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Circulating cytokines in Norwegian patients with psoriatic arthritis determined by a multiplex cytokine array system

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Objectives. **Serum cytokines play an important role in the pathogenesis of psoriatic arthritis (PsA) by initiating and perpetuating various cellular and humoral autoimmune processes. The aim of this study was to describe a broad spectrum of T- and B-cell cytokines, growth factors and chemokines in patients with PsA and healthy individuals.**

Methods. **A novel protein array system, denoted as multiplex cytokine assay was utilized to measure simultaneously the levels of 23 circulating cytokines of patients with PsA and healthy individuals. Additionally, correlational clustering and discriminant function analysis (DFA), two multivariate, supervised analysis methods were employed to identify a subset of biomarkers in order to describe potential functional inter-relationships among these pathological cytokines and identify biomarkers with prognostic and diagnostic utility.**

Results. **Univariate analysis demonstrated that serum levels of a complex set of immune and inflammatory modulating cytokines are significantly up-regulated in patients with PsA relative to unaffected controls including interleukin (IL)-10, IL-13, interferen (IFN)-α, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor [CCL3 macrophage inflammatory protein (MIP)-1α], CCL4 (MIP-1β) and CCL11 (Eotaxin), while granulocyte-colony stimulating factor was significantly reduced in PsA patients. Correlational clustering was able to discriminate among, and hence subclassify, patients with varying levels of disease activity, which may prove useful in guiding therapy in these apparently phenotypically distinct disease subsets. DFA identified EGF, IFN-α, VEGF, CCL3 (MIP-1α) and IL-12p40 as analytes with the strongest discriminatory power among various PsA patients and controls.**

Conclusions. **Our findings suggest that these factors modulate PsA pathology and the articular involvement in a synergistic manner. Identifying factors could be used in the development of clinical diagnostic tests, which are valuable to guide evidence-based diagnosis and disease management of PsA.**

KEY WORDS: Psoriatic arthritis, Circulating cytokines, Hierarchical clustering, Discriminant function analysis, Disease activity, Multiplex cytokine array system.

Introduction

Arthritides, associated with psoriasis is a heterogeneous disease entity consisting of monoarticular, oligoarticular and/or polyarticular type of peripheral joint involvement. The disease is also characterized by erosive manifestations, bony periosteal reaction, exuberant proliferation at sites of enthesis and the presence of bony ankylosis [1]. Psoriatic arthritis (PsA) has been diagnosed in ~20% of those who have psoriasis and manifests in most patients between the ages of 20 and 50 yrs [1]. In addition to the peripheral joint synovitis and spontaneous joint fusion, patients present with dermatological manifestations of psoriasis, rheumatoid factor (RF) seronegativity and human leucocyte antigen (HLA) associations [2]. The pathological process of skin and joint lesions in PsA is primarily mediated by autoimmune inflammatory reaction and a pathogenic interplay of genetic and environmental factors [3].

The prominent inflammatory lymphocytic infiltrate in the skin dermal papillae, the joint stroma and at inflammatory enthesis reflects a complex cytokine profile in PsA [1]. Cytokines, as molecular mediators of the inflammatory process in PsA including tumour necrosis factor (TNF)-α [4, 5], have previously been described as crucial factors in the pathogenesis of the disease. Other cytokines, such as interleukin (IL)-10, IL-12, IL-13, IL-18 and vascular endothelial growth factor (VEGF), have also been described to have a pathogenic role in PsA [6–9]. These studies suggest that cytokines secreted from activated T- and B-cells and other immunocompetent cells induce proliferation and activation of the synovial and epidermal fibroblasts leading to clinical manifestations of arthritis

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in these patients. The importance of this class of disease mediators is underscored by the highly efficacious action of anti-cytokine-based biological therapies in PsA and other inflammatory arthritides [10, 11]. Circulating cytokines therefore reflect the activation status of ongoing inflammatory processes and the evaluation of serum cytokines is a good indicator and a reliable surrogate marker of disease activity in inflammatory arthritides [12, 13].

Clinical studies of cytokine function in inflammatory arthritis can be controversial and even contradictory. The roles of cytokines in health and disease can be blurred due to the complexity of cytokine mechanics, including kinetics of expression, mode of induction, regulation of receptor expression, competition for occupancy, and synergy of action, all critical aspects of their net effect. Moreover, there are differences in pathological manifestations, disease severity and drug response among patients that must be considered in cohort studies of cytokine function to accurately define their role in health and disease [14].

Broad-based proteomic screening methods are beginning to reveal the rich repertoires of cytokines at play in a given form of autoimmune diseases or inflammatory arthritides [15, 16]. Moreover, increasingly sophisticated multi-variant analyses methods have been developed to aid in our understanding of the complex regulatory networks of these disease mediators [17–19].

The aim of this study was to first catalogue a broad spectrum of lymphokines, monokines, chemokines, growth and angiogenic factors in the periphery of PsA patients, then model putative regulatory networks among these factors, using multivariate biostatistical methods and clinical correlations. In doing so, we have verified the presence of previously characterized and identified novel cytokines up-regulated in PsA sera, mediators with both diagnostic and mechanistic potential. We have also characterized likely interplay among these cytokines using multivariate analysis methods that model network-like behaviours among biological variables.

Materials and methods

Patients and controls

The study population consisted of 43 Norwegian patients with PsA of polyarticular type who were recruited from the out-patient clinic at the Department of Rheumatology, Haukeland University Hospital, Bergen, Norway (24 females and 19 males; mean age 53.77 yrs, range 26–74) fulfilling the diagnosis of PsA according to the criteria of Moll and Wright [20]. Also 25 age- and sex-matched healthy controls were enrolled in the study. These controls were healthy blood donors, without any sign of arthritis, psoriasis or ongoing inflammation. Controls were medication-free for at least 3 months prior to this study. To assess the present disease activity, clinical examination of 52 joints was performed (the EULAR 44 joint count added to DIP joints of hands). Skin affection by psoriasis was assessed using the PASI score [21]. Before inclusion in the study, informed consent was signed by each patient.

