

## EXTENDED REPORT

# Proteomic analysis of secreted proteins in early rheumatoid arthritis: anti-citrulline autoreactivity is associated with up regulation of proinflammatory cytokines

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**Objectives:** To identify peripheral blood autoantibody and cytokine profiles that characterise clinically relevant subgroups of patients with early rheumatoid arthritis using arthritis antigen microarrays and a multiplex cytokine assay.

**Methods:** Serum samples from 56 patients with a diagnosis of rheumatoid arthritis of <6 months' duration were tested. Cytokine profiles were also determined in samples from patients with psoriatic arthritis (PsA) and ankylosing spondylitis (n=21), and from healthy individuals (n=19). Data were analysed using Kruskal–Wallis test with Dunn's adjustment for multiple comparisons, linear correlation tests, significance analysis of microarrays (SAM) and hierarchical clustering software.

**Results:** Distinct antibody profiles were associated with subgroups of patients who exhibited high serum levels of tumour necrosis factor (TNF) $\alpha$ , interleukin (IL)1 $\beta$ , IL6, IL13, IL15 and granulocyte macrophage colony-stimulating factor. Significantly increased autoantibody reactivity against citrullinated epitopes was observed in patients within the cytokine "high" subgroup. Increased levels of TNF $\alpha$ , IL1 $\alpha$ , IL12p40 and IL13, and the chemokines eotaxin/CCL11, monocyte chemoattractant protein-1 and interferon-inducible protein 10, were present in early rheumatoid arthritis as compared with controls (p<0.001). Chemokines showed some of the most impressive differences. Only IL8/CXCL8 concentrations were higher in patients with PsA/ankylosing spondylitis (p=0.02).

**Conclusions:** Increased blood levels of proinflammatory cytokines are associated with autoantibody targeting of citrullinated antigens and surrogate markers of disease activity in patients with early rheumatoid arthritis. Proteomic analysis of serum autoantibodies, cytokines and chemokines enables stratification of patients with early rheumatoid arthritis into molecular subgroups.

Rheumatoid arthritis is an autoimmune disease that involves multiple molecules and pathways. Autoantibodies and cytokines represent classes of immune cell-secreted proteins postulated to have a variety of roles in rheumatoid arthritis, from regulating the initiation and perpetuation of chronic inflammatory responses to joint destruction.<sup>1–3</sup> However, the precise mechanisms leading to the expression of autoantibodies and cytokines in early rheumatoid arthritis are not completely understood.

Although only scant evidence exists that autoantibodies are directly pathogenic in rheumatoid arthritis, they represent important markers for diagnosis and classification of rheumatoid arthritis.<sup>2</sup> By contrast, autoantibodies have been observed infrequently in other types of arthritis.<sup>4</sup> Proinflammatory cytokines such as tumour necrosis factor (TNF) $\alpha$  and interleukin (IL)1 probably play important parts in regulating immune activation, driving the inflammatory process and promoting joint destruction in a variety of inflammatory joint diseases.<sup>5</sup> Chemokines are chemotactic cytokines produced by fibroblast-like synoviocytes, cells of the innate immune system and other immunoregulatory cells, and there is solid evidence that, among their many roles, they are important potentiators of autoimmune arthritis.<sup>4,6</sup> As expression of cytokines and chemokines in synovial tissue occurs early in the course of rheumatoid arthritis,<sup>7,8</sup> they are under evaluation as biomarkers in early rheumatoid arthritis. The advent of proteomics technologies has enabled large-scale analysis of proteins to identify biomarkers that delineate disease subtypes of

rheumatoid arthritis, and to gain insights into the mechanisms underlying these subtypes. We recently developed and applied antigen microarrays for the diagnosis and classification of rheumatoid arthritis and early rheumatoid arthritis.<sup>9,10</sup> We described 1536-feature arthritis antigen arrays containing 225 peptides and proteins representing candidate autoantigens in rheumatoid arthritis.<sup>9</sup> Antigens included a wide variety of native and in vitro citrullinated proteins and peptides, which were robotically printed to the surface of microscope slides, where the binding of serum autoantibodies was detected.<sup>9,11</sup>

In this paper, we describe a multiplex analysis of serum cytokines using an optimised cytokine bead assay, and integration of these datasets with previously determined antigen array-derived autoantibody signatures.<sup>9</sup> We tested the following hypotheses: (1) cytokines and chemokines derived from subsets of immunoregulatory cells are selectively upregulated in early rheumatoid arthritis; and (2) classes of cytokines are associated with distinct patterns of autoantibody reactivity. Our results provide new insights into associations of anti-citrulline autoantibody responses with production of proinflammatory cytokines, highlight the potential of autoantibodies

**Abbreviations:** ARAMIS, Arthritis, Rheumatism, and Aging Medical Information System; CCP2, cyclic citrullinated peptide 2; FDR, false discovery rate; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IP-10/CXCL10, interferon-inducible protein 10; MCP-1/CCL2, monocyte chemoattractant protein-1; PsA, psoriatic arthritis; SAM, significance analysis of microarrays; TNF, tumour necrosis factor

and cytokines as biomarkers, and suggest a role for chemokines as additional biomarkers in early rheumatoid arthritis.

