured by our assay may not be biologically active [15].

Discussion

Our analysis of inflammatory SF and blood plasma has identified not only differences in cytokine levels between patients with RA and other inflammatory arthritides, but heterogeneity among RA SFs that may, in part, be explained by drug therapy. We have identified significant differences in the SF cytokine profiles of patients receiving different drug regimens, and importantly, identified three cytokines with significantly different levels of expression in those patients who went on to respond to anti-TNF therapy compared with patients who did not. These cytokines may be useful biomarkers of a patient's potential to respond to a specific biologic therapy. The different RA SF cytokine profiles clustered into six groups based on

FIG. 2 Effect of drug therapy and patient response on SF cytokine levels. SF from RA patients receiving DMARDs ($n = 16$) contained significantly higher concentrations of (**A**) IL-1β, (**B**) IL-2, (**C**) IL-10, (**D**) IL-17 and (**E**) GM-CSF compared with patients receiving anti-TNF therapy ($n = 22$) ($P < 0.05$). SF from patients who did not achieve a response to anti-TNF therapy ($n = 12$) had significantly elevated levels of IL-6 (**F**) compared with responders ($n = 10$) ($P < 0.05$). However, SF from responders contained significantly higher pre-therapy concentrations of (**G**) IL-2 and (**H**) G-CSF ($P < 0.05$). Box plots represent the interquartile range, the median is represented by a horizontal bar and the whiskers represent the maximum and minimum values.



different levels of disease activity, and may be indicative of different underlying cellular pathologies within the joints of the patients.

While there is considerable published data measuring cytokine concentrations in SF using single cytokine assays, few groups have published data measuring levels of multiple cytokines in different SF samples. A study by Raza *et al*. [11], which characterized the cytokine profiles of early RA (<3 months duration), only included nine patients with established disease in their RA comparison group. They found that early RA SF had a cytokine profile that not only indicated T-cell and stromal cell activity, but was transient and not seen in established disease. They found that patients who had an eventual diagnosis of RA had significantly higher concentrations of IL-2, IL-4, IL-13, IL-17, IL-15, basic fibroblast growth factor and epidermal growth factor compared with other patients. Of the SFs analysed in our study, only one was from an RA patient in the first 3 months of active disease. However, it is interesting to note that this patient had elevated concentrations of IL-2 (339.7 pg/ml), IL-4 (788.0 pg/ml) and IL-17 (640.9 pg/ml) compared with other patients with established disease. Indeed, this patient had by far the highest IL-17 concentration observed (next highest was 54.29 pg/ml).

DMARDs modulate the inflammatory response in RA by directly influencing the activity of immune cells. MTX is the most widely prescribed DMARD, and has multiple effects on the immune response [17]. Its reported effects include inhibition of IL-8 synthesis, inhibition of lymphocyte proliferation, increased apoptosis of T cells and inhibition of osteoclast formation [18–21]. MTX has also been reported to abrogate delayed neutrophil apoptosis, chemotaxis and reactive oxygen species (ROS) production.[22–24] Other DMARDs, such as LEF and AZA, may decrease the production of pro-inflammatory mediators such as IL-6 and PGE, and increase the production of anti-inflammatory mediators, such as IL-10 and IL-1ra [25]. It is interesting, therefore, that our analysis shows significant differences between the levels of some

