Serum Cytokine Profile of Unaffected First-degree Relatives of Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Various cytokines have been implicated in the pathogenesis of rheumatoid arthritis (RA) and it is known that elevations in multiple cytokines occur prior to disease onset. The objective of our study is to determine whether the cytokine profile in unaffected first-degree relatives (FDR) of patients with RA is distinct from healthy controls.

Methods. The sera of patients with RA, their unaffected FDR, and healthy controls were measured for 27 cytokines using a luminex-based multiplexed immunoassay. Subjects were also tested for rheumatoid factor and 6 different anticitrullinated protein/peptide antibodies (ACPA). Discriminant analysis was performed to define the cytokine profile of the 3 study groups.

Results. Fourteen patients with RA, 37 unaffected FDR, and 27 healthy controls were enrolled. All patients with RA and 49% of FDR were ACPA-positive. Patients with RA had elevated levels of inflammatory cytokines compared to FDR and controls. Lower interleukin 13 (IL-13) levels were independently associated with RA. IL-4 was significantly lower in FDR compared to controls. Using discriminant analysis based on the levels of all cytokines measured, RA, FDR, and healthy controls could be distinguished with 91% accuracy. ACPA-positive subjects had higher levels of IL-9, but lower levels of IL-12p70 and macrophage inflammatory protein-1β. No individual cytokine was associated with ACPA-positivity in FDR, but the entire cytokine profile accurately distinguished ACPA-positive from ACPA-negative FDR.

Conclusion. The cytokine profiles of patients with RA, healthy controls, unaffected ACPA-positive FDR, and ACPA-negative FDR appear to be distinct. (First Release Jan 15 2014; J Rheumatol 2014;41:280–5; doi:10.3899/jrheum.130539)

Key Indexing Terms:
RHEUMATOID ARTHRITIS FIRST-DEGREE RELATIVE CYTOKINES
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Rheumatoid arthritis (RA) is an inflammatory arthritis that is preceded by a period of autoimmunity characterized by elevations in RA-associated antibodies, inflammatory markers, and cytokines[1,2,3,4]. The cause of RA remains unknown; however, several genetic and environmental factors have been identified as risk factors[5]. Because first-degree relatives (FDR) of patients with RA share some of these genetic and environmental factors, studying this population may further elucidate mechanisms of RA pathogenesis.

We and others have shown that unaffected FDR of patients with RA are more likely to express anticitrullinated protein/peptide antibodies (ACPA) than are healthy controls[6,7,8,9]. ACPA are highly specific for RA and predict radiographic joint damage[10]. In mouse models of inflammatory arthritis, ACPA are arthritogenic[11]. However, it is currently unknown whether ACPA predicts RA development in FDR.

Similar to ACPA, retrospective studies revealed that various cytokines were increased in patients with RA prior to disease onset[2,3,4]. Many cytokines are implicated in RA pathogenesis, but the utility of serum levels of cytokines in predicting RA is unclear. In our study we revealed that patients with RA, their unaffected FDR, and healthy controls appear to have unique cytokine profiles.

MATERIALS AND METHODS

Study population. Patients with RA were recruited from St. Joseph's Health Care (London, southwestern Ontario, Canada) and were included in the study if they had ≥ 1 FDR residing in southwestern Ontario, they met the 1987 American College of Rheumatology (ACR) or 2010 ACR/European League Against Rheumatism criteria for RA, and were ACPA-positive. FDR had no previous diagnosis of RA. Healthy controls consisted of employees of the University of Western Ontario and St. Joseph's Health

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Care. All subjects were examined by 1 of 3 experienced rheumatologists (28-joint count). FDR and healthy controls were excluded if they had ≥ 1 swollen joint. All subjects were white. Written consent was obtained from all subjects enrolled and the protocol was approved by the research ethics board of the University of Western Ontario.