## PATIENTS AND METHODS

### Patients and sera

All rheumatoid arthritis and control serum samples were obtained under Stanford University Institutional Review Board approved protocols and with informed consent. Samples from patients with ankylosing spondylitis and psoriatic arthritis ( $n = 21$ ), and from healthy individuals ( $n = 19$ ), were provided by a clinical reference laboratory (RDL, Los Angeles, California, USA). Owing to limitations in the number of arrays run in individual experiments, the Arthritis, Rheumatism, and Aging Medical Information System (ARAMIS) cohort samples studied comprised 56 randomly selected serum samples from 793 patients in the ARAMIS early rheumatoid arthritis inception cohort,<sup>12</sup> collected from patients with a clinical diagnosis of rheumatoid arthritis (according to the revised American College of Rheumatology 1987 criteria)<sup>13</sup> for a duration of <6 months. We used a randomisation algorithm for selection of 56 serum samples from the ARAMIS sample bank. The baseline characteristics of this subgroup of patients with early rheumatoid arthritis were assessed and found comparable with those of the whole cohort of patients (table 1<sup>9</sup>), and their autoantibody responses had been previously characterised by antigen microarray assays.<sup>9</sup>

### Cytokine assay

The human 22-cytokine Beadlyte kit (Upstate, Charlottesville, Virginia, USA) and the Luminex xMAP 100IS platform (Luminex, Austin, Texas, USA) were used according to the manufacturers' protocols, except for using 50% of the recommended serum and buffer volumes. For all experiments reported in this paper, unless stated otherwise, an additional blocking reagent optimised for sandwich immunoassays (HeteroBlock, Omega Biologicals, Bozeman, Montana, USA) was added to the serum sample buffer to achieve 3 µg/ml final concentration. Immunodepletion was performed by incubation of 100 µl of serum with 25 µl of protein L-sepharose beads (Pierce Biotechnologies, Rockford, Illinois, USA) for 30 min at 4°C, followed by 30-s centrifugation at 14k revolutions per minute and removal of the supernatant for cytokine analysis. Calibration controls and recombinant standards were used as specified by the manufacturer. Linear correlation coefficients and Mann–Whitney U test statistics were calculated using InStat software. Cytokine concentrations were plotted, and  $p$  values calculated by Kruskal–Wallis tests with Dunn's multiple comparisons using Prism software.

### Microarrays

Protocols for array production and data analysis were presented in prior work<sup>9, 14</sup> and are available online ([www.stanford.edu/group/robinsonlab](http://www.stanford.edu/group/robinsonlab)). Previously generated antigen array datasets<sup>9</sup> were integrated with newly generated cytokine array datasets for the analysis of associations between autoantibody profiles and cytokine profiles. Arrays were scanned using the GenePix4000 Scanner (Molecular Devices Corporation, Union City, California, USA). Median pixel intensities of features and background were determined using GenePix Pro V.3 software.

### Array data analysis

Median net digital fluorescence units represent median values from 4–8 identical features on each array, and were normalised to the median intensity of 12–20 anti-immunoglobulin (Ig)M features. SAM<sup>15</sup> identified antigens with statistical differences in array reactivity between subgroups of early rheumatoid arthritis, stratified on the basis of cytokine levels (fig 1).

Normalised median array values were mathematically adjusted and input into SAM, and results were selected based on false discovery rates (FDRs) and numerator thresholds to identify differentially targeted antigens that exhibited the greatest fold change in reactivities: fig 1A, FDR<0.08 and a numerator threshold of 1.3; fig 1B, FDR<0.14 and a numerator threshold of 1.9; fig 1C, FDR<0.41 and a numerator threshold of 1.9; fig 1D, FDR<0.11 and a numerator threshold of 1.2; fig 1E, FDR<0.21 and a numerator threshold of 1.9; and fig 1F, FDR<0.22 and a numerator threshold of 1.5.

SAM results were arranged as per relationships using Cluster software, and Cluster results displayed using TreeView software.<sup>15</sup> The CCP2 ELISA kits (Immunoscan RA Mark 2, Eurodiagnostica, Malmoe, Sweden) were used in accordance with the instructions of the manufacturer.