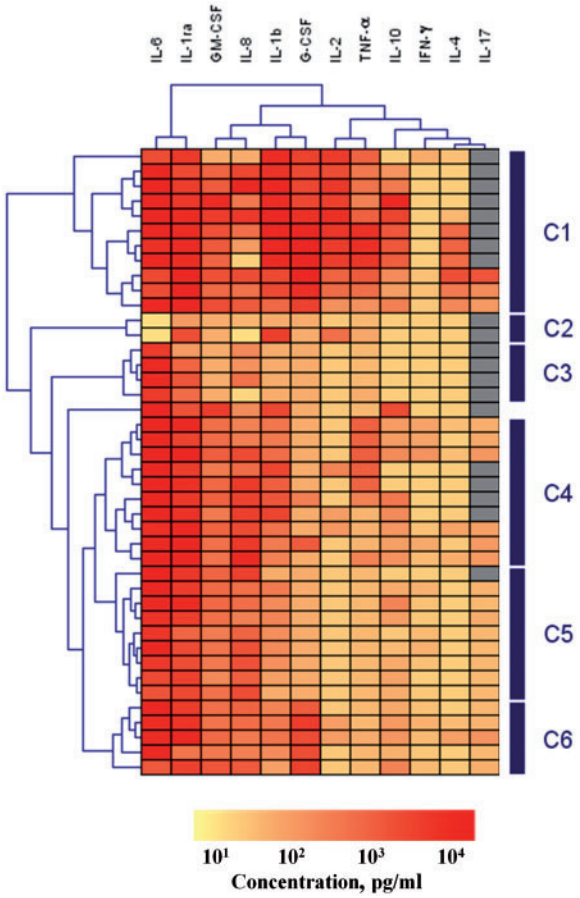


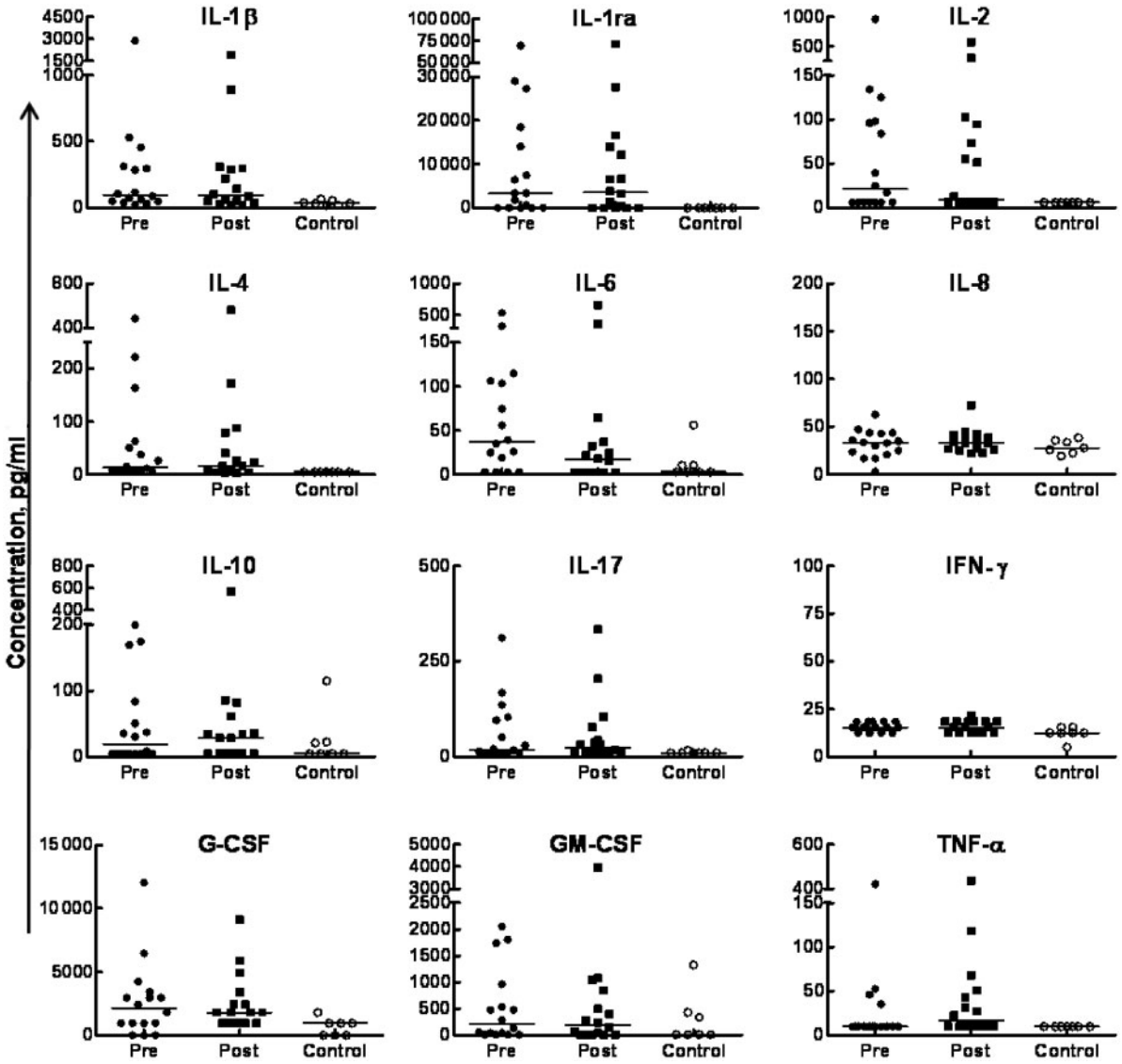
Fig. 3 Cluster analysis of SF from RA patients. Cluster analysis using Genesis software [12] revealed that RA SFs cluster into six groups. Analysis of variance with Kruskal–Wallis test and Dunn's post-analysis test revealed significant differences in the levels of cytokines in each cluster, which may be indicative of different underlying disease pathology. IL-17 was only measured in 22 of 42 samples (grey shading = not measured).

