Analysis of Complex Biomarkers for Human Immune-Mediated Disorders Based on Cytokine Responsiveness of Peripheral Blood Cells

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The advent of improved biomarkers promises to enhance the clinical care for patients with rheumatoid arthritis (RA) and other immune-mediated disorders. We have developed an innovative approach to broadly assess the cytokine responsiveness of human PBMCs using a multistimulant panel and multiplexed immunoassays. The objective of this study was to demonstrate this concept by determining whether cytokine profiles could discriminate RA patients according to disease stage (early versus late) or severity. A 10-cytokine profile, consisting of IL-12, CCL4, TNF-α, IL-4, and IL-10 release in response to stimulation with anti-CD3/anti-CD28, CXCL8 and IL-6 in response to CMV and EBV lysate, and IL-17A, GM-CSF, and CCL2 in response to human heat shock protein 60, easily discriminated the early RA group from controls. These data were used to create an immune response score, which performed well in distinguishing the early RA patients from controls and also correlated with several markers of disease severity among the patients with late RA. In contrast, the same 10-cytokine profile assessed in serum was far less effective in discriminating the groups. Thus, our approach lays the foundation for the development of immunologic “signatures” that could be useful in predicting disease course and monitoring the outcomes of therapy among patients with immune-mediated diseases. The Journal of Immunology, 2010, 184: 7297–7304.

Among the greatest challenges in managing patients with rheumatoid arthritis (RA) and other immune-mediated disorders is how to determine whether an individual patient has attained an optimal response to therapy (1–3). This is important because patients with suboptimal control of inflammation may suffer chronic pain (4), impaired quality of life (5), disability (6), progressive joint damage (7), extra-articular complications and comorbidity (8), and increased mortality (9). Evaluating the adequacy of treatment response in RA is problematic because the conventional assessments of disease activity suffer from limited sensitivity to detect low levels of articular as well as systemic inflammation (10). Consequently, patients judged clinically to be in “remission” over time can have structural joint deterioration because of subclinical disease activity (11). The limited capacity to detect systemic inflammation may explain in part the increased burden of atherosclerosis and cardiovascular disease suffered by patients with RA and other rheumatic diseases (12, 13). Thus, therapeutic decision-making for individuals with RA might be enhanced by the development of improved biomarkers, which could assist clinicians in assessing disease activity, the outcomes of therapy, and predicting long-term disease outcomes.

In an effort to identify a multivariate immunologic biomarker for use in managing RA, we have developed an approach to broadly assess the functional activity of PBMCs based on the responsiveness of cytokine production ex vivo. A previous study by de Jager et al. (14) discussed the potential use of simultaneously detecting the release of 15 cytokines by PBMCs into culture supernatants after stimulation with Ag or mitogens to monitor cellular immune responses in patients with inflammatory diseases. In this study, we advance a sophisticated and comprehensive analytical approach, which makes several contributions to studying human immunology. First, the methodology involves a unique panel of stimuli designed to elicit diverse responses of the innate and adaptive immune systems, which may optimize the identification of discerning cytokine profiles. As suggested by de Jager et al. (14), we used high-throughput technology to analyze ex vivo cytokine release with multiplexed immunoassays. Second, we developed a statistical methodology to account for variability because of extraneous patient and assay effects and to identify the most informative cytokine profile. Third, we integrated the resulting cytokine data to create an immune response score that may have enhanced discriminative and predictive power.

In this study, our objective was to demonstrate that multiplexed analysis of ex vivo cytokine responsiveness could identify an immunologic signature that discriminates patient groups, according to clinical differences as well as theoretical constructs of disease severity (construct validity). Therefore, we tested the hypothesis that a distinct cytokine response profile can differentiate patients,
both with early and late RA, from controls. We also tested the hypothesis that an immune response score can stratify a relatively diverse group of patients with long-standing RA, according to clinical and laboratory indicators of disease severity.

Materials and Methods

Study design and participants

Patients with RA as defined by the American College of Rheumatology classification criteria (15) were included from two prospective cross-sectional studies at our institution. All participants were recruited in parallel during the period August 2006 to June 2008. First, we included patients with recently diagnosed disease (early RA) from the outpatient clinic of the Division of Rheumatology. Second, we included patients with long-standing RA (‘late’ RA) who had been recruited from the community of Olmsted County, Minnesota, to study the relationships between cytokine response profiles and cardiac disease. Healthy volunteers with no history of inflammatory or autoimmune diseases were recruited by advertisements on campus bulletin boards. The procedures for blood sampling and transport were similar for all subjects. The study was approved by the Mayo Foundation Institutional Review Board and was conducted according to the principles of the Declaration of Helsinki. All patients provided written informed consent prior to participating in this study.

PBMC isolation, cell culture, and stimulation panel

A single experienced laboratory technician (M.A.S.) performed all experiments, which had identical procedures for all subjects. Venous blood samples were harvested and maintained at room temperature. Within 1–2 h, fresh PBMCs were isolated by Ficol density gradient centrifugation. The PBMCs were stimulated in tissue culture under eight separate conditions using a panel of stimuli, mAbs to the CD3 receptor and the costimulatory molecule CD28 (anti-CD3/anti-CD28) (Dynabeads Human T-Activator; Invitrogen, Carlsbad, CA) as well as a plant lectin, PHA (Sigma-Aldrich, St. Louis, MO), were used to cross-link signaling receptors and thereby activate T cells under conditions without requiring Ag presentation. Staphylococcal enterotoxin A and B (Toxin Technology, Sarasota, FL) are bacterial CpG oligonucleotides capable of crosslinking II molecules on APCs to TCRs, activating naive and memory T cells independently of Ag (16). Combined CMV and EBV lyses (CMV/EBVs) (Advanced Biotechnologies, Columbia, MD) were selected to induce T cell responses in an Ag-dependent manner with strong cell-mediated and humoral responses (17, 18).