Assays for RA-associated antibodies. Details of assays were as described[6]. In brief, serum samples were obtained from all enrolled subjects at the time they were assessed by a rheumatologist and were tested for rheumatoid factor (RF) and 6 different ACPA. RF was measured by nephelometry at the immunology laboratory of London Health Sciences Centre. A positive test was defined as > 21 U/ml. Anticyclic citrullinated peptide 2 (anti-CCP2) antibodies and antibodies to modified citrullinated vimentin (anti-MCV) were tested using an ELISA from Euroimmun AG and OrgenTec, respectively. As per the manufacturers' specifications, positive values for anti-CCP2 were ≥ 5 U/ml and for anti-MCV, ≥ 21 U/ml. JED is a proprietary synthetic citrullinated cyclic peptide. JED was used in ELISA to determine IgG, IgA, and IgM anti-JED antibodies. IgG anticitrullinated fibrinogen (cFib) was also determined by ELISA[12]. Positive values were defined as follows: > 8.76 relative U/ml (RU/ml) for IgG anti-JED, > 2.32 RU/ml for IgG anti-cFib, > 0.051 optical densities (OD) for IgA anti-JED, > 0.11 OD for IgM anti-JED[6]. Subjects positive for any of the above ACPA were classified as expressing ≥ 1 ACPA.

Cytokine quantification. Laboratory assistants were blinded to subject group. Cytokine levels were measured in serum samples using a luminex-based multiplexed immunoassay (Bio-Rad Laboratories) as per product specifications. The following 27 cytokines were measured: interleukin 1α (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), basic fibroblast growth factor, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage (GM)-CSF, platelet-derived growth factor-β, vascular endothelial growth factor (VEGF), IFN-γ inducible protein 10, monocyte chemoattractant proteins 1 (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and eotaxin. Samples were run in duplicate (< 5% variability within assay). Normalization of cytokine data was not performed, but assays were standardized as per product specifications. Testing of RA cases, FDR, and controls was not done on separate plates. RF blocking agents were not used; however, only 3 of 78 subjects had RF levels > 100 U/ml and a previous study reported RF interference with multiplexed immunoassays more commonly with levels > 100 U/ml[13]. Removing the 3 subjects from the analysis did not significantly change results. Also, RF levels were not significantly correlated with cytokine levels for the majority of cytokines when accounting for age, sex, smoking history, and presence of ACPA (data not shown).

Statistical analyses. Statistical analyses were performed using SAS 9.3. Cytokine levels were log-transformed to better approximate a normal distribution. ANOVA was used to compare continuous variables and the chi-squared test was used to compare categorical variables. Bonferroni correction was conducted for comparisons of the levels of each cytokine in patients with RA, FDR, and healthy controls ($p < 0.0167$ was considered significant). Multiple logistic regression using forward stepwise selection was conducted to determine variable associations with the presence of ACPA, RA versus FDR and healthy controls, and FDR versus healthy controls. Variables tested were cytokines, age, sex, smoking history, and RF. For RA and FDR regression analyses, the presence of ≥ 1 ACPA was also tested. Variables were included in the regression model if associated with the outcome of interest with a $p < 0.1$ controlling for other variables. Associations with the outcomes of interest were considered significant if $p < 0.05$. Discriminant analysis using the log-transformed cytokine data generated 2 discriminant functions based on nonoverlapping contributions of the cytokines, which classified the 3 study groups: patients with RA, unaffected FDR, and healthy controls[14]. Statistical analyses described above were determined *a priori*. Analyses excluding RF-positive patients, cross-validation of the discriminant analysis, and stepwise discriminant analysis were performed posthoc. For stepwise discriminant analysis, cytokines were included if the p value associated with the F-test for significance from analyses of covariance was < 0.05.

RESULTS

Characteristics of enrolled study subjects are described in Table 1. There were 14 RA patients with ≥ 1 FDR (referred to as probands), 37 unaffected FDR, and 27 healthy controls. There were 3 sets of twins discordant for RA. There was no significant difference in the sex distribution of the 3 groups. Healthy controls were younger (mean age 40 yrs) than FDR (mean age 56 yrs) and RA subjects (mean age 58 yrs; $p < 0.0001$). However, age was not independently associated with cytokine levels (data not shown). Also, healthy controls reported lower rates of current or previous smoking (7.4%) than patients with RA (69.2%) and FDR (54.3%; $p < 0.0001$). Smokers had significantly higher levels of IL-12p70, IL-13, G-CSF, and eotaxin (data not shown); removal of these cytokines from the discriminant analyses described below minimally affected results (< 6% difference).