General laboratory and immunolaboratory assessments included erythrocyte sedimentation rate, C-reactive protein, white blood cell count, haemoglobin, anti-nuclear antibody and RF. Clinical and laboratory characteristics of the patients included in the study are summarized in Table 1.

Serum samples

Blood samples were obtained from both patients and unaffected controls after informed consent and treated anonymously throughout the analysis. Blood was collected in endotoxin-free silicone-coated tubes without additive. The blood samples were allowed to clot at room temperature for 30 min before centrifugation (3000 rpm, 4°C, 10 min), the serum was removed and stored at −80°C until analysed.

Multiplex cytokine assay

Serum levels of cytokines and chemokines, including IL-1β, IL-2, IL-4, IL-5, IL-6, CXCL8 (IL-8), IL-10, IL-12 (p40), IL-13, IL-15, IL-17, interferon (IFN)-α and IFN-γ, TNF-α, epidermal growth factor (EGF), VEGF, basic fibroblast growth factor (FGF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), CCL2 [monocyte chemoattractant protein (MCP)-1]/(MCAF), CCL3 [macrophage inflammatory protein (MIP)-1α], CCL4 (MIP-1β) and CCL11 (Eotaxin) were measured using a bead-based immunofluorescence assay (Luminex Inc. Austin, TX, USA) using multiplex cytokine reagents supplied by Biosource International, Camarillo, CA, USA as previously described [16, 22]. Briefly, a sandwich immunoassay-based protein array system (Biosource International), which contains dyed microspheres conjugated with a monoclonal antibody specific for a target protein was used in this assay. Serum samples were thawed and run in duplicates. Antibody-coupled beads were incubated with the plasma sample (antigen) after which they were incubated with biotinylated detection antibody before finally being incubated with streptavidin–phycoerythrin. A broad sensitivity range of standards (Biosource International), ranging between 1.95 and 32 000 pg/ml were used to help enable the quantitation of a dynamic wide range of cytokine concentrations and provide the greatest sensitivity. This captured bead-complexes were then read by the Bio-Plex array reader (Bio-Rad Laboratories, Hercules, CA), which uses Luminex fluorescent bead-based technology (Luminex Corporation Austin, TX, USA) with a flow-based dual laser detector with real-time digital signal processing to facilitate the analysis of up to 100 different families of colour-coded polystyrene beads and allow multiple measurements of the sample ensuring in the effective quantification of cytokines.

Validation of the multiplex assays was performed using single protein ELISAs (Biosource International). Values obtained from multiplex assay analytes were highly correlative (Spearman's rank correlation coefficient, 0.97 ± 0.03) when compared with individual ELISAs for particular cytokines.

Serum levels of 23 cytokines were compared among PsA patients and unaffected control individuals, also among various subsets of patients with PsA. The cytokines assayed included modulators of several key aspects of disease pathology including regulation of inflammation, cellular and humoral immunity, leukocyte trafficking, cell growth and angiogenesis. To facilitate functional interpretation of results, cytokines were sorted into four functional groups in the graphical representations of these analyses. This included a group denoted 'cellular cytokines' which drive, albeit not exclusively, cytotoxic and anti-viral responses (e.g. IL-1β, IL-2, IL-12p40, IL-15, IL-17, TNF-α, IFN-α, IFN-γ), 'humoral cytokines' (e.g. IL-4, IL-5, IL-6, IL-10, IL-13), 'growth and angiogenic factors' (e.g. EGF, VEGF, FGF basic, G-CSF, GM-CSF) and 'chemokines' [e.g. CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL11 (Eotaxin) and CXCL8 (IL-8)].

Statistical analysis

The concentrations of analytes in these assays were quantified using a calibration or standard curve. A 5-parameter logistic regression analysis was performed to regress a known serial dilution of analyte *vs* median fluorescent intensity read from a flow cytometer. The resulting equation was then used to predict the concentration of the unknown samples [23].

TABLE 1. Clinical and laboratory characteristics of patients with psoriatic arthritis