Three molecules containing pathogen-associated molecular patterns were selected to induce innate cytokine responses via Toll-like receptor signaling pathways. CMV/EBVs contain ligands for TLR2, TLR3, and TLR9 (19, 20), and bacterial CpG oligonucleotides are ligands for TLR9 (21); these molecules activate cytokine production in B cells and innate immune effectors (i.e., monocytes and dendritic cells). Human heat shock protein 60 (HSP60) (StressGen Biotechnologies, Victoria, British Columbia, Canada) is an endogenous ligand for TLR2 and TLR4 that is released by damaged tissues and capable of crosslinking II molecules on APCs to TCRs, activating naive and memory T cells independently of Ag (16). Three molecules containing pathogen-associated molecular patterns were selected to induce innate cytokine responses via Toll-like receptor signaling pathways. CMV/EBVs contain ligands for TLR2, TLR3, and TLR9 (19, 20), and bacterial CpG oligonucleotides are ligands for TLR9 (21); these molecules activate cytokine production in B cells and innate immune effectors (i.e., monocytes and dendritic cells). Human heat shock protein 60 (HSP60) (StressGen Biotechnologies, Victoria, British Columbia, Canada) is an endogenous ligand for TLR2 and TLR4 that is released by damaged tissues and capable of crosslinking II molecules on APCs to TCRs, activating naive and memory T cells independently of Ag (16). Combined CMV and EBV lyses (CMV/EBVs) (Advanced Biotechnologies, Columbia, MD) were selected to induce T cell responses in an Ag-dependent manner with strong cell-mediated and humoral responses (17, 18).

For each stimulation condition, $4 \times 10^5$ PBMCs were cultured in 200 μl medium (RPMI 1640 medium plus 10% FBS plus 1% penicillin/streptomycin/glutamine) containing the stimulant (or media alone) in quadruplicate wells of a microtiter plate. The final concentrations of each stimulant in cell culture were based on our established protocols and published work as follows: anti-CD3/anti-CD28, 0.5 $\times 10^{-8}$ beads/cell; Hib (1 1 ratio of beads to PBMCs per manufacturer’s instructions); 5 μg/ml PHA (27); 10 ng/ml staphylococcal enterotoxin A with 10 ng/ml staphylococcal enterotoxin B (28); 1 μg/ml CMV with 1 μg/ml EBV (29); 10 μg/ml CpG (30); 1 μg/ml HSP60 (24); and 1 μg/ml PMA with 700 ng/ml ionomycin (27). The PBMCs were incubated at 37°C in 5% CO2 for 24 h; the super- natants were subsequently harvested, transferred to a storage plate, and frozen at −80°C for later analysis.

Differential leukocyte counts

The results of differential leukocyte counts were collected from electronic laboratory records when available. These results were included if the blood counts were drawn within 14 d of the blood draws for the cytokine profiles. Data were available for a sample of the RA patients but none of the controls.

Multiplexed cytokine immunoassays

A panel of 17 cytokines and chemokines was analyzed using a multiplexed approach with commercially available human 17-plex kits. The following cytokines were assessed: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-10, IL-12, IL-13, IL-17A, IFN-γ, TNF-α, CCL2 (MCP-1), CCL4 (MIP-1β), G-CSF, and GM-CSF. For serum samples, we used the Bio-Plex 17-plex kit (Bio-Rad, Hercules, CA) and determined the cytokine concentrations using the Bio-Plex 200 reader (Bio-Rad). For the PBMC culture supernatants, we used a customized platform obtained from Meso Scale Discovery (Gaithersburg, MD) and determined the cytokine concentrations using the Sector 2400 instrument (Meso Scale Discovery). This technology was chosen because it performed better than the Bio-Plex at values near the upper limit of detection (data not shown).

Cytokine concentrations were determined based on a standard curve generated on each plate using the manufacturer-supplied reagents. For each of the cytokines, the interassay coefficient of variation was 9–15%, except for IL-7, which had a coefficient of variation of 22%.

Statistical analysis

The distributions of the patient characteristics were analyzed descriptively using the mean ± SD for continuous variables or numbers and percentages for categorical variables. To test for differences between the groups, we used the t test for continuous variables or the χ2 test for categorical variables. Differences in the peripheral blood leukocyte counts between the patients with early and late disease were tested for statistical significance using the Wilcoxon rank-sum test.

The analytic methods were required to explicitly account for the multiplicity and interrelatedness of the cytokine data, along with the blocking induced by multiple patients and assays per reaction plate. All cytokine concentrations were log transformed. Mixed effects models were used to normalize the data and to estimate and test for differences between the groups. The analyses resulted in fold differences (95% confidence intervals) in the geometric mean cytokine concentrations between the groups; p values for differences between the early RA, late RA, and controls subjects were determined using two degrees of freedom tests. The models included fixed effects for age, sex, and stimulation and random effects for subject and plate; the values as presented are therefore adjusted for age, sex, and assay effects. To identify biomarker profiles that reliably discriminated patient groups while controlling for the likelihood of spurious findings, we selected cytokines into the profile based on the magnitude and significance of differences among the groups and, more importantly, on potential immunologic mechanisms of cytokine production.

A multivariate technique was developed to construct an immune response score with characteristics suitable for class prediction. Standard stepwise regression methods, which remove “nonsignificant” variables from the model, were not used because deletion of nonsignificant variables from the final score may increase prediction error and decrease generalizability (31). Although the individual coefficients may be more variable when collinear cytokines are included, the overall score is more stable. To create the immune response score, the selected cytokines were converted to Z scores (each cytokine value subtracted by the mean of the control group and divided by the SD of the control group), and the Z-scores were added (or subtracted depending on the direction of differences between the control and early RA groups). For ease of interpretation, the score was rescaled so the minimum score was 0, and the optimal cutoff value for discriminating the groups was 50. The score then dichotomized, and differences between the resulting patient groups were tested as appropriate.