The majority of patients with RA were positive for RF (64.3%) and all were positive for at least 1 ACPA. About half the unaffected FDR also expressed ACPA (48.7%). ACPA positivity in healthy controls was significantly lower (20%). RF positivity was rare in FDR (8.1%) and absent in controls. In the total study population, 3 patients with RA had RF levels > 100 U/ml.

Sera from all subjects were analyzed for 27 cytokines using a multiplex immunoassay (Table 2). Patients with RA had significantly higher cytokine levels in 10/27 cytokines (37.0%) and 12/27 (44%) cytokines compared to healthy controls and FDR, respectively ($p < 0.0017$). Levels of eotaxin, IL-10, and IL-8 were higher in FDR compared to healthy controls ($p < 0.0017$), while IL-4, IL-17, and IL-7 levels were lower in FDR compared to controls ($p < 0.0017$).

To determine independent associations of the various cytokines with RA, FDR, and ACPA, multiple logistic regression was performed (Table 3). As expected, a history of smoking was significantly associated with RA (OR 7.08; 95% CI 1.57–31.83; $p = 0.0006$). IL-13 levels were lower in RA compared to FDR and healthy controls (OR 0.17; 95% CI 0.06–0.47; $p = 0.0107$). IL-4 levels were lower in FDR compared to healthy controls (OR 0.015; 95% CI < 0.01–0.55, $p = 0.0224$). IL-9 was associated with ACPA in both RA and FDR subjects: OR 4.56; 95% CI 1.18–17.66. IL-12p70 and MIP-1β were negatively associated with ACPA (OR 0.09; 95% CI 0.02–0.48; $p = 0.0048$ and 0.03; 95% CI < 0.01–0.39; $p = 0.0066$, respectively). IL-6 (OR 8.01; 95% CI 0.93–68.69, $p = 0.0578$) and a history of smoking (OR 5.17; 95% CI 0.93–28.82, $p = 0.061$) was associated with ACPA positivity, but did not reach statistical significance. No variable independently predicted ACPA positivity in FDR.

Table 1. Characteristics of enrolled patients with rheumatoid arthritis (RA), unaffected first-degree relatives (FDR), and healthy controls. Values are n/total N (%) unless otherwise indicated.

	RA	FDR	Healthy Controls	p
Age, yrs, mean (SD)	58 (10.4)	56 (11.8)	40 (15.0)	< 0.0001
Females	10/14 (71.4)	24/37 (64.9)	20/27 (74.1)	0.7189
Ever smoker	9/13 (69.2)	19/35 (54.3)	2/27 (7.4)	< 0.0001
RF	9/14 (64.3)	3/37 (8.1)	0/10 (0)	< 0.0001
≥ 1 ACPA	14/14 (100)	18/37 (48.7)	2/10 (20.0)	< 0.0002

RF: rheumatoid factor; ACPA: anticitrullinated protein/peptide antibodies.

Table 2. Log transformed cytokine levels (range) for patients with rheumatoid arthritis (RA), unaffected first-degree relatives (FDR), and healthy controls.