Determination of the shared epitope status and other parameters for the ARAMIS cohort have been described elsewhere.<sup>13</sup>

## RESULTS

### Broad up regulation of diverse serum cytokines and chemokines in early rheumatoid arthritis

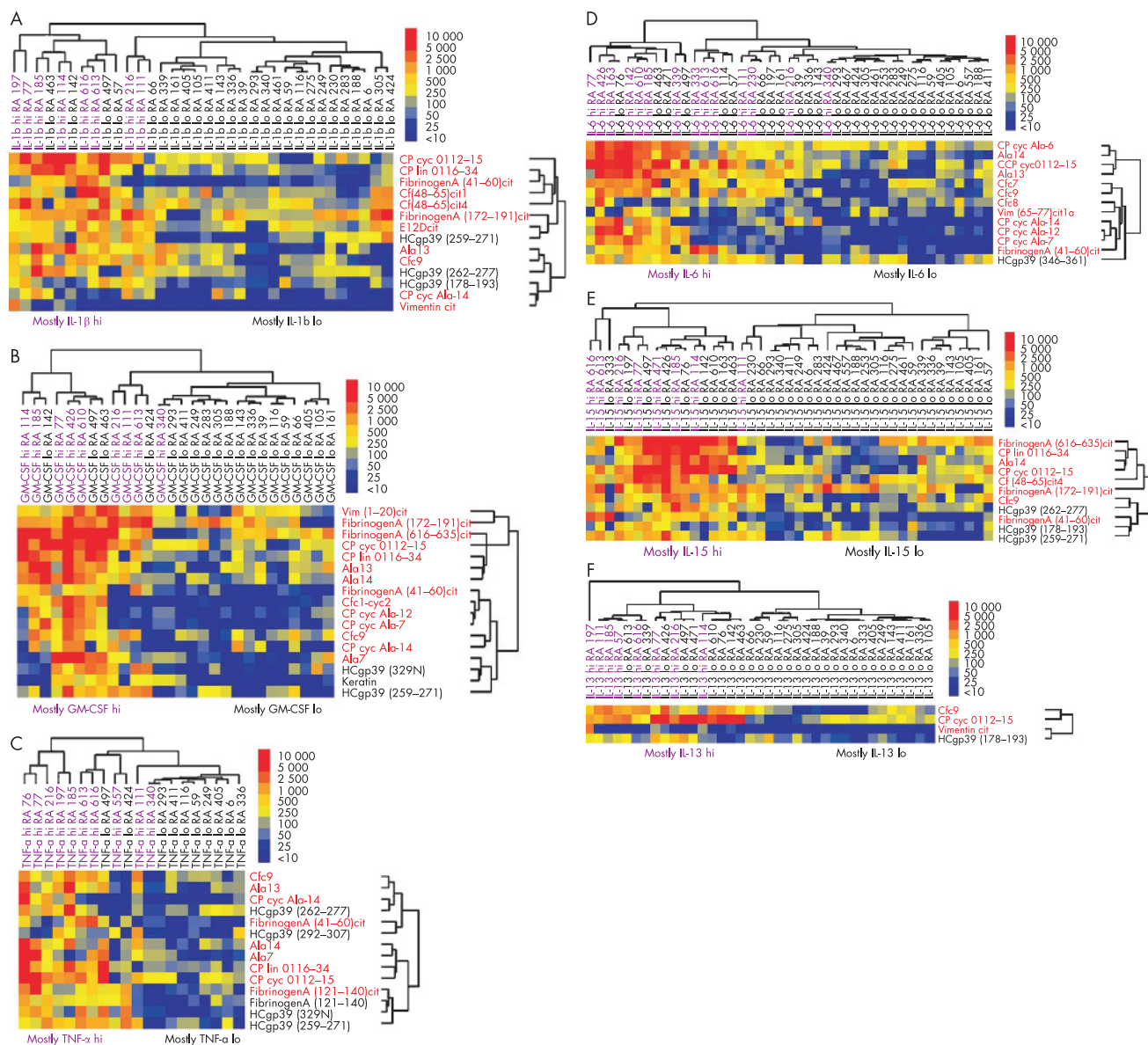
We measured cytokine concentrations in a cohort of patients with early rheumatoid arthritis and controls, using an optimised 22-plex cytokine assay (see supplementary fig A at <http://ard.bmjournals.com/supplemental>). In fig 2, the complete dataset of measurements derived from the early rheumatoid arthritis samples is represented as a hierarchical cluster heatmap for easy visualisation of cytokine signatures in individual patients. Strikingly, samples with detectable cytokines often had increased levels of several cytokines, including both the classical T helper (Th)1 (interferon (IFN) $\gamma$  and IL12) and Th2 (IL10 and IL13) cytokines (fig 2A).

Two major umbrella clusters of patients emerged, a "high" cytokine/high inflammatory cluster and a "low" cytokine/low inflammatory cluster. The "high" cytokine/high inflammatory cluster was comprised of 21 (37.5%) patients with early rheumatoid arthritis, characterised by a multi-cytokine signature. Figure 1B summarizes the marked differences in features between patients of these umbrella clusters. Of note, only laboratory (CCP ELISA, rheumatoid factor, eosinophil sedimentation rate, C reactive protein), and not the available clinical parameters of disease activity (HAQ scores, global assessment scores) differed markedly between patients of the two clusters.