Results

Patient characteristics

We recruited 25 patients with early RA, 60 with late RA, and 15 healthy volunteers as controls (Table I). The patients with early RA, who were nearly a decade younger on average than the patients with late RA, were newly diagnosed (mean disease duration, 0.2 y) and beginning initial disease-modifying therapy. The early patients had highly active disease as demonstrated by high values for C-reactive protein (CRP), the Health Assessment Questionnaire (HAQ) disability index, and pain scores. In contrast, the patients with late RA were more diverse but, on average, had lower disease activity, pain, and disability and had normal acute phase reactants. The patients with early RA had a higher frequency of anti-citrullinated protein Abs (ACPsAs) and a similar frequency of erosive disease (as
determined by radiologist reports of available x-rays) as compared with the patients with late disease.

**Selection of the cytokine response profile**

We tested for differences in 136 values (8 stimulation conditions × 17 cytokines) between the groups. The analyses showed statistically significant differences for 58 of 136 (43%) of the stimulated cytokine values among the three groups at a significance level of 0.05 (data not shown). The large number of statistically significant differences indicated that these profiles easily distinguished the RA groups, both early and late, from controls.

The results of the analyses revealed clear, recurring profiles of immune response most characteristic of either T cell responses or myeloid lineage responses (Table II). The cytokine profiles showed consistency in the direction of effect for cytokines within canonical classes. For example, the profiles of IFN-γ, IL-4, IL-10, and TNF-α release by PBMCs in response to stimulation with anti-CD3/anti-CD28 were all significantly reduced in the early RA group as compared with controls (with the exception of IFN-γ, which was nonsignificant at p = 0.2 and hence not included in the final profile). Considerable redundancy was present in the profiles of several cytokines among the different stimuli; for example, the responses of PBMCs to anti-CD3/anti-CD28 and staphylococcal enterotoxins A and B, or CpG and HSP60, were often similar.

Our strategy was to incorporate several cytokines in the context of a particular stimulation to describe the immunologic responses of PBMCs to anti-CD3/anti-CD28 and staphylococcal enterotoxins A and B, or CpG and HSP60, were often similar.

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Our strategy was to incorporate several cytokines in the context of a particular stimulation to describe the immunologic responsiveness of a theoretical immune compartment. Then, cytokine profiles for particular stimulants were assimilated to create the cytokine response profile. Ultimately, we selected IL-12, CCL4, TNF-α, IL-4, and IL-10 with anti-CD3/anti-CD28 to assess T cell-mediated responses, IL-6 and CXCL8 with CMV/EBV to assess adaptive and innate immune responses, and IL-17A, GM-CSF, and CCL2 with HSP60 to assess IL-17A–producing cell and innate immune responses (Table II).

**Cytokine response profiles in early or late RA as compared with controls**

Next, we investigated the fold differences in the 10-cytokine profiles for patients with early RA as compared with controls (Fig. 1A). In response to stimulation with anti-CD3/anti-CD28, the production of IL-12, CCL4, TNF-α, IL-4, and IL-10 were significantly reduced in the early RA group as compared with controls, each by >50%. In response to CMV/EBV, the production of CXCL8 was significantly decreased, whereas the production of IL-6 was significantly increased as compared with controls. In response to stimulation with HSP60, a different pattern was evident, with significantly elevated production of IL-17A and GM-CSF and marginally elevated CCL2 as compared with controls.

The 10-cytokine profiles were significantly different for the patients with late RA (Fig. 1B) compared with controls. In response to stimulation with anti-CD3/anti-CD28, the release of IL-12 and IL-10 were significantly decreased in the group with late RA, but the release of CCL4, IL-4, and TNF-α were not significantly different from controls. As compared with the early RA group

### Table I. Characteristics of the patients with early or late RA and controls

| Variable                  | Controls (n = 15) | Early RA (n = 25) | Late RA (n = 60) | p Value |
|---------------------------|------------------|------------------|-----------------|---------|
| Age (y)                   | 44.6 ± 16.8      | 50.9 ± 14.1      | 60.3 ± 9.1      | <0.0001 |
| Sex, no. (%)              |                  |                  |                 | 0.15    |
| Female                    | 8 (53%)          | 18 (72%)         | 47 (78%)        |         |
| Male                      | 7 (47%)          | 7 (28%)          | 13 (22%)        |         |
| Disease duration (y)      |                  | 0.2 ± 0.3        | 13.6 ± 5.6      | <0.0001 |
| CRP (mg/l)                |                  | 40.3 ± 44.6      | 4.1 ± 4.7       | <0.0001 |
| HAQ disability index (0–3)|                  | 1.4 ± 0.7        | 0.5 ± 0.5       | <0.0001 |
| VAS pain (0–100 mm)       |                  | 52.4 ± 29.1      | 30.9 ± 22.9     | 0.006   |
| ACPA positive, no. (%)    |                  | 18 (72%)         | 26 (43%)        | 0.016   |
| RF positive, no. (%)      |                  | 17 (68%)         | 33 (55%)        | 0.27    |
| Erosions, no. (%)         |                  | 6 (27%)          | 25 (44%)        | 0.18    |
| MTX use, no. (%)          |                  | 9 (36%)          | 29 (48%)        | 0.30    |
| Prednisone use, no. (%)   |                  | 9 (36%)          | 19 (32%)        | 0.70    |
| Prednisone dose, mg       |                  | 9.2 ± 5.7        | 5.8 ± 5.6       | 0.12    |
| Other DMARDs, no. (%)     |                  | 5 (20%)          | 23 (38%)        | 0.10    |
| Anti-TNF use, no. (%)     |                  | 0 (0%)           | 14 (23%)        | 0.008   |

Values are mean ± SD, except as indicated otherwise. DMARD, disease-modifying antirheumatic drug; VAS, visual analog scale.