Cytokine	RA, n = 14	FDR, n = 37	Healthy Controls, n = 27
General activation			
IL-1β	2.41 (0.41–4.7)*	1.36 (0.63–2.72)†	1.59 (0.18–3.59)
IL-1ra	5.36 (4.5–6.09)	5.41 (4.62–6.43)	5.45 (4.37–6.73)
IL-2	3.07 (0.56–6.28)*	1.52 (0.11–3.83)†	1.49 (0.56–3.90)
TNF-α	4.80 (2.08–7.14)*	3.06 (1.32–5.72)†	2.97 (2.08–5.45)
IL-6	3.91 (2.08–6.15)*	2.67 (1.75–4.44)†	2.53 (0.76–3.87)
IL-15	2.78 (0.69–5.48)*	1.56 (0.67–3.43)†	1.26 (–0.31–3.15)
Th1-related			
IL-12p70	4.05 (1.45–6.55)	3.40 (1.89–4.70)	3.27 (2.20–4.45)
IFN-γ	6.10 (4.33–8.34)	5.11 (4.17–6.70)†	5.42 (1.89–6.87)
Th2-related			
IL-4	2.15 (1.38–3.37)	1.83 (1.40–2.52)†	2.09 (0.16–2.92)‡
IL-5	1.44 (–0.26–4.86)	0.79 (–1.02–1.94)	0.81 (–1.66–2.92)
IL-9	5.21 (2.36–8.78)*	3.92 (1.69–8.11)†	3.62 (2.50–4.46)
IL-13	2.15 (0.68–4.15)	1.34 (–0.24–2.87)†	1.41 (–0.46–3.27)
Eotaxin	5.95 (4.87–7.60)*	5.23 (4.39–6.27)†	4.62 (2.25–5.76)‡
Th17-related			
IL-17	3.91 (3.12–4.79)*	4.09 (3.18–5.59)	4.36 (3.33–4.75)‡
Treg-related			
IL-10	2.16 (0.87–4.07)*	1.44 (0.77–2.29)†	0.83 (–1.14–2.92)‡
Bone marrow-derived			
IL-7	3.03 (1.55–4.89)	2.39 (0.49–4.00)†	2.87 (1.75–4.38)‡
GM-CSF	2.50 (–0.22–5.39)	1.36 (–0.22–5.37)	1.01 (–0.22–4.53)
G-CSF	3.74 (2.53–7.04)	3.31 (2.53–4.22)	3.41 (2.58–4.53)
Stromal, angiogenic			
FGF	3.50 (0–5.81)	3.20 (0–4.85)	3.85 (2.41–4.52)
PDGF-β	9.21 (8.40–9.87)	9.48 (8.86–10.44)	9.56 (8.00–10.18)
VEGF	4.58 (3.40–6.15)	4.93 (3.66–6.90)	4.75 (3.59–5.69)
Chemokines			
IL-8	3.05 (2.19–4.92)*	2.79 (1.92–3.75)	2.45 (1.55–4.33)‡
IP-10	7.45 (6.43–8.66)*	7.11 (6.50–8.20)	6.78 (4.58–7.95)
MCP-1	1.92 (0.08–4.53)	2.94 (0–4.60)	2.97 (0.17–4.34)
MIP-1α	1.93 (0.41–4.17)	1.15 (–0.94–3.47)	1.23 (–0.03–3.15)
MIP-1β	4.92 (4.19–5.89)	4.92 (3.81–6.09)	4.74 (3.14–5.72)
RANTES	8.79 (7.86–10.27)	9.48 (8.15–11.84)	9.21 (8.35–11.84)

* $p < 0.0167$ for RA versus healthy controls. † $p < 0.0167$ for RA versus unaffected FDR. ‡ $p < 0.0167$ for unaffected FDR versus healthy controls. IL-1ß: interleukin 1 beta; IL-1Ra: IL-1 receptor antagonist; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ; GM-CSF: granulocyte macrophage-colony stimulating factor; G-CSF: granulocyte colony-stimulating factor; FGF: fibroblast growth factor 2; PDGF-β: platelet-derived growth factor-beta; VEGF: vascular endothelial growth factor; IP-10: IFN-γ-inducible 10-kd protein; MCP-1: monocyte chemotactic protein 1; MIP-1α: macrophage inflammatory protein 1 α; MIP-1β: macrophage inflammatory protein 1 ß; RANTES: regulated on activation, normal T cell expressed and secreted.

Table 3. Multiple logistic regression models* for variables associated with rheumatoid arthritis (RA), unaffected first-degree relatives (FDR), and the presence of anticitrullinated protein/peptide antibodies (ACPA).