Linear (Pearson's) correlation analysis showed strong correlations between the Th1 cytokines (eg, IFN $\gamma$  and IL12,  $R = 0.91$ ) and Th2 cytokines (eg, IL4 and IL10,  $R = 0.79$ ). Moderate to strong correlations were also observed between Th1 and Th2 cytokines—for example, IFN $\gamma$  and IL10 ( $R = 0.65$ ), IFN $\gamma$  and IL6 ( $R = 0.65$ ), and IL12p70 and IL10 ( $R = 0.84$ ). Anti-CCP2 ELISA positivity correlated moderately to strongly with positive rheumatoid factor results (Spearman's  $R = 0.76$ , 95% confidence interval (CI) 0.62 to 0.86;  $p < 0.001$ ). Correlations were also observed between CRP levels and IL6 concentrations ( $R = 0.42$ ; 95% CI 0.17 to 0.63;  $p = 0.001$ ), and between CRP and MIP-1 $\alpha$ /CCL3 concentrations ( $R = 0.38$ ; 95% CI 0.13 to 0.59;  $p = 0.003$ ).

### Removing or blocking of heterophilic antibodies is essential for quantitative measurements of cytokines in rheumatoid factor seropositive sera

Bead-based multiplex cytokine assays have been validated by others, using both human blood (serum or plasma) and human peripheral blood mononuclear cell culture supernatants.<sup>16, 17</sup> Multiplex assays were more reproducible and reliable than conventional ELISA-based measurements.<sup>17, 19–21</sup> However,