### Table II. Distributions of cytokine concentrations for the selected ex vivo cytokine response profile among patients with early or late RA as compared with controls

| Cytokine | Stimulation | Controls (n = 15) | Early RA (n = 25) | Late RA (n = 60) |
|----------|-------------|------------------|------------------|------------------|
| IL-12    | Anti-CD3/anti-CD28 | 22.7 (19.0, 34.5) | 13.7 (8.5, 22.1) | 17.2 (11.7, 25.8) |
| CCL4     | Anti-CD3/anti-CD28 | 112,709 (35167, 305774) | 36,696 (19,877, 67,245) | 60,664 (45,030, 99,887) |
| TNF-A    | Anti-CD3/anti-CD28 | 6,689 (5,020, 12,959) | 2,795 (1,045, 7,219) | 4,114 (2,637, 8,029) |
| IL-4     | Anti-CD3/anti-CD28 | 63.1 (37,9, 108.6) | 31.1 (9.8, 83.4) | 52.6 (25.2, 114.3) |
| IL-10    | Anti-CD3/anti-CD28 | 4,114 (1,570, 9,576) | 1,460 (453, 4,966) | 2,069 (1,007, 5,602) |
| CXCL8    | CMV/EBV      | 994 (438, 1,909) | 414 (250, 557) | 403 (293,1, 539.6) |
| IL-6     | CMV/EBV      | 35,288 (21,005, 57,747) | 63,664 (29,634, 132,442) | 70,161 (46,238, 108,898) |
| IL-17A   | HSP60        | 52 (0.3, 119.4) | 13.4 (0.7, 82.7) | 35.7 (26.8, 122.4) |
| GM-CSF   | HSP60        | 14.0 (4.6, 196.8) | 25.7 (34.1, 184.1) | 82.4 (44.6, 201.7) |
| CCL2     | HSP60        | 6,639 (2,211, 22,423) | 9,475 (3,257, 31,247) | 12,034 (7,498, 28,071) |

Values are the geometric means (25th percentile, 75th percentile) for the concentrations (picograms per milliliter) of individual cytokines in the supernatants of stimulated PBMC cultures according to stimulant and group. These results were obtained by taking the means of log-transformed cytokine concentrations using normalized data from mixed effects models and then converting back to the raw scale.
stimulants (Fig. 2). The profiles of the early RA group (Fig. 2A) and the late RA group (Fig. 2B) were nearly identical. In the group with early RA, the basal production of GM-CSF and CCL2 was significantly increased as compared with controls (Fig. 2A). In the group with late RA, the only differences from early RA were the significantly increased responses of CCL4 and IL-17A as compared with controls (Fig. 2B). Thus, because most of the cytokine responses in both patient groups were not significantly different from controls, the unstimulated profiles were less useful in differentiating the patient groups.

**Comparison with cytokine profiles in the serum of patients with early or late RA as compared with controls**

Because serum multicytokine profiles have been shown to discriminate patients with RA and controls by other investigators, we compared the profiles of the same 10 cytokines that we previously assessed in PBMC culture supernatants with the profiles in serum samples (Fig. 3). Patients with early RA had significantly elevated serum levels of IL-12, TNF-α, IL-10, IL-6, and IL-17A, between 2- and 5-fold in magnitude, as compared with controls (Fig. 3A). In contrast, patients with late RA, who had lower average disease

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**Figures**

**FIGURE 1.** Multiplexed analysis of ex vivo cytokine production by stimulated PBMCs discriminated patients with both early and late RA from controls. We measured the profiles of 10 cytokines released into the culture supernatants by patient PBMCs in response to stimulation under three separate conditions using multiplexed immunoassays. The values represent the fold differences in the geometric means (●) and associated 95% confidence intervals (vertical lines) among the 25 patients with early RA (A) or 60 patients with late RA (B) as compared with 15 controls (horizontal dotted reference lines). For these plots, we included cytokines that showed statistically significant differences at α = 0.05 between RA patients and controls; the observed differences were tested for statistical significance using mixed effects models, adjusting for patient age, sex, and cytokine plate. See text for further description.

**FIGURE 2.** Multiplexed analysis of ex vivo cytokine production by unstimulated PBMCs was less effective in discriminating patients with early or late RA as compared with controls. In this study, we assessed the profiles of 10 cytokines released by patient PBMCs cultured in media alone without the use of additional stimulants. The values represent the fold differences in the geometric means (●) and associated 95% confidence intervals (vertical lines) among the 25 patients with early RA (A) or 60 patients with late RA (B) as compared with 15 controls (horizontal dotted reference lines). Differences in the values were tested for statistical significance at the level α = 0.05 using mixed effects models, adjusting for patient age, sex, and cytokine plate. See text for further description.

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(Fig. 1A), the average impairments in immune responsiveness to anti-CD3/anti-CD28 stimulation appeared to be ameliorated in the late RA group. In response to stimulation with CMV/EBV, the production of CXCL8 was significantly decreased, yet the production of IL-6 was significantly increased as compared with controls (Fig. 1B). These responses were almost identical to those of the early RA group (Fig. 1A). In response to stimulation with HSP60, the release of IL-17A and GM-CSF were significantly increased to 12.9- and 9.6-fold higher, respectively, in the patients with late RA as compared with controls, and the release of CCL2 was also significantly elevated (Fig. 1B). As compared with the group with early RA (Fig. 1A), these HSP60 responses appeared to be higher in the group with late RA.