pro-inflammatory cytokines in SF from patients receiving DMARDs and those receiving anti-TNF therapy. Those cytokines that decreased in the SF from patients receiving anti-TNF drugs are downstream products of TNF-stimulated T cells and macrophages, and the observation that these SF cytokines are lower in anti-TNF patients supports the pivotal role played by TNF-α in the progression and persistence of inflammation in a subgroup of RA patients. Another interesting finding is that patients who were unresponsive to anti-TNF therapy (12 months post-therapy) had significantly higher concentrations of IL-6 in their SF. This observation lends support to recently published data, which showed elevated IL-6 gene expression in synovial biopsies from non-responders to adalimumab therapy [26]. IL-6 is an emerging target for drug therapy in inflammatory diseases, and tocilizumab (anti-soluble IL-6 receptor) has been developed as a biologic therapy to block IL-6 signalling. Data from the RADIATE (Research on Actemra Determining Efficacy After Anti-TNF Failures) study shows that tocilizumab is effective in decreasing disease activity in a significant number of patients with RA who have previously failed anti-TNF therapy [5], and this drug is now approved for the treatment of RA. Our analysis also found that SF from patients who went on to achieve an adequate response to anti-TNF therapy contained significantly elevated concentrations of IL-2 and G-CSF. Production of IL-2 by T cells, and G-CSF by synovial fibroblasts, can be stimulated *in vitro* by TNF-α [26, 27], and therefore the observation that these cytokines were absent in SF from non-responders suggests that TNF-α may not be the pivotal cytokine in these patients. The presence of T-cell-derived cytokines in the pre-therapy SF of responders suggests they may have T-cell-driven inflammation, which responds to TNF-α blockade through restoration of Treg function. TNF-α has been shown to suppress Treg function through decreased expression of FOXP3, an effect that can be reversed by TNF-α blockade [28]. The absence of T-cell-derived cytokines in SF from non-responders suggests they may not have T-cell-driven disease, and that their persistent inflammation is dominated by different cellular microenvironments within their synovial joints. The presence of IL-2 and G-CSF in SF of responders, and the elevation of IL-6 in the SF of non-responders, may therefore represent a biomarker of a response to anti-TNF therapy. A prospective study would clarify whether the ratio of these cytokines could be used to predict responsiveness to anti-TNF therapy. Clearly such a study could be designed such that potential confounding factors such as previous and concurrent drug therapy, disease duration, etc., could be accounted for in the interpretation.

Cluster analysis of RA SF highlighted differences in local inflammatory profiles from patients with the same disease classification. Cluster 1 SFs were high in IL-1β, IL-2, IL-4, G-CSF, GM-CSF and TNF-α, which regulate the activity of lymphocytes, macrophages, neutrophils and fibroblasts [29–33]. High concentrations of IL-2 and IL-4 in these synovial environments support a humoral immune response, B-cell activation and autoantibody production. SFs falling within Cluster 4 contained high levels of IL-6 and IL-17. IL-6 is secreted by synoviocytes and fibroblast-like synovial cells when cultured *in vitro* in the presence of TNF-α and IL-17 [26, 34]. It is therefore possible that these patients were experiencing a flare of arthritis dominated by the activity of Th17 lymphocytes and synoviocytes. We also identified clusters of SFs with much lower levels of inflammation, possibly indicating lower disease activity and/or underlying OA.

This work also highlights the differences in the levels of circulating blood plasma cytokines compared with the levels found at the site of inflammation. Cytokine levels in blood plasma from patients about to start anti-TNF therapy were measured, and by virtue of their imminent progression onto anti-TNF, these individuals represent RA patients with very active disease. Despite this, some of

FIG. 4 Cytokine concentrations in blood plasma from 16 RA patients before (filled circle) and 12 weeks after (filled square) anti-TNF therapy and from 8 healthy controls (open circle). IL-6 was the only cytokine that significantly decreased following initiation of anti-TNF therapy ($P < 0.05$). RA plasma contained significantly higher concentrations of IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-17, G-CSF and IFN-γ compared with healthy controls ($P < 0.05$). The median concentration of each cytokine is represented by a horizontal bar.



the cytokines measured were below the detection limit of the assay. Most surprisingly, TNF-α was only detected in 4 of 16 plasma samples, although this is not a new observation and has been reported by others [15, 16]. Indeed, IL-6 was the only cytokine measured that was significantly decreased following anti-TNF therapy. The low serum concentration of some cytokines in these patients, all of whom had high disease activity, highlights the importance of measuring cytokines in bodily fluids taken directly from the site of inflammation. In particular, the concentration of IL-6, IL-8 and TNF-α in RA SF appears to be many times greater than the circulating blood plasma levels of these cytokines.

Rheumatology key messages

- Drug regimen may explain the heterogeneity of RA SF cytokine profiles.
- High IL-6 and low G-CSF and IL-2 may be a potential biomarker of non-response to anti-TNF therapy.

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