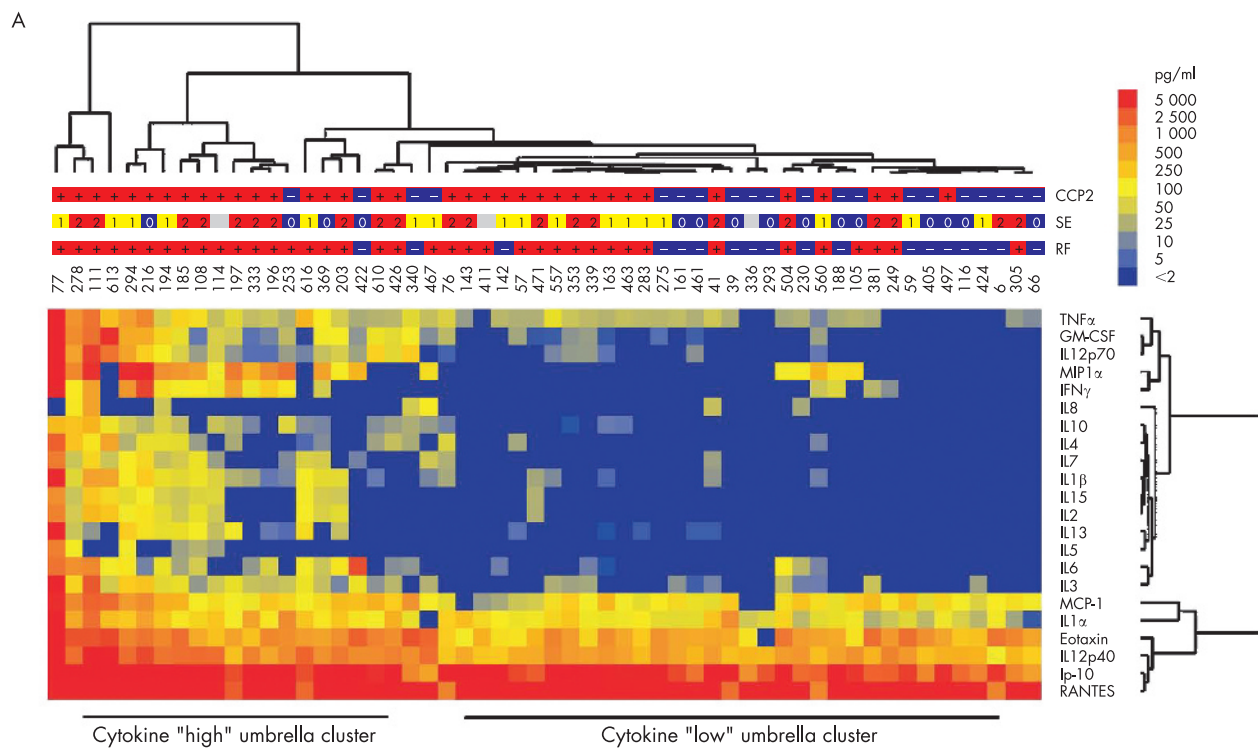


**Figure 1** Differential targeting of citrullinated epitopes in subpopulations of patients with early rheumatoid arthritis with divergent serum cytokine levels. Autoantibody reactivity was determined by antigen arrays, and cytokine concentrations were determined by a bead-based multiplex cytokine assay in 56 early rheumatoid arthritis serum samples. Pairwise significance analysis of microarrays (SAM) was performed to identify antigen features with statistically significant differences in arthritis array reactivity that were associated with serum cytokine levels. Specific analyses include comparisons of female patients with rheumatoid arthritis who had increased or immeasurable serum levels of interleukin (IL)1β (A), granulocyte macrophage colony-stimulating factor (GM-CSF) (B), tumour necrosis factor (TNF)α (C), IL6 (D), IL15 (E) and IL13 (F). Hierarchical clustering was applied to arrange the patients and SAM-identified antigen features (dendrograms on the top and right, respectively). The labels below the cluster images indicate the general locations of the clustering of the sample type being compared. The labels to the right of the cluster images indicate the general locations of antigens, with the citrullinated antigens shown in red type.

concerns exist for both assays regarding the accuracy of measurements in blood or synovial fluid when interfering factors such as heterophilic antibodies are present.<sup>22</sup> Heterophilic antibodies such as rheumatoid factors are defined as antibodies with multispecific activities directed against poorly defined antigens.<sup>22</sup> Multiple studies have shown that blocking or depletion of heterophilic antibodies results in major reductions in read-out levels from cytokine immunoassays, suggesting that heterophilic antibodies including rheumatoid factor can result in false-positive signals in ELISAs and other immunoassays.<sup>8, 24, 25</sup>

In our preliminary experiments, we observed a striking association of increased serum concentrations of multiple

cytokines with rheumatoid factor seropositivity (data not shown). To determine whether rheumatoid factor was causing false elevations in signal in our multiplex cytokine assay, we depleted the serum of immunoglobulins by incubation of 100 μl of serum with 25 μl of protein L-sepharose beads for 30 min at 4°C. Depletion of immunoglobulins resulted in substantial reduction in signal in several rheumatoid factor seropositive samples (supplementary fig A). Measurements in rheumatoid factor seronegative samples were not affected (see supplementary fig C in <http://ard.bmjournals.com/supplemental>, sample 12; also data not shown). Importantly, a substantial fraction of rheumatoid factor seropositive samples showed very low or undetectable concentrations of several cytokines (fig 2A).



**B**

	Cytokine high umbrella cluster (n=21); medians	Cytokine low umbrella cluster (n=35); medians	p Value (Mann-Whitney U test)
Age	54	53	
CRP	1.10 mg/dl	0.33 mg/dl	0.001
ESR	38.5 mm Hg	19 mm Hg	0.045
RF titre	285.0	32.1	<0.001
CCP titre	472.2	28.0	0.024
HAQ	1.25	1.125	NS
GLOBAL	33	45	NS

**C**

Linear correlation analysis		
Cytokine	Anti-CCP2 and cytokine R; (95% CI)	p Value
TNF $\alpha$	0.39 (0.15 to 0.59)	0.003
IL10	0.38 (0.13 to 0.48)	0.004
IL1 $\beta$	0.37 (0.12 to 0.58)	0.005
IL1 $\alpha$	0.36 (0.10 to 0.57)	0.007
IL2	0.36 (0.10 to 0.57)	0.007
IL4	0.35 (0.10 to 0.56)	0.008
IL12p70	0.34 (0.08 to 0.56)	0.01
IP10/CXCL10	0.32 (0.06 to 0.54)	0.02
GM-CSF	0.31 (0.05 to 0.53)	0.02
IFN $\gamma$	0.28 (0.014 to 0.50)	0.04
IL6	0.22 (-0.05 to 0.46)	NS
IL13	0.22 (-0.05 to 0.46)	NS
IL15	0.22 (-0.05 to 0.46)	NS

Anti-CCP2 and RF titre: Spearman R=0.76; 95% CI 0.62 to 0.86, p<0.001

**Figure 2** Blood cytokine profiles stratify patients with early rheumatoid arthritis. We applied a bead-based array using an optimised protocol to profile cytokines in rheumatoid arthritis serum samples. (A) Array results are displayed as a heat map after hierarchical clustering of all data points to visualise the spectrum of cytokine levels for each patient. Columns represent individual patients, labelled on the top. Red represents the highest cytokine values. For each patient, the number of copies of the shared epitope, rheumatoid factor status and CCP2 ELISA reactivity are indicated across the top of the panel. Rows representing individual cytokine levels are labelled on the right side of the panel. (B) Comparison between disease activity parameters of patients in the cytokine “high” and “low” umbrella clusters, respectively. (C) Linear correlation analysis was performed to determine correlations between anti-CCP2 and cytokine concentrations. Correlation coefficients for selected individual pairs are displayed in descending order.