Patient No.	Age	Sex	Type of psoriasis	Therapy	No. of swollen joints	No. of tender joints	WBC	HgB	ESR	CRP	RF	ANA
1	45	F	PV	MTX	2	12	5.8	12.8	8	5	0	0
2	40	M	PV	COX-2 MTX	0	0	6.4	16.1	10	11	0	0
3	26	F	PV	–	0	11	6.6	13.9	4	5	0	0
4	58	F	PV	COX-2 MTX	1	2	9.2	14.9	26	20	0	0
5	57	F	PV	COX-2	3	15	4.9	12.7	39	5	1	1
6	73	M	PV	NSAID	4	22	9.3	16.1	2	5	0	0
7	33	F	PV	COX-2 MTX	2	26	10.4	12.2	10	5	0	0
8	69	F	PP	NSAID	3	20	6	11.7	7	5	0	0
9	72	M	PV	COX-2	4	9	4.9	13.8	19	5	0	0
10	58	F	PV	–	2	21	4.7	13.8	21	5	0	0
11	48	M	PV	COX-2 MTX	4	5	15.1	15.6	20	5	0	0
12	34	M	PV	NSAID	0	6	6.7	16	2	5	0	0
13	57	M	PV	COX-2 LEF	8	29	4.8	15.4	5	5	0	0
14	61	F	PV	NSAID MTX	1	7	701	14.5	3	5	0	0
15	51	M	PV	NSAID MTX SUL	10	14	7.3	13.8	10	16	1	0
16	53	M	PV	COX-2	5	16	6.6	14.6	15	23	0	0
17	67	M	PV	NSAID	9	37	9	15.1	5	5	0	0
18	55	F	PP	COX-2 MTX PRED	2	5	10.7	14.9	3	5	0	0
19	50	M	PV	NSAID HYDCL	2	10	8.1	14.1	24	25	0	0
20	55	F	PV	NSAID LEF	5	27	–	–	–	–	0	0
21	50	F	PV	COX-2 SUL	2	13	5.4	14	3	5	0	0
22	46	M	PV	COX-2	0	15	4.8	14.4	10	5	0	0
23	56	F	PV	NSAID	8	23	5	15	15	5	0	0
24	65	F	PV	COX-2 SUL	1	24	5.9	12.7	11	5	0	0
25	32	F	PV	COX-2 MTX TNF	1	6	7.3	13.8	13	5	0	0
26	56	M	PV	COX-2	1	8	6.1	16.3	1	5	0	0
27	59	M	PV	NSAID MTX	1	7	8.6	15.2	1	5	0	0
28	70	M	PV	COX-2	0	27	6.1	16.6	1	5	0	0
29	61	F	PP	NSAID SUL	7	8	4.6	12.4	27	16	0	0
30	44	M	PV	NSAID	4	4	6.3	14.9	3	5	1	0
31	74	F	PV	–	4	7	10.2	13.8	27	34	0	0
32	54	F	PV	NSAID	8	13	6.4	13.1	14	18	0	1
33	47	M	PV	COX-2 MTX	0	23	8.5	14.4	3	5	0	0
34	35	M	PV	NSAID MTX	0	0	6.7	15.3	1	5	0	0
35	45	F	PV	NSAID LEF	0	9	8.4	12.6	31	15	0	0
36	46	F	PV	NSAID MTX	7	15	4.7	12.2	32	5	0	0
37	68	F	PV	NSAID	1	4	9.6	14.4	20	10	0	0
38	54	F	PV	COX-2	2	2	10.4	14	7	5	0	0
39	56	M	PV	–	0	2	7.6	15.1	3	5	0	0
40	74	M	PV	–	0	4	3.8	14.4	10	5	0	0
41	57	F	PV	NSAID MTX	2	5	7.2	13.1	17	5	0	0
42	58	F	PV	COX-2	11	9	4.9	13.2	6	5	0	0
43	43	F	PV	MTX	1	7	6.5	13.3	6	5	0	0

ANA, anti-nuclear antibody (0 = negative, 1 = positive) (norm 0.00–0.99); COX-2, selective cyclo-oxigenase-2 inhibitor; CRP, C-reactive protein (norm < 10); ESR, erythrocyte sedimentation rate (norm 0–20 mm/hr); HYDCL, hydroxy-chloroquine; F, female; HgB, haemoglobin (norm: 13.2–16.6 g/dl for men and 11.6–16.0 for women); M, male; MTX, methotrexate, NSAID, non-steroidal anti-inflammatory drugs (unselective), LEF, leflunomide; PP, pustulosis palmoplantaris; PRED, prednisolone (2.5 mg/day); PV, psoriasis vulgaris; RF, rheumatoid factor (0 = negative, 1 = positive: Waaler ≥ 128); SUL, sulfasalazine, TNF, TNF-blocking agent (Infliximab); WBC, white blood cell count (norm 3.5–11.0 10^9/l).

Statistical differences in measured values were analysed using a Mann–Whitney U-test. *P*-values <0.05 were considered statistically significant. All *P*-values reported are nominal due to the exploratory nature of this analysis. Matlab R13 (Natick, MA, USA) and Statistica v6 (Tulsa, OK, USA) were used to perform all statistical analyses.

Sample size

Group sample sizes of 25 and 43 achieve 97% power to detect a difference of −30.0 between the null-hypothesis, where both group means are 30.0 and the alternative hypothesis, where the mean of cytokine X is 60.0 with estimated group SDs of 30.0 and 30.0, and with a significance level (α) of 0.05000 using a two-sided Mann–Whitney U-test assuming that the actual distribution is uniform.

Multivariate analysis

Hierarchical clustering using Pearson's correlation coefficient as the distance metric [24, 25] was used to assess cytokine similarity. Next, the resulting 'distance matrix' (here representing correlational strength) was sorted by cytokine to align the most similar cytokines closer to one another. The resorted distance matrix was then plotted like a heat map using SigmaPlot 2001 (SPSS Inc., Chicago, IL, USA). Discriminant function analysis (DFA) was used for selection of the set of analytes that maximally discriminate among the groups studied ($\alpha = 0.10$), built in a step-wise manner, as described previously [18, 19]. A DFA creates a discriminate function, denoted by a root, which is an equation consisting of a linear combination of analytes used for the prediction of group membership. Once the analytes are selected by DFA, a leave-one-out cross-validation is performed to protect against over-fitting. The best cross-validated model is then reported. The final discriminatory