Variable**	β Coefficient	OR (95% CI)	p
RA†			
IL-13	–1.77	0.17 (0.06, 0.47)	0.0006
Smoking history	1.96	7.08 (1.57, 31.83)	0.0107
VEGF	0.97	2.64 (0.94, 7.44)	0.0659
FDR‡			
IL-4	–4.23	0.015 (< 0.01, 0.55)	0.0224
ACPA			
IL-12p70	–2.43	0.09 (0.02, 0.48)	0.0048
MIP-1β	–3.42	0.03 (< 0.01, 0.39)	0.0066
IL-9	1.52	4.56 (1.18, 17.66)	0.0279
IL-6	2.08	8.01 (0.93, 68.69)	0.0578
Smoking history	1.64	5.17 (0.93, 28.82)	0.061

* Stepwise selection was performed and variables were included in the models if $p < 0.1$. ** Cytokine variables are continuous (log transformed cytokine levels). † Compared to FDR and healthy controls. ‡ Compared to healthy controls. IL: interleukin; VEGF: vascular endothelial growth factor; MIP: macrophage inflammatory protein.

Canonical discriminant analysis was performed to differentiate patients with RA, their FDR, and healthy controls based on their cytokine values. Using the variances from the values of all the tested cytokines, 2 discriminant functions (Can1 and Can2) were derived. These functions accounted for 87% of the variance of the dataset. The model predicted group membership with an accuracy of 91%. When all RF-positive subjects were excluded from the discriminant analysis, cytokine levels distinguished FDR from healthy controls with an accuracy of 82%. Figure 1 shows a plot of each subject's calculated value for Can1 (X axis) and Can2 (Y axis). Group means for Can1 and Can2 were easily distinguishable: for RA, Can1 = 3.66 and Can2 = –1.24; for FDR, Can1 = 0.68 and Can2 = 0.97; for healthy controls, Can1 = –2.94 and Can2 = –0.62. Similarly, ACPA-positive FDR were distinguished from ACPA-negative FDR based on their cytokine profiles (Figure 2).

Using stepwise discriminant analysis, the following cytokines were found to contribute most to RA, FDR, and healthy control group differentiation: IL-4, IL-10, VEGF, TNF-α, eotaxin, RANTES, and IL-7. These 7 cytokines distinguish the groups with 86% accuracy (81% accuracy for cross-validation analysis; Appendix).

DISCUSSION

We investigated the cytokine profile of a white population of unaffected FDR of patients with RA that had a high proportion of ACPA-positive individuals. Using a multiplex immunoassay and discriminant analysis, we found that