**Table 1** Baseline characteristics of the Arthritis, Rheumatism, and Aging Medical Information System patients analysed on arthritis arrays and with a multiplex cytokine assay (n = 56)\*

Age, median (range), years	53.5 (19–78)
Female sex, n (%)	43 (77)
RF seropositive, n (%)	38 (68)
CRP median (range), mg/dl	0.50 (0.09–15.7)
Median (range) disability score	1.125 (0–2.375)
Median (range) educational level score	12 (8–17)
Treatment with conventional DMARD, n (%)	23 (41)
Shared epitope present, no (%)	38 (68)

CRP, C-reactive protein; DMARD, disease-modifying anti-rheumatic drug; RF, rheumatoid factor.

HeteroBlock is a reagent optimised to prevent rheumatoid factor from bridging capture and detection antibodies in sandwich immunoassays. Heteroblock was used previously to reduce non-specific binding of rheumatoid factor to primary and secondary antibodies in cytokine ELISAs.<sup>25</sup> In our experiments, 3 µg/ml of HeteroBlock in serum diluent yielded effects comparable to immunoglobulin depletion by protein L-sepharose precipitation (see supplementary fig B in <http://ard.bmjournals.com/supplemental>). Our observations are in line with recent experiments by de Jager *et al*, who showed that near-complete (89%) depletion of plasma IgM rheumatoid factor by protein L-sepharose and additional blocking with 10% rodent serum before bead-based multiplex cytokine analysis resulted in reduced non-specific binding and more accurate recovery rates of cytokines.<sup>18</sup>

#### Serum concentrations of the cytokines IL1 $\alpha$ , TNF $\alpha$ , IL12p40 and IL13 are increased in patients with early rheumatoid arthritis compared with controls

Comparisons of cytokine concentrations between early rheumatoid arthritis, PsA/ankylosing spondylitis and controls were performed using Kruskal–Wallis test, with post-test analysis by Dunn’s multiple comparisons. Serum concentrations of the following cytokines were significantly increased in patients with early rheumatoid arthritis: IL1 $\alpha$  ( $p < 0.001$ ), TNF $\alpha$  ( $p < 0.001$ ) and IL12p40 ( $p < 0.001$ ; fig 3A,C,D), and IL13 ( $p = 0.02$ , data not shown). Significant differences were not observed for IL6, for which median concentrations did not differ between PsA/ankylosing spondylitis and early rheumatoid arthritis (fig 3B).

#### Serum concentrations of the chemokines interferon-inducible protein 10 (IP-10/CXCL10), monocyte chemoattractant protein-1 (MCP-1/CCL2) and eotaxin/CCL11 were raised in patients with early rheumatoid arthritis compared with controls

Median serum concentrations of three chemokines were higher in early rheumatoid arthritis: IP-10/CXCL10 ( $p < 0.001$ ), eotaxin/CCL11 ( $p < 0.001$ ) and MCP-1/CCL2 ( $p = 0.001$ ; fig 3E–G). IL-8/CXCL8 was the only cytokine with higher median concentrations in patients with PsA/ankylosing spondylitis compared with those with early rheumatoid arthritis ( $p = 0.02$ ; fig 3H). Although included in the multiplex cytokine assay, we did not analyse data for the chemokine RANTES/CCL5, because a large number of measurements were out of range of quantification due to saturation of the beads with this cytokine at the standard serum dilution of 1:1 (data not shown).