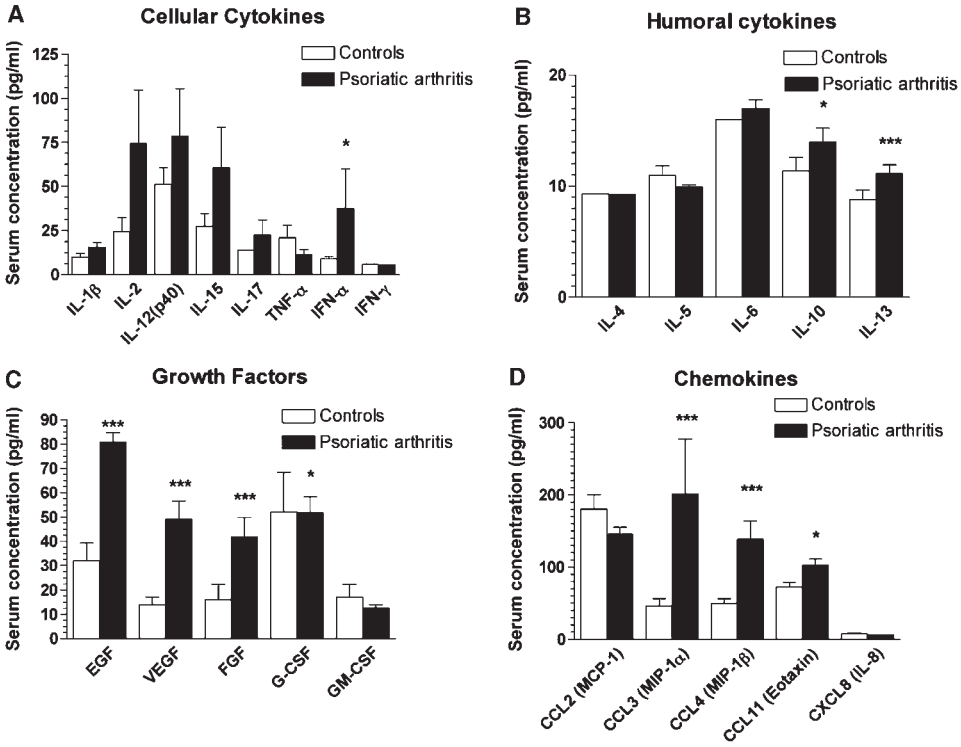

FIG. 1. Comparison of various serum cytokine levels between PsA patients ($n = 43$) and controls ($n = 25$). Bars show the mean in pg/ml and SEM. (**A**) Levels of circulating cellular cytokines. (**B**) Levels of humoral cytokines. (**C**) Levels of growth factors. (**D**) Circulating chemokine levels. *Significantly different from compared values by Mann–Whitney U-test ($P < 0.05$).

power of each analyte is characterized by a partial Wilk's Lambda coefficient; 1.0 (no discriminatory power) to 0.0 (perfect discriminatory power). The discriminatory potential of a DFA analysis is best realized through a simple scatter plot of the roots.

Simulation studies

A total of 500 Monte Carlo simulation studies were performed on the hierarchal cluster analysis to estimate the strength of random correlations. The simulations can be defined as follows:

(i) Estimate the average and SD of each analyte in each group (normal or PsA).
(ii) Generate 43 or 25 numbers (depending on whether the normal or PsA group is being examined) from a normal distribution using the estimates found in 1) for each analyte.
(iii) Create a hierarchical cluster from the simulated data (see above for details).
(iv) Repeat 1–3 500 times.
(v) Determine the 95% percentile of correlations and denote it as ρ.

All values greater than ρ have <5% chance of being caused by random fluctuations in the data.

Results

Univariate comparison of serum cytokines in patients with PsA and unaffected controls

Among the 'cellular cytokines', only IFN-α ($P = 0.039$) was significantly increased in PsA patients compared with the levels found in healthy individuals (Fig. 1A). Among humoral cytokines, the levels of IL-10 ($P = 0.0107$) and IL-13 ($P = 0.0006$) were significantly elevated in patients (Fig. 1B), as were the chemokines CCL3 (MIP-1α) ($P < 0.0001$), CCL4 (MIP-1β) ($P < 0.0001$) and CCL11 (Eotaxin) ($P = 0.0101$), and the growth and angiogenic factors EGF ($P < 0.0001$), VEGF ($P < 0.0001$), FGF ($P < 0.0001$) (Fig. 1C and D). G-CSF was significantly decreased ($P = 0.014$) in the patient group compared with controls (Fig. 1C).

Comparison of serum cytokine levels among PsA patients with variation in disease severity

Patients were divided based on swollen joint counts. Group 1 consisted of 8 patients with counts of 0; group 2 (12 patients), 1–3, and group 3 (12 patients), ≥4. Increases in the average levels IL-2, IL-12(p40), IL-15, IFN-α and CCL3 appeared elevated in patients of group 3 relative to groups 1 and 2 (Fig. 2A–D). These results suggest that a more active Th1/cellular immunity may be at play in a subset of patients with a more severe disease. Accordingly, serum levels of IL-2 appear to correlate with increasing disease severity among the three groups (Fig. 2A–D). When the levels of these differential analytes were evaluated using discriminant functional analysis, IFN-α, IL-15 and IL-2 were identified as being significantly discriminatory between the three groups with a P-value of 0.012, 0.026 and 0.069, respectively (Table 2), suggesting the potential role of a T-cell signature in the aetiopathogenesis of the disease.

Characterization of cohort profiles by hierarchical clustering

Hierarchical clustering was used to identify sets of cytokines whose expression changes are linearly related within a given population, i.e. sets of cytokines whose expression levels among individuals within a population are correlated. In this method, correlation coefficients between pairs of cytokines are positive for cytokines that are consistently at the same level in individuals of a given population under study, negative for cytokines that