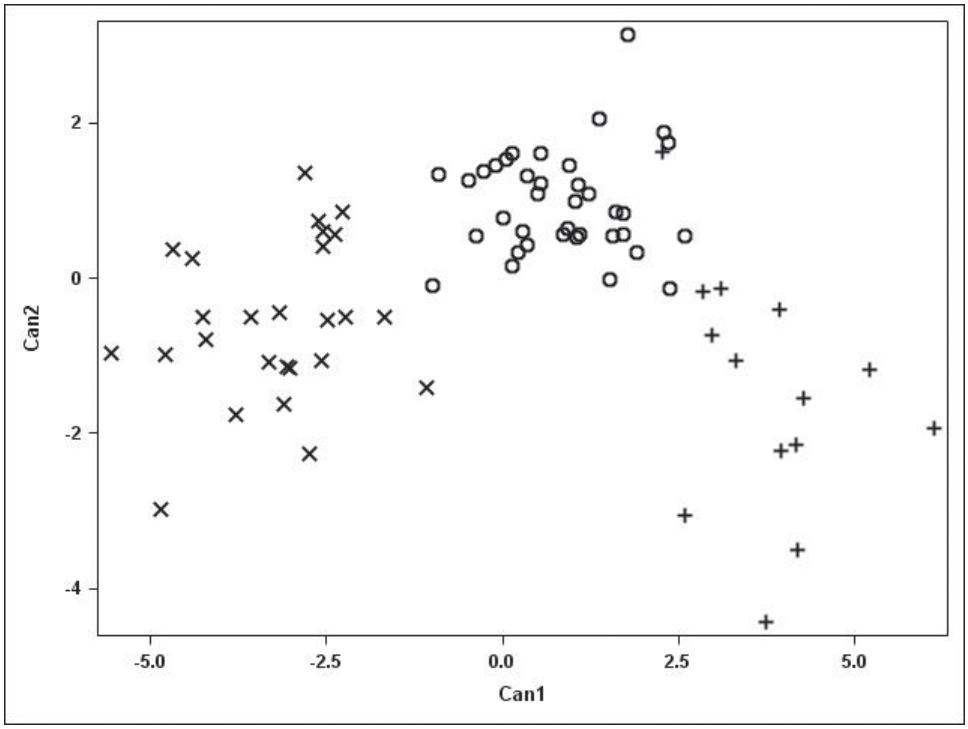

Figure 1. Canonical discriminant analysis for differentiating patients with rheumatoid arthritis (RA), unaffected first-degree relatives (FDR) of patients with RA, and normal subjects based on their cytokine profile. Canonical discriminant functions (Can1 and Can2) were generated using the standardized coefficients derived from the levels of all individual cytokines determined by a multiplex immunoassay. Can1 and Can2 differentiate patients with RA (+), unaffected FDR of patients with RA (o), and normal subjects (x). Group membership is predicted with an accuracy of 91% using this model.

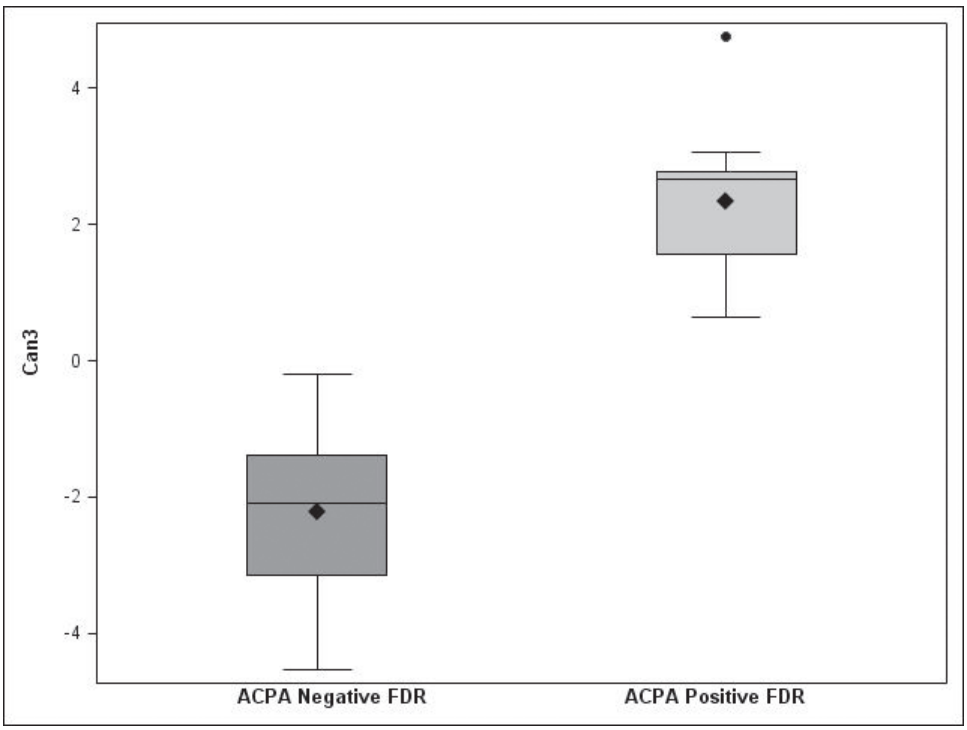

Figure 2. Canonical discriminant analysis for differentiating anticitrullinated protein/peptide antibodies (ACPA)-negative first-degree relatives (FDR) from ACPA-positive FDR based on their cytokine profile. A canonical discriminant function (Can3) was generated using the standardized coefficients derived from the levels of all individual cytokines determined by a multiplex immunoassay. Can3 differentiates ACPA-negative FDR (dark shaded box) and ACPA-positive FDR (light shaded box); mean = diamonds; outliers = circle).