#### Integration of autoantibody profiles with cytokine concentrations

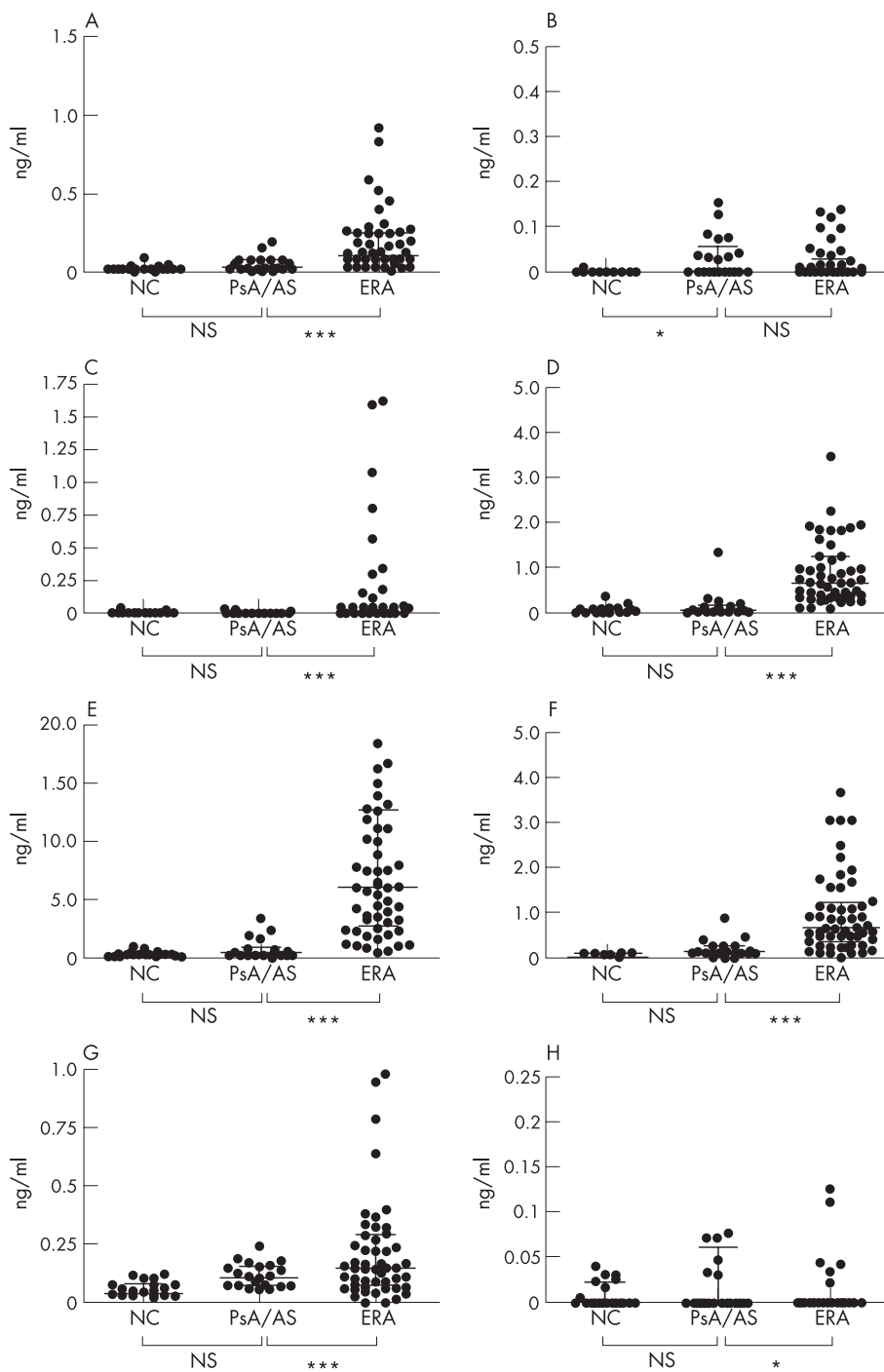
To integrate cytokine profiles with autoantibody profiles, we performed a pairwise SAM analysis of arthritis array results from patients stratified based on the presence of raised and low/undetectable serum concentrations of cytokines. In this cross-sectional dataset, we determined which cytokines were associated with distinct antibody profiles and surrogate markers of disease activity and severity (CRP and HAQ) in early rheumatoid arthritis. Cut-offs for sample classification into the high cytokine subgroup were defined as samples above the 75th centile. Patients were stratified according to sex and rheumatoid factor. These analyses showed significantly increased autoantibody reactivity against citrullinated epitopes in patients in the high cytokine subgroup, and subset analysis of women alone showed even stronger correlations (fig 1A–F). IL1 $\beta$  “low”, IL6 “low” and IL15 “low” subgroups of patients exhibited lower reactivity against citrullinated epitopes (fig 1A,D,E), and a trend towards targeting certain native epitopes including collagen type II, heterogenous nuclear ribonucleoprotein B and heterogenous nuclear ribonucleoprotein peptides (data not shown).

Comparison of the two major clusters shown in fig 2A showed significantly higher CRP ( $p = 0.001$ ), ESR ( $p = 0.045$ ), CCP ( $p = 0.024$ ) and RF titres ( $p = 0.001$ ) within the “high” cytokine group (fig 2B). This observation indicates that the multi-cytokine signature defining the “high” cytokine cluster represents an additional biomarker in patients with more active disease, as measured by standard laboratory markers of disease activity.

#### DISCUSSION

We describe the application of arthritis antigen microarrays and a bead-based cytokine assay to profile secreted immunoregulatory proteins, including autoantibodies and cytokines, in blood derived from patients with early rheumatoid arthritis. We identified proteomic patterns of differential antigen recognition and cytokine production that differentiated a high inflammatory from a low inflammatory subtype of early rheumatoid arthritis. Several citrullinated epitopes and a few native human cartilage gp39 peptides were preferentially targeted by autoantibodies in patients with high serum levels of the proinflammatory cytokines TNF $\alpha$ , IL1 $\beta$ , IL6, IL15 and granulocyte macrophage colony-stimulating factor (GM-CSF), as well as IL13, and these patients possessed features predictive for the development of more severe arthritis. Moreover, our results suggest an important role for the downstream amplifiers of inflammatory responses in early rheumatoid arthritis, as three major chemokines were upregulated in patients with rheumatoid arthritis over controls, namely IP-10/CXCL10, a ligand of CXCR3 associated with Th1-type reactions,<sup>26</sup> eotaxin/CCL11, a ligand of CCR3 associated with Th2-type reactions,<sup>27</sup> and MCP-1/CCL2.