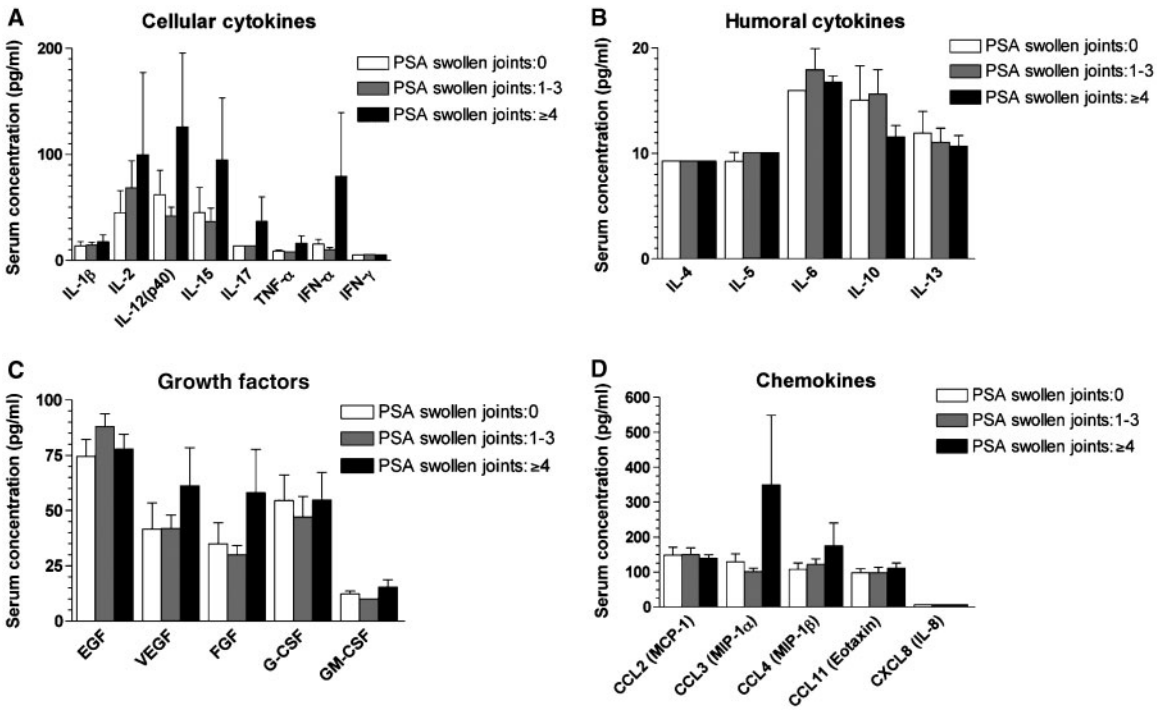

FIG. 2. Comparison of various serum cytokine levels among PsA patients with various affected joint counts. Bars show the mean in pg/ml and SEM. (**A**) Levels of circulating cellular cytokines. (**B**) Levels of humoral cytokines. (**C**) Levels of growth factors. (**D**) Circulating chemokine levels. *Significantly different from compared values by Mann–Whitney U-test ($P < 0.05$).

TABLE 2. Cytokines identified by discriminant function analysis as being significantly discriminatory among PsA patients with various affected joint counts

	Wilk's Lambda	*P*-value
IFN-α	0.8385	0.0121
IL-15	0.7879	0.0263
IL-2	0.7295	0.0690

are at opposite levels (one is high, while one is low) and neutral when there is a lack of a correlation in levels. Coregulated sets of variables will display similar trends in variance that can be identified and used for cluster assignment.

The results of these analyses were represented in graphical outputs, denoted as heatmaps. In a heatmap, correlated cytokines are grouped together. Moreover, heatmaps utilize colour mapping of correlation coefficients to facilitate visual assessment of these values among a large set of variables. Analytes with positive correlations are represented in graded shades of red and negative correlations in graded shades of blue, allowing these groups of correlated variables to be readily identified by visual inspection as relatively monochromatic clusters.

Several gross differences between the heatmaps from healthy individuals and PsA patients were observed (Fig. 3A and B). The most pronounced was a large cluster of highly correlative cytokines identified in patients, which contained IL-2, IL-12(p40), IL-15, TNF-α, MIP-1β, MIP-1α, IFN-α and FGF that was not present in healthy controls. These results reflect a disease-specific change in the regulation of these cytokine levels in the periphery. A subset of these cytokines was identified in the above univariate analysis as being differentially expressed. The high correlation values of these cytokines within individual PsA patients suggests that in addition to being 'up-regulated' as identified, they participate in processes that result from coordination of their levels. Given the collection of cytokines in this cluster, these results underscore the likely functional relevance of Th1/cellular immunity in this disease, an immune response bias shown to be highly relevant in the skin-aspects of PsA [26–28].

In order to further facilitate visual characterization of the difference in cluster patterns between patient and control cluster heatmaps, an analysis, denoted as 'extraction heatmap' method, was developed in which the heatmaps between the control and PsA matrices were subtracted from each other and the differences between the two visually represented (Fig. 3C).

Multiple Monte Carlo simulations were used to define the threshold ρ below which significant correlations are unlikely to appear by chance, thus providing a measure of the statistical differences in the correlational coefficients characterized. Using the results of these simulation studies as a reference, the most significant changes in correlation values between patients and controls were observed for CCL3 (MIP-1α), CCL4 (MIP-1β), FGF, CCL11 (Eotaxin), IFN-α, IL-2, TNF-α, IL-15 and IL-12p40, suggesting that changes in regulation of these cytokines are highly characteristic of PsA (Fig. 3D). These results could be used to develop a multivariate-based diagnostic test for the disease, a test that is likely to be significantly more powerful in terms of disease discrimination than those developed from simple univariate studies of up- and down-regulation of peripheral cytokines.