"Unstimulated" cytokine profiles of PBMCs in patients with early or late RA as compared with controls

To assess the value of the multistimulant panel, we next evaluated the cytokine profiles of PBMC in media alone without any additional
activity, had no significant differences compared with controls (Fig. 3B). These data suggested that serum cytokine profiles are substantially less sensitive to low disease activity states than ex vivo profiles.

Comparison of peripheral leukocyte differentials among the patients with early or late RA

Next, we compared the peripheral blood leukocyte differential counts between the patients with early and late disease to evaluate the possibility that changes in the distribution of peripheral immune cell types might explain the differences in the cytokine response profiles between the groups (Fig. 4). We observed a small but statistically significant increase in total leukocytes in the patients with early RA as compared with controls; however, there were no significant differences between the groups with late RA as compared with controls. Statistical significance ($\alpha = 0.05$) of the fold changes was tested using mixed effects models with adjustment for patient age, sex, and cytokine plate. See text for further description.

Development of an immune response score

Finally, we created an immune response score, which integrated the variation for each individual cytokine of our selected cytokine response profile into a continuous index of immune response (Fig. 5). The cutoff of $\geq 50$ accurately classified 17 of 19 early RA patients and 12 of 13 controls, indicating that the immune response score performed very well in discriminating the early RA group from controls. Among the 60 patients with late RA group, 35 had an immune response score of $\geq 50$ and 25 had a score of $< 50$, showing...
greater heterogeneity among the late RA group, with some indi-
guals having immune profiles more similar to early RA patients
and others having profiles more similar to controls.

We assessed the construct validity of the immune response score
by testing whether subgroups of the patients with late RA defined by
ichotomous levels of the immune response score differed in several
clinical indicators of disease severity (Table III). The groups had
imilar distributions for age, sex, and disease duration. However,
the group with higher immune response scores had higher levels of
disability as defined using the HAQ (p = 0.05) and higher pro-
portions of rheumatoid factor (RF) (p = 0.048), erosive disease
(p = 0.025), and methotrexate (MTX) use (p < 0.001). The group
with higher immune response scores tended to have higher CRP
(p = 0.09) and to be taking TNF blockers (p = 0.08). Notably, there
was no association between prednisone use and the score.

In a sensitivity analysis, we assembled another ex vivo cytokine
profile that evaluated only those 10 cytokines with the most sta-
istically significant differences (all p < 0.01) among the patient
groups and controls, without considering potential mechanisms.
This profile included release of IL-12, CCL4, TNF-α, IL-4, and
IL-10 in response to anti-CD3/anti-CD28; CXCL8 release in re-
response to CMV/EBV; GM-CSF production in media alone; G-
CSF release in response to HSP60; and IL-7 release in response
to PMA with ionomycin. The immune response score based on this
profile performed as well as the former score in discriminating
the early RA group from controls but correlated poorly with mark-
ers of disease severity among the late RA group (data not shown).

Discussion

RA is a systemic inflammatory autoimmune disease orchestrated by
diverse cellular mediators of the innate and adaptive immune sys-
tems, producing a myriad of cytokines and chemokines to initiate and
amplify inflammation in diarthrodial joints, leading ultimately
to destruction of articular bone and cartilage (32, 33). In this study,
we have developed an innovative strategy of biomarker discovery
for immune-mediated disorders based on the premise that the re-
sponsiveness of the various canonical subsets of the immune system
is an important determinant of disease severity and therapeutic
outcomes. We have demonstrated that multiplexed analysis of ex
vivo cytokine production by stimulated PBMCs with the develop-
ment of a multicytokine immune response score effectively dis-
criminated the patients with RA, particularly the early RA group,
from controls. Among the more diverse group with late RA—with
wider variation in disease activity and severity—we demonstrated
evidence for construct validity by showing significant correlations
of the immune response score with clinical indicators of disease
severity. The findings underscore the potential clinical use of our
approach in identifying immunologic “signatures” for predicting
disease course and monitoring the outcomes of therapy in patients
with immune-mediated inflammatory diseases.

Several points illuminate the potential significance of this ap-
proach. Circulating PBMCs experience the unique microenviron-
ments in many body tissues of an individual. The particular milieu
present likely influences the responsiveness of cytokine production
by interacting with genetic variation and affecting epigenetic control
of immunoregulatory pathways. In RA, environmental factors, such
as tobacco smoking, are known to interact with a patient’s genotype
to influence disease pathogenesis and severity (34). In view of this,
it is noteworthy that the level of ex vivo cytokine production by
peripheral blood immune cells can be affected by multiple envi-
ronmental factors, such as infectious exposures and place of resi-
dence (35). Thus, ex vivo cytokine profiling may capture informa-
tion on relevant but unknown environmental factors, which
could impact disease phenotype. Our approach also assesses the
systemic immune reactions to local, articular inflammation. The
phenotype of the systemic immune compartment can be polarized
as compared with that of the primary disease site, for example,
with low Th1 cell activity in the peripheral blood of RA patients
as compared with high Th1 activity in their affected joints (36). The
systemic immune phenotype may therefore be closely associated, if
divergently, with the underlying immunopathology. The developed
approach involves mixed populations of cells and, while precluding
elucidation of the cellular mechanisms involved, allows cell–cell
interactions as well as autocrine and paracrine signaling to influ-
ence the cytokine responses, potentially creating biomarker assays
with greater discriminative power. The use of a panel to induce
“dynamic” cellular responses may offer higher sensitivity to detect
abnormal immune function as compared with the use of only a few
stimuli because differences in a particular cytokine can often be
detected using one stimulant but not another (37). This method
may be useful not only to assess current disease status, including
systemic disease activity, but also to assess the potential for future
disease progression. Finally, multivariate analyses can be used to
construct a prediction model, such as the immune response score
reported in this paper, to classify the likelihood of particular out-
comes. Such a classifier could provide higher predictive accuracy
as compared with any single cytokine response as has been sug-
gested in RA and other immune-mediated diseases (38–41).