cytokine profiles could distinguish FDR from patients with RA and healthy controls with an accuracy of > 90%. Our findings are consistent with those of El-Gabalawy, *et al*[14], who investigated unaffected FDR from a North American Native population and a white population. We also found that cytokine levels could accurately distinguish ACPA-positive FDR from ACPA-negative FDR.

As expected, patients with RA had elevations of multiple cytokines compared to healthy controls, including pro-inflammatory cytokines that are known to play an important role in RA pathogenesis, such as IL-1β, TNF-α, and IL-6[14]. Although these cytokines have been shown to be consistently elevated in patients with RA from multiple studies, levels of other cytokines have varied significantly depending on the study[2,3,4]. An advantage of using multiplex immunoassays is that levels of different cytokines are measured simultaneously. Consequently, results reflect the level of cytokines relative to each other at the time of sampling. In multivariable analysis, IL-13 levels were significantly lower in RA compared to the other groups. Similarly, when controlling for other variables, IL-4 was significantly lower in FDR compared to healthy controls. Both IL-13 and IL-4 are Th2 cytokines, which downregulate the production of proinflammatory cytokines and attenuated arthritis in a mouse model[15].

Although MCP-1 has previously been shown to be a strong independent predictor of FDR compared to healthy controls[14], we did not find a difference in the levels of this cytokine in FDR compared to controls. We also did not identify any independent predictors for ACPA-positivity in FDR, which was consistent with the findings from El-Gabalawy, *et al*[14]. In contrast, another study that investigated RF or ACPA-positive FDR reported several cytokines that were associated with antibody positivity, including IL-2, IL-6, IL-9, GM-CSF, and IFN-γ[16]. A small proportion of these FDR were ACPA-positive (8%); therefore, this population is different from our study, which may contribute to the discrepancy in the results. In addition, our study was limited by the small sample size.

Cytokine profiling can be a useful tool to determine potential therapeutic targets or decipher disease stage/activity. Yet, caution must be exercised when interpreting cytokine data. Serum levels often do not reflect tissue levels. In addition, RF may interfere with immunoassays[13]. In our population, the cytokine profile accurately distinguished FDR from healthy subjects when all RF-positive subjects were excluded. Fluctuations in cytokine levels are known to occur nonspecifically, such as during infection; thus multiple samplings over time may be more informative of disease activity. The immune response

depends upon the balance of cytokines, rather than the absolute levels of individual cytokines. Therefore, investigating relative cytokine levels as done in this study may better identify autoimmunity by monitoring the ratio of proinflammatory to antiinflammatory cytokine levels.

We have shown that unaffected FDR of patients with RA have a cytokine profile that can be accurately distinguished from healthy controls. Forty-nine percent of FDR were ACPA-positive and these subjects were distinguishable from ACPA-negative FDR by their cytokine profile. Prospective followup of FDR will be important to identify biomarkers that predict RA development.

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APPENDIX. Stepwise discriminant analysis. Cytokines were included if $p < 0.05$.

Cytokine	F Test From Analyses of Covariance	p
IL-4	23.68	< 0.0001
IL-10	15.98	< 0.0001
VEGF	12.23	< 0.0001
TNF-α	6.52	0.0025
Eotaxin	6.42	0.0028
RANTES	4.44	0.0153
IL-7	3.67	0.0307
Accuracy of group discrimination	86%	
Accuracy of group discrimination from cross-validation	81%	

IL: interleukin; VEGF: vascular endothelial growth factor; TNF-α: tumor necrosis factor-α; RANTES: regulated on activation normal T cell expressed and secreted.