Identification of serum cytokine levels correlated with disease activity

Multivariate analyses can better distinguish variables that are characteristic of disease subsets when heterogeneous populations are analysed than the univariate analyses utilized above. This is in part because univariate analyses are based on identifying differences in the averages among populations, while hierarchical clustering distinguishes patterns on an individual basis and are thus unaffected by heterogeneity in the overall cytokine levels

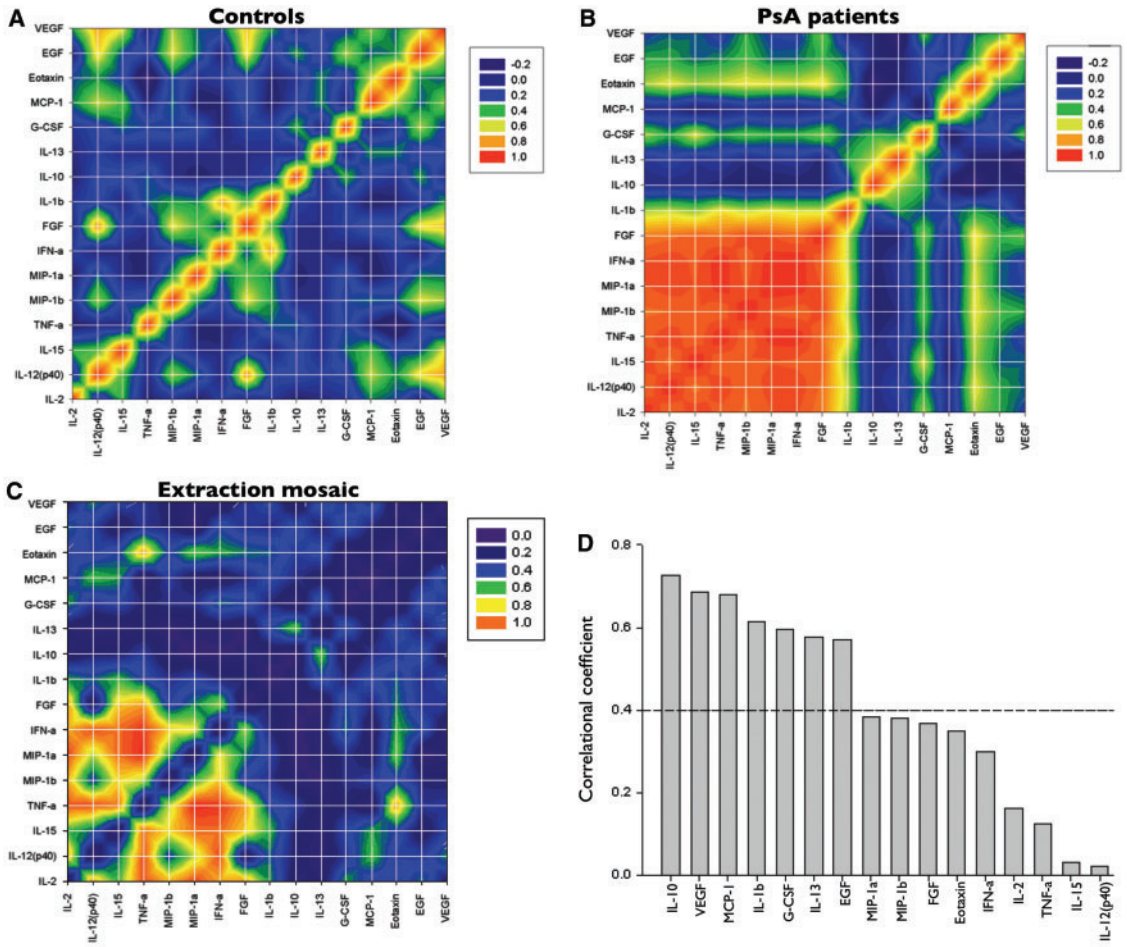

FIG. 3. Hierarchical clustering comparing PsA patients and controls. A heatmap representation of the correlational coefficients are graphed. Cytokines with positive correlations are represented in graded shades of red and negative correlations in graded shades of blue. The same order of the analytes along axis is used for all the three heatmaps. (**A**) Controls, (**B**) PsA patients and (**C**) extraction heatmap differences between the heatmap representations of patients and controls were isolated and depicted in an extraction heatmap. Representation of the level of correlations is listed on the side of the graph. (**D**) Graphical representation of Monte Carlo simulations that were used to define the threshold ρ above which significant correlations are unlikely to appear.

within a given subgroup. Accordingly, although differences in single cytokine values described earlier did not reach statistical significance, differences in the coordinated regulation of serum cytokine levels among the three disease activity subsets described earlier (swollen joint counts: 0, 1–3, ≥4) were readily observed using hierarchical clustering (Fig. 4A–C). These differences in cytokine patterns among disease severity subgroups can serve as a 'cytokine fingerprint' for the development of prognostic tests and also help guide an understanding of the mechanistic differences among these groups. For example, the group 3 heatmap (swollen joint counts: ≥4) was most distinct from more mild disease subgroups in that it exhibited a high positive correlation of several pro-inflammatory cytokines (Fig. 4C *vs* Fig. 4A and B, large clusters, lower left corner). These results demonstrate that these cytokines are present in the periphery as a unit in individuals with severe disease, and may therefore have a cooperative function.

Discriminant function analysis (DFA)

DFA is a multivariate discrimination method that uses observed changes in variables to characterize the most discriminatory variables amongst groups. The diagnostic power of these discriminatory variables is enhanced, as they are used additively to create the class discrimination algorithms, denoted roots, derived from this method. A 'root' is a linear combination of variables with constant coefficients. DFA includes in the roots variables that minimize group overlap in a multidimensional plot of root values (Fig. 5). Root values for individuals within each of the three populations analysed formed non-overlapping clusters, suggesting that the results of the DFA can be used for developing cytokine-based serum diagnostic criteria that could distinguish these groups through a clinical blood test. The cytokines EGF, IFN-α, VEGF, CCL3 (MIP-1α) and IL-12p40 were identified as having the highest discriminatory activity. This is a subset of the cytokines identified by univariate methods distinguishing patients from controls. Also, the all but one of these cytokines were characterized as discriminatory by hierarchical clustering, highlighting the potential significance of these mediators in PsA.

Discussion

Inflammatory infiltrate of lymphocytes in the dermal papillae of the skin and the stromal lining of the joints in PsA results in the collagenase cleavage of cartilage collagen early in the disease and is achieved through the release of various chemokines and cytokines through cytokine-driven production of proteases [2].