Although previous studies of RA patients have reported ab-
normalities for cytokines that we have evaluated individually, ours

| Variable                      | Score < 50 (n = 25) | Score ≥ 50 (n = 35) | p Value |
|-------------------------------|--------------------|--------------------|---------|
| Age                           | 58.3 ± 9.5         | 61.7 ± 8.8         | 0.16    |
| Sex, no. (%) female/male      | 223 (88%) (12%)    | 2510 (71%) (29%)   | 0.12    |
| Disease duration, y           | 13.3 ± 6.5         | 13.8 ± 4.9         | 0.76    |
| CRP, mg/l                     | 2.9 ± 3.6          | 5.0 ± 5.3          | 0.09    |
| Pain VAS (0–100 mm)           | 26.8 ± 22.3        | 33.8 ± 23.2        | 0.26    |
| HAQ disability index > 1      | 2 (8%)             | 10 (29%)           | 0.05    |
| ACPA positive, no. (%)        | 9 (36%)            | 17 (49%)           | 0.33    |
| RF positive, no. (%)          | 10 (40%)           | 23 (66%)           | 0.048   |
| Erosive disease, no. (%)      | 6 (25%)            | 19 (54%)           | 0.025   |
| MTX use, no. (%)              | 5 (20%)            | 24 (69%)           | <0.001  |
| Prednisone use, no. (%)       | 6 (24%)            | 13 (37%)           | 0.28    |
| Other DMARD use, no. (%)      | 8 (32%)            | 15 (43%)           | 0.39    |
| Anti-TNF use, no. (%)         | 3 (12%)            | 11 (31%)           | 0.08    |

Values are mean ± SD except as indicated otherwise.
DMARD, disease-modifying antirheumatic drug; VAS, visual analog scale.
is among the first to assimilate these immune abnormalities into a multivariable biomarker. We noted decreased production of IL-12, CCL4, IL-4, IL-10, and TNF-α following stimulation of the PBMCs with anti-CD3/anti-CD28, suggesting changes in the peripheral T cell compartment. Previous studies have reported abnormalities of CD4+ T cell function in patients with early RA, including defective Th1 (i.e., IFN-γ) and Th2 (i.e., IL-4) immune responses (42–45). Furthermore, we observed among patients with RA significantly increased release of IL-6 with CMV/EBV and of GM-CSF with HSP60, suggesting changes in the myeloid compartment, such as increased activity of a proinflammatory subset of monocytes or dendritic cells. Our results extend the previous observation in RA that ex vivo responsiveness to stimulation with TLR ligands (including HSP60) is increased, as indicated by proliferative capacity (46), IL-6 release (47), or TNF-α release (48, 49). Also consistent with our data, the expression of IL-17A mRNA is reported to be increased in PBMCs of patients with RA as compared with osteoarthritis (50). Prior work supports the use of ex vivo immune biomarkers to monitor treatment responses with the evidence that disease-modifying therapy may lead to recovery of peripheral T cell and innate immune responses during follow-up (37, 48, 51, 52). Finally, previous studies have shown that pretreatment cytokine responsiveness of stimulated PBMCs may be useful in predicting outcomes of antirheumatic therapy (48, 52).

Our approach appears to have several advantages over existing biomarker approaches for rheumatic diseases. As we have shown, serum biomarker profiles are prone to high variability, likely related to the complex matrix of the serum samples, including the potential for nonspecific binding by heterophilic Abs (53). In addition, many cytokines have diurnal variation, so the time of blood sampling can significantly impact the cytokine levels in serum. The degree of “leakage” of particular cytokines from the joints into the bloodstream may determine how useful they are as biomarkers (3). These factors should impact ex vivo PBMC-based assays much less. Not surprisingly, the serum profiles we observed differed significantly from the ex vivo profiles. For example, severe RA is correlated with higher serum levels of TNF-α, IFN-γ, IL-4, and IL-10 (54, 55), whereas the production of these cytokines by stimulated PBMCs was significantly reduced in our study. We showed that the serum cytokine profiles were less discriminative than the ex vivo cytokine profiles, suggesting that PBMC-based assays are more useful as biomarkers. Additionally, the differences in routine blood leukocyte counts could not explain the differences in cytokine production observed among the groups. In future studies, we will perform immunophenotyping to determine whether differences in the distributions of lymphoid or myeloid subtypes can explain our findings. An important insight from our data is that ex vivo cytokine profiles offer unique and clinically relevant information that is not available from routine laboratory techniques.

In conclusion, we have developed an innovative approach to identify complex biomarkers for human immune-mediated disorders by analyzing the responsiveness of cytokine production by peripheral blood cells. We have demonstrated this concept with a 10-cytokine immune response score that may identify patients with more severe or less treatment responsive RA. However, this score is currently not useful as a biomarker, and additional steps are necessary to refine the approach and to establish the clinical applicability of the findings. By design, this study does not have systematically collected data for joint counts, global assessments, or composite disease activity scores, so correlative analyses of the cytokine assays with validated measures of disease activity are required. Prospective studies should determine whether the developed approach adds value beyond current clinical or serological assessments in predicting treatment outcomes for patients with RA or other inflammatory diseases. Although the assays appear to have adequate reproducibility overall, further studies should test alternative methods of blood cell isolation and stimulation to facilitate the development of practical assays with the level of standardization necessary for clinical translation. Future clinical trials of RA and other immune-mediated disorders should endeavor to collect and store PBMCs in a manner conducive to the development of similar strategies of immune monitoring.