Little is known about the association of serum autoantibodies with serum cytokines and chemokines. In a study involving a very small number of patients with rheumatoid arthritis, a generalised up regulation of serum cytokine concentrations over that of controls was observed.<sup>28</sup> Another recent study on early undifferentiated arthritis showed correlations between raised concentrations of multiple cytokines with clinical subtypes and anti-CCP ELISA antibody responses in early rheumatoid arthritis.<sup>28</sup> In contrast to Hitchon *et al*,<sup>29</sup> we did not observe statistically significant increases in the classical Th2 cytokine IL4 in rheumatoid arthritis serum compared with controls. This discrepancy might be due to our more aggressive sample treatment with HeteroBlock, which corrected for false elevations of several cytokines in rheumatoid factor seropositive samples (fig 2A, see supplementary fig B in <http://ard.bmjournals.com/supplemental>; also data not shown). Moreover, we



**Figure 3** Comparison of cytokine concentrations in healthy individuals, patients with psoriatic arthritis (PsA) and ankylosing spondylitis, and early rheumatoid arthritis. Serum samples from patients with early rheumatoid arthritis (n = 56), PsA and ankylosing spondylitis (n = 21), and from healthy subjects (n = 19) were analysed by multiplex cytokine assay. Representative results are shown for (A) interleukin (IL)1 $\alpha$ , (B) IL6, (C) tumour necrosis factor (TNF) $\alpha$ , (D) IL12p40, (E) interferon-inducible protein 10 (IP-10/CXCL10), (F) eotaxin/CCL11, (g) monocyte chemoattractant protein (MCP-1/CCL2) and (h) IL8/CXCL8. Horizontal bars represent medians with centiles for each column.

observed raised serum levels of both classical Th1 (IFN $\gamma$  and IL12) and Th2 (IL10 and IL13) cytokines in about one third of patients with early rheumatoid arthritis (clustering to the left of the heatmap in fig 2A). Our results are consistent with the observation that dichotomous Th1 and Th2 T cells, although delineated in mice, have not been readily identified in humans.<sup>30</sup>

As patients in the ARAMIS cohort were not treated with anti-cytokine or other biological treatments at the time blood samples were obtained, serum concentrations in these samples may be more reflective of systemic levels of proinflammatory cytokines than in patients treated with cytokine-antagonising biological agents.<sup>31</sup> Serum cytokine levels may reflect the level

of immune cell activation in involved joints<sup>3</sup> or lymphoid tissues. Our findings suggest that generation of autoantibodies against citrullinated epitopes and other antigens is linked predominantly to the production of high levels of proinflammatory cytokines by activated T cells, macrophages and other cells in rheumatoid arthritis. For instance, we observed associations of GM-CSF levels with anti-citrulline reactivity (fig 1B). As GM-CSF has been implicated in up regulation of class II MHC on human monocytes,<sup>32 33</sup> it is plausible that an immunological link between GM-CSF production, autoantigen presentation and induction of autoantibody production exists.

Recent work has suggested that chemokines have a prominent role in rheumatoid arthritis, and hence these modulators were

proposed, together with their respective receptors, as targets for next generation therapeutics.<sup>6, 34–36</sup> Remarkably, our findings show that three chemokines, IP-10/CXCL10, eotaxin/CCL11 and MCP-1/CCL2, were strikingly increased in early rheumatoid arthritis serum compared with control serum (fig 3E–G). Chemokines such as IP-10/CXCL10 may have pivotal roles in recruiting activated T cells to sites of inflammation, and were proposed as key mediators of T cell polarisation in animal models of Th1-type autoimmunity.<sup>37, 38</sup> Other chemokines, including IL8/CXCL8 and MCP-1/CCL2,<sup>39, 40</sup> and GM-CSF<sup>41</sup> are linked to TNF $\alpha$  via positive feedback loops, and are additive or even synergistic in their pathophysiological effects. Although expression of macrophage-associated chemokines has been described in the synovium of patients with early rheumatoid arthritis, their precise role in early synovitis, and their potential role as biomarkers in early rheumatoid arthritis, remains ill-defined.<sup>7, 8</sup> Longitudinal studies in a larger cohort of early rheumatoid arthritis including preclinical samples are under way to further elucidate the evolution of cytokine, chemokine and autoantibody signatures in early rheumatoid arthritis.

Multiplex assays enable cost-effective simultaneous measurements of serum cytokines, chemokines and autoantibodies. As robust assays become available for use in clinical laboratories, we expect that proteomic analyses will become a mainstay in the evaluation of patients with rheumatoid arthritis and other autoimmune diseases for assessing prognosis, guiding treatment and monitoring response to treatment.

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For supplementary figures see at <http://ard.bmjournals.com/supplemental>

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Competing interests: None.

All rheumatoid arthritis and control serum samples were obtained under Stanford IRB (Stanford University Institutional Review Board, Stanford University, Stanford CA 94305) approved protocols and with informed consent.

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