The aim of this study was to describe subpopulations of circulating cytokines likely to drive a broad spectrum of

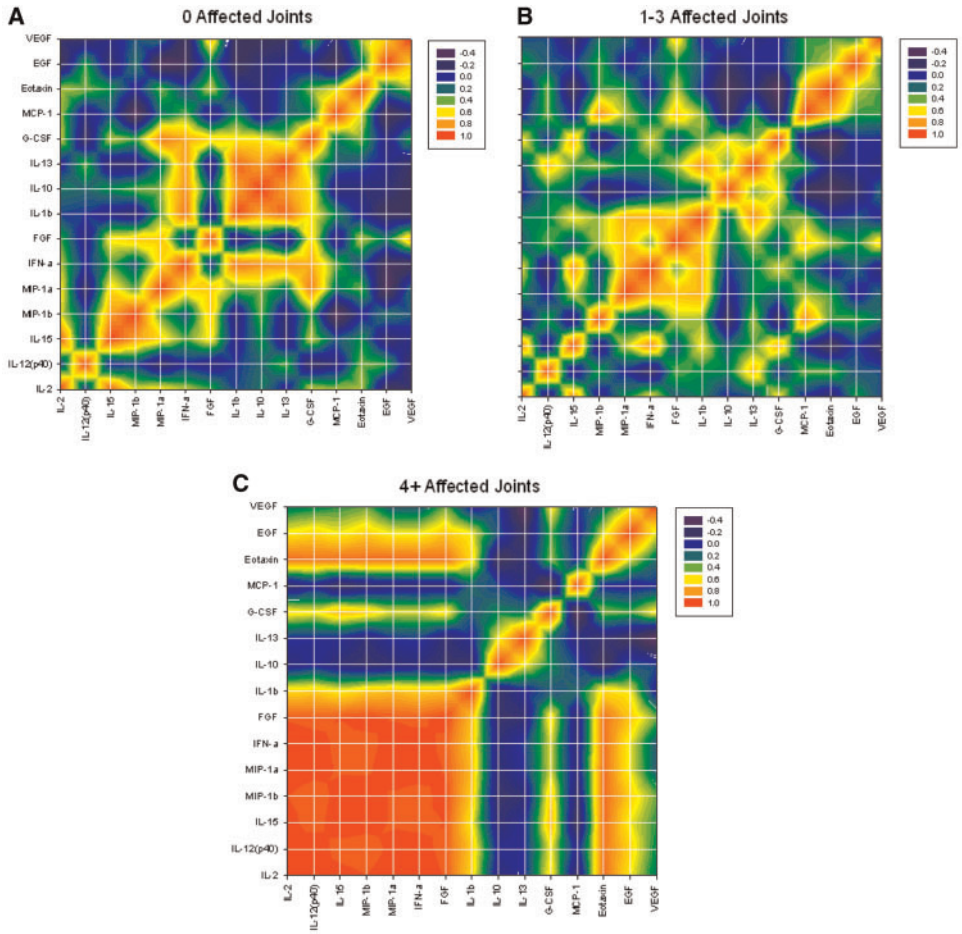

FIG. 4. Hierarchical clustering comparing PsA patients with various affected joint counts. A heatmap representation of the correlational coefficients are graphed. Cytokines with positive correlations are represented in graded shades of red and negative correlations in graded shades of blue. Representations of the level of correlations are listed on the side of the graphs. (**A**) PsA patients with 0 affected joints, (**B**) PsA patients with 1–3 affected joints and (**C**) PsA patients with ≥4 affected joints.

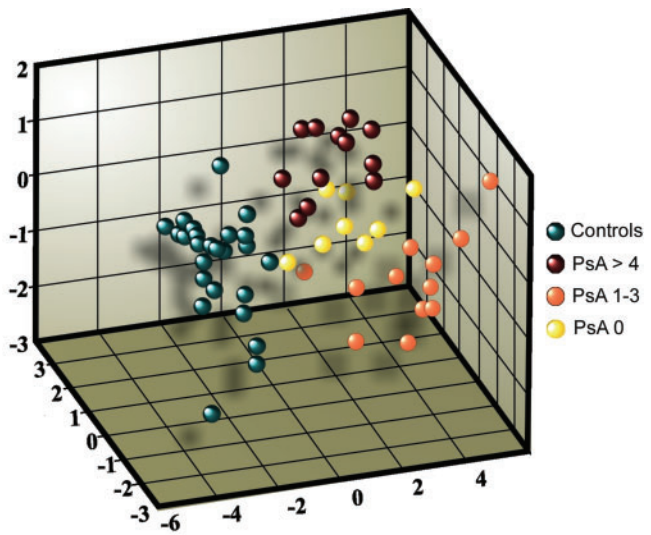

FIG. 5. A graphical representation of the discriminatory potential of discriminant function analysis (DFA). The analysis was used to select variables that maximally discriminate among the cohort, represented by a multidimensional plot of root values.

autoimmune processes in patients with PsA. Besides the description of the differences in the circulating cytokine network between healthy individuals and patients, we further subcategorized the patients based on their affected joint counts as a reflection of the disease severity. We have identified a subset of cytokines that significantly differ between controls and PsA, as well as differentiate between various subsets of patients with PsA. We have characterized different cytokine profiles according to the present disease activity, compared these disease groups and pinpointed special subsets of cytokines that are attributed to the particular disease subset.

The key cytokines that discriminate between PsA and healthy individuals were IL-10, IL-13, IFN-α, EGF, VEGF, FGF, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL11 (Eotaxin) and G-CSF. These findings support the hypothesis that a broad spectrum of impaired immune functions are involved in the pathogenesis of PsA, affecting cellular, humoral immune responses and various leucocyte functions. Although a few of the cytokines related to PsA have been described previously [9–11], the majority of these significant cytokines have not been identified in PsA. With the advanced multiplex cytokine array system, a novel and distinct PsA-related serum cytokine pattern has been revealed that discriminated between patients and healthy individuals.