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Disclosures

J.M.D., K.L.K., and S.E.G. have filed a provisional patent application pertaining to the technology described in this study.

References

1. Cohen, S. B., M. D. Cohen, J. J. Cush, R. M. Fleischmann, P. J. Mease, M. A. Schill, L. S. Simon, and A. L. Weaver. 2005. Unresolved issues in identifying and overcoming inadequate response in rheumatoid arthritis: weighing the evidence. J. Rheumatol. Suppl. 81: 4–30, quiz 31–34.
2. Bykerk, V. 2009. Unmet needs in rheumatoid arthritis. J. Rheumatol. Suppl. 82: 47–46.
3. Smolen, J. S., D. Aletaha, J. Grisar, K. Redlich, G. Steiner, and O. Wagner. 2008. The need for prognosticators in rheumatoid arthritis: biological and clinical markers: where are we now? Arthritis Res. Ther. 10: 208.
4. Wolfe, F., and K. Michaud. 2007. Assessment of pain in rheumatoid arthritis: minimal clinically significant difference, predictors, and the effect of anti-tumor necrosis factor therapy. J. Rheumatol. 34: 1674–1683.
5. Wiles, N. J., D. G. Scott, E. M. Barrett, P. Merry, E. Arie, K. Gaffney, A. J. Silman, and D. P. Symmons. 2001. Benchmarking: the five year outcome of rheumatoid arthritis assessed using a pain score, the Health Assessment Questionnaire, and the Short Form-36 (SF-36) in a community and a clinic based sample. Ann. Rheum. Dis. 60: 956–961.
6. Wolfe, F., and A. M. Chathey. 1991. The assessment and prediction of functional disability in rheumatoid arthritis. Rheumatology. 30: 1298–1307.
7. Smolen, J. S., C. Han, D. M. van der Heijde, P. Emery, J. M. Bathon, E. Keystone, R. N. Maini, J. R. Kalden, D. Aletaha, D. Baker, et al. 2009. Radiographic changes in rheumatoid arthritis patients attaining different disease activity state with methotrexate monotherapy and infliximab plus methotrexate: the impacts of remission and tumour necrosis factor blockade. Ann. Rheum. Dis. 68: 823–827.
8. Turesson, C., W. M. O’Fallon, C. S. Crowson, S. E. Gabriel, and E. L. Matteson. 2003. Extra-articular disease manifestations in rheumatoid arthritis: incidence trends and risk factors over 46 years. Ann. Rheum. Dis. 62: 722–727.
9. Gabriel, S. E., C. S. Crowson, H. M. Kremers, M. F. Doran, C. Turesson, W. M. O’Fallon, and E. L. Matteson. 2003. Survival in rheumatoid arthritis: a population-based analysis of trends over 40 years. Arthritis Rheum. 48: 54–58.
10. Wolfe, F., J. J. Rasker, M. Boers, G. A. Wells, and K. Michaud. 2007. Minimal disease activity, remission, and the long-term outcomes of rheumatoid arthritis. Arthritis Rheum. 57: 935–942.
11. Brown, A. K., P. G. Comaghan, Z. Karim, M. A. Quinn, K. Ikeda, C. G. Peterfy, E. Hensor, R. J. Wakefield, P. J. O’Connor, and P. Emery. 2008. An explanation for the apparent dissociation between clinical remission and continued structural deterioration in rheumatoid arthritis. Arthritis Rheum. 58: 2958–2967.
12. Sattar, N., D. W. McCarey, H. Capell, and I. B. McInnes. 2003. Explaining how “high-grade” systemic inflammation accelerates vascular risk in rheumatoid arthritis. Circulation. 108: 2957–2963.
13. Ku, I. A., J. B. Imboden, P. Y. Hsu, and P. Ganz. 2009. Rheumatoid arthritis: model of systemic inflammation driving atherosclerosis. Circ. J. 73: 977–985.
14. de Jager, W., H. te Velthuis, B. J. Prakken, W. Kuis, and G. T. Rijkers. 2003. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin. Diagn. Lab. Immunol. 10: 133–139.
15. Arnett, F. C., S. M. Edworthy, D. A. Bloch, D. J. McShane, J. F. Fries, N. S. Cooper, L. A. Healey, S. R. Kaplan, M. H. Liang, H. S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 31: 315–324.
16. Cameron, S. B., M. C. Nawijn, W. W. Kuan, H. F. Savelkoul, and A. W. Chow. 2001. Regulation of helper T cell responses to staphylococcal superantigens. Eur. Cytokine Netw. 12: 210–222.
17. Sinclair, E. D., Black, C. L., Eppling, A. C., Carvidi, S. Z., Joselewicz, B. M., Breed, and M. A. Jacobson. 2004. CMV antigen-specific CD4+ and CD8+ T cell IFN-g expression and proliferation responses in healthy CMV-seropositive individuals. *Viral Immunol.* 17: 445–454.

18. Wang, Y., Y. Y. Zhang, L. Y. Zha, Z. J. Zhu, Z. Q. Tang, Y. Jiang, L. Peng, G. Li, and X. H. Zhang. 2004. T-helper and T-cytotoxic cell subsets monitoring during active cytomegalovirus infection in liver transplantation. *Transplant. Proc.* 36: 1498–1499.

19. Compton, T., E. A. Kurt-Jones, K. W. Boehme, J. Belko, E. Latz, D. T. Golenbock, and R. W. Finberg. 2003. Human cytomegalovirus activates inflammatory cytokine responses via CD41 and Toll-like receptor 2. *J. Virol.* 77: 4585–4596.

20. Tabetta, K., P. Georgel, E. Jansen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, et al. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl. Acad. Sci. USA* 101: 3516–3521.

21. Rutz, M., J. Metzger, T. Gellert, P. Luppa, G. B. Lipford, H. Wagner, and S. Bauer. 2005. Heat shock protein 60 activates B cells via the TLR4-MyD88 pathway. *J. Immunol.* 175: 3594–3602.

22. Fabbios, R. M., P. Ahmad-Nejad, C. da Costa, T. Miethke, C. J. Kirschning, M. Cohen-Sfady, M., G. Nussbaum, M. Pevsner-Fischer, F. Mor, P. Carmi, A. Zanin-Zhorov, O. Lider, and I. R. Cohen. 2005. Heat shock protein 60 activates B cells via the TLR4-MyD88 pathway. *J. Immunol.* 175: 3594–3602.

23. Allen, E. M., S. P. Young, R. H. Michell, and P. A. Bacon. 1995. Altered T lymphocyte signaling in rheumatoid arthritis. *Eur. J. Immunol.* 25: 1547–1554.

24. Nascimbeni, M., E. C. Shin, L. Chiriboga, D. E. Kleiner, and B. Rehermann. 2002. CpG motifs in bacterial DNA and their immune effects. *Cancer Res.* 62: 1498–1499.

25. Sinclair, E., D. Black, C. L. Eppling, A. Carvidi, S. Z. Joselewicz, B. M. Breed, and M. A. Jacobson. 2004. CMV antigen-specific CD4+ and CD8+ T cell IFN-g expression and proliferation responses in healthy CMV-seropositive individuals. *Viral Immunol.* 17: 445–454.

26. Wang, Y., Y. Y. Zhang, L. Y. Zha, Z. J. Zhu, Z. Q. Tang, Y. Jiang, L. Peng, G. Li, and X. H. Zhang. 2004. T-helper and T-cytotoxic cell subsets monitoring during active cytomegalovirus infection in liver transplantation. *Transplant. Proc.* 36: 1498–1499.

27. Compton, T., E. A. Kurt-Jones, K. W. Boehme, J. Belko, E. Latz, D. T. Golenbock, and R. W. Finberg. 2003. Human cytomegalovirus activates inflammatory cytokine responses via CD41 and Toll-like receptor 2. *J. Virol.* 77: 4585–4596.

28. Tabetta, K., P. Georgel, E. Jansen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, et al. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl. Acad. Sci. USA* 101: 3516–3521.

29. Allen, E. M., S. P. Young, R. H. Michell, and P. A. Bacon. 1995. Altered T lymphocyte signaling in rheumatoid arthritis. *Eur. J. Immunol.* 25: 1547–1554.

30. Nascimbeni, M., E. C. Shin, L. Chiriboga, D. E. Kleiner, and B. Rehermann. 2002. CpG motifs in bacterial DNA and their immune effects. *Cancer Res.* 62: 1498–1499.

31. Sinclair, E., D. Black, C. L. Eppling, A. Carvidi, S. Z. Joselewicz, B. M. Breed, and M. A. Jacobson. 2004. CMV antigen-specific CD4+ and CD8+ T cell IFN-g expression and proliferation responses in healthy CMV-seropositive individuals. *Viral Immunol.* 17: 445–454.

32. Fabbios, R. M., P. Ahmad-Nejad, C. da Costa, T. Miethke, C. J. Kirschning, M. Cohen-Sfady, M., G. Nussbaum, M. Pevsner-Fischer, F. Mor, P. Carmi, A. Zanin-Zhorov, O. Lider, and I. R. Cohen. 2005. Heat shock protein 60 activates B cells via the TLR4-MyD88 pathway. *J. Immunol.* 175: 3594–3602.

33. Allen, E. M., S. P. Young, R. H. Michell, and P. A. Bacon. 1995. Altered T lymphocyte signaling in rheumatoid arthritis. *Eur. J. Immunol.* 25: 1547–1554.

34. Nascimbeni, M., E. C. Shin, L. Chiriboga, D. E. Kleiner, and B. Rehermann. 2002. CpG motifs in bacterial DNA and their immune effects. *Cancer Res.* 62: 1498–1499.

35. Sinclair, E., D. Black, C. L. Eppling, A. Carvidi, S. Z. Joselewicz, B. M. Breed, and M. A. Jacobson. 2004. CMV antigen-specific CD4+ and CD8+ T cell IFN-g expression and proliferation responses in healthy CMV-seropositive individuals. *Viral Immunol.* 17: 445–454.

36. Wang, Y., Y. Y. Zhang, L. Y. Zha, Z. J. Zhu, Z. Q. Tang, Y. Jiang, L. Peng, G. Li, and X. H. Zhang. 2004. T-helper and T-cytotoxic cell subsets monitoring during active cytomegalovirus infection in liver transplantation. *Transplant. Proc.* 36: 1498–1499.

37. Compton, T., E. A. Kurt-Jones, K. W. Boehme, J. Belko, E. Latz, D. T. Golenbock, and R. W. Finberg. 2003. Human cytomegalovirus activates inflammatory cytokine responses via CD41 and Toll-like receptor 2. *J. Virol.* 77: 4585–4596.

38. Tabetta, K., P. Georgel, E. Jansen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, et al. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl. Acad. Sci. USA* 101: 3516–3521.

39. Allen, E. M., S. P. Young, R. H. Michell, and P. A. Bacon. 1995. Altered T lymphocyte signaling in rheumatoid arthritis. *Eur. J. Immunol.* 25: 1547–1554.

40. Nascimbeni, M., E. C. Shin, L. Chiriboga, D. E. Kleiner, and B. Rehermann. 2002. CpG motifs in bacterial DNA and their immune effects. *Cancer Res.* 62: 1498–1499.