Since limited system information can be obtained from univariate analysis on the synergistic and/or antagonistic

multifaceted network of cytokines driving the immune/autoimmune processes, a more complex approach utilizing various multivariate statistical analyses was used in this study. Hierarchical clustering was utilized to identify the changes in cytokine networks, as this method clusters cytokines, whose relative values are correlated on individual basis within a population. In this analysis, CCL3 (MIP-1α), CCL4 (MIP-1β), FGF, CCL11 (Eotaxin), IFN-α, IL-2, TNF-α, IL-15 and IL-12p40 were found to distinguish between PsA patients and healthy individuals. Finally, DFA pinpointed EGF, IFN-α, VEGF, CCL3 (MIP-1α) and IL-12p40 to discriminate among controls and subsets of PsA patients. The parallel evaluation of univariate analysis, hierarchical clustering and DFA, besides others, revealed CCL3 (MIP-1α) to be the major distinctive cytokine between PsA patients and healthy individuals. CCL3 (MIP-1α) is involved in the cell activation of human granulocytes and appears to be involved in acute neutrophilic inflammation. Also, it stimulates the production of reactive oxygen species in neutrophils and the release of lysosomal enzymes. Furthermore, CCL3 (MIP-1α) induces the synthesis of other pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α in fibroblasts and macrophages [29–32]. These findings support the idea that this chemokine can play a crucial pro-inflammatory role in the pathogenesis of PsA and strongly associated with arthritis development.

Further investigations, utilizing the multiplex cytokine assay may be used to describe the *in situ* cytokine milieu in PsA synovial fluid (SF)/synovium. Recently, the expression of pro-inflammatory cytokines in the synovial biopsy of PsA patients has been described [33]. PsA synovium was characterized by a predominant expression of IL-10, IL-15, IFN-γ, IL-1β, and TNF-α [33].

In the study by Partsch *et al.*, the concentrations of T cell-derived cytokines in the synovial fluids (SFs) of patients with PsA in comparison with rheumatoid arthritis (RA) and osteoarthritis (OA) was described [34]. They found that the pattern of T cell-derived cytokines in PsA SFs was similar to that of RA SFs. However, both the frequency and the concentrations of cytokines were lower in PsA SFs than in RA SFs. The presence of Th1 and Th2 cell-derived cytokines in PsA SFs suggests the presence of activated T cells in the inflamed joint tissues and their participation in the immuno-inflammatory events [34].

The assessment of pro-inflammatory cytokines in the SF of patients with PsA in comparison with RA and OA showed that the levels of TNF-α, IL-1β, and IL-8 were significantly higher in PsA SF than in OA SF, although lower than in RA SF [35]. The pattern of expression of pro-inflammatory cytokines seen in PsA is similar to that in RA. Since PsA is also a destructive arthropathy, cytokines, in particular TNF-α and IL-1, may be the principle factors in joint destruction [35].

Early immune vascular remorphology and dysregulated angiogenesis have been hypothesized as prominent features linked to the specific up-regulation of growth factors in PsA [2]. Our data is consistent with this hypothesis with angiogenic growth factors like EGF and VEGF being identified as significantly discriminatory between patients and unaffected controls, further reflecting the role of these players in the molecular and cellular mechanisms responsible for the vascular remorphology and the formation of pathological new bone in PsA.

Our results imply that a complex disorder of secreted serum cytokine levels driving various immuno-competent cell types can be found in PsA patients. Also, different patterns of circulating cytokines were detected according to different disease activities of PsA. We assume that the utilization of the multiplex cytokine array system in PsA provides a powerful tool to sub-categorize the disease and, along with common clinical and laboratory parameters, help to evaluate disease activity.

In this study, we focused on static time points to describe the cytokine profile of the patients, with similar disease activity and have compared them with unaffected healthy controls. Serial follow-ups of these patients may result in fluctuations in cytokine levels that sometimes accompanies disease flare or remissions as is evident from profiles identified in other ongoing studies (our unpublished observations). This validates the utility of the multiplex assay as an important application to monitor therapeutic response to various treatment profiles in patients with PsA.

A future important application of this technology will be to evaluate the circulating cytokine pattern and thereby help sub-categorizing patients with PsA and develop this technology to become a powerful diagnostic tool. By the simultaneous monitoring of general laboratory values and serum multiplex cytokine levels prognostic, therapeutic assessments and monitoring can be achieved [e.g. anti-CCL3 (MIP-1α) therapy]. Recently, other biological agents have been studied in PsA. A group of drugs is being developed, which inhibit T cells by blocking the 'second signal' of T-cell activation (e.g. alefacept, efalizumab, abatacept) [36]. In these studies, ~20–50% of the patients with PsA appeared to be responders to these therapies [36]. Opposed to these empirical therapies, for the first time, our results clearly identified a subset of PsA patients with a strong T cell-mediated pathology; therefore by utilizing correlational clustering and DFA excellent candidates for anti-T cell therapy can be selected based on their cytokine profiles.

Long, empirical therapies can be replaced by optimized combination therapies through personalized pro-inflammatory cytokine targeting and planned advanced cost–benefit strategies. The utilization of the multiplex cytokine array system will aid in the diagnosis and therapy design in PsA, and will provide an advanced disease management in the future.

Rheumatology	Key message
	• Utilizing a broad spectrum bead-based immunoassay and multivariate analysis, we have identified unique correlations among soluble immune modulators in patients with PsA. In doing so we have generated distinct profiles of factors that parallels with various affected joint counts, therefore providing new insights into the pathophysiology of PsA.

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