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## Rheumatoid Factor as a Potentiator of Anti–Citrullinated Protein Antibody–Mediated Inflammation in Rheumatoid Arthritis

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**Objective.** The co-occurrence of rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) positivity in rheumatoid arthritis (RA) is well described. However, the mechanisms underlying the potential interaction between these 2 distinct autoantibodies have not been well defined. The aim of this study was to evaluate the epidemiologic and molecular interaction of ACPAs and RF and its association with both disease activity and measures of RA-associated inflammation.

**Methods.** In a cohort of 1,488 US veterans with RA, measures of disease activity and serum levels of cytokines and multiplex ACPAs were compared between the following groups of patients: double-negative (anti-cyclic citrullinated peptide [anti-CCP]–/RF–), anti-

CCP+/RF–, anti-CCP–/RF+, or double-positive (anti-CCP+/RF+). Additional studies were performed using an in vitro immune complex (IC) stimulation assay in which macrophages were incubated with ACPA ICs in the presence or absence of monoclonal IgM-RF, and tumor necrosis factor  $\alpha$  production measured as a readout of macrophage activation.

**Results.** Compared with the double-negative subgroup (as well as each single-positive subgroup), the double-positive subgroup exhibited higher disease activity as well as higher levels of C-reactive protein and inflammatory cytokines (all  $P < 0.001$ ). In vitro stimulation of macrophages by ACPA ICs increased cytokine production, and the addition of monoclonal IgM-RF significantly increased macrophage tumor necrosis factor  $\alpha$  production ( $P = 0.003$  versus ACPA ICs alone).

**Conclusion.** The combined presence of ACPAs and IgM-RF mediates increased proinflammatory cytokine production in vitro and is associated with increased systemic inflammation and disease activity in RA. Our data suggest that IgM-RF enhances the capacity of ACPA ICs to stimulate macrophage cytokine production, thereby providing a mechanistic link by which RF enhances the pathogenicity of ACPA ICs in RA.

Rheumatoid arthritis (RA) is often characterized by the presence of circulating autoantibodies. Rheumatoid factor (RF) was first described more than 50 years ago (1–3) and is detectable in nearly 70% of patients with RA, although its presence is not specific for RA (4,5). Nevertheless, whether RF plays a role in RA pathogenesis has remained unclear. More recently, attention has been given to anti-citrullinated protein antibodies (ACPAs). Anti-cyclic citrullinated peptide (anti-CCP) assays are commonly used to test for ACPAs, which are detectable in ~70% of patients with

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RA and are highly specific for RA (6). In addition to their use in diagnostic criteria, RF and ACPAs provide important prognostic information.

Previous studies have examined the diagnostic utility of RF and ACPAs (7,8), and multiple studies have demonstrated that higher concentrations of both autoantibodies are associated with a more aggressive disease course marked by increased disease activity and reduced rates of remission (9–11). However, there has been little investigation into the mechanisms by which these autoantibodies could interact and/or contribute to RA pathogenesis. Although several *in vitro* (12–14) and *in vivo* (15) studies have identified a potential role for ACPAs in disease pathogenesis, the role of RF in RA pathogenesis remains elusive. In this study, we sought to investigate the role of RF as a contributor to the RA inflammatory burden, both independently and in synergy with ACPAs.

## PATIENTS AND METHODS

**Patient samples and clinical measures.** Study subjects included US veterans enrolled in the Veterans Affairs Rheumatoid Arthritis (VARA) registry, with sites across the US (16). The registry has received Institutional Review Board approval at each site, and the Stanford Institutional Review Board approved the biomarker and *in vitro* analyses performed using RA samples. All patients satisfied the 1987 American College of Rheumatology classification criteria for RA (17) and provided informed written consent and Health Insurance Portability and Accountability Act authorization. The study group comprised 1,488 veterans with RA (89% male); detailed characteristics of the cohort are shown in Table 1. Banked serum samples were available for a representative population of 1,466 patients and were used for multiplex cytokine and autoantibody analyses.

In addition to banked sera, VARA collects clinical data including the baseline and longitudinal 28-joint Disease Activity Score (DAS28) values (18), as well as baseline measurements of ACPAs, obtained using a Diastat second-generation CCP-2 antibody enzyme-linked immunosorbent assay (ELISA) (positivity  $\geq 5$  units/ml; Axis-Shield). VARA also collects RF (positivity  $\geq 15$  IU/ml) and high-sensitivity C-reactive protein (hsCRP) test results, as determined by nephelometry (Siemens). HsCRP concentrations were not available at followup visits. Although VARA is a multicenter project, standardized autoantibodies are measured at the laboratory of a single investigator (GMT). HLA-DRB1 genotyping was conducted as previously described (19). Patients were categorized as being positive or negative for HLA-DRB1 shared epitope (SE)-containing alleles and HLA-DR3 alleles.

Followup measures include tender and swollen joint counts in 28 joints, erythrocyte sedimentation rate (ESR; mm/hour), pain score (range 0–10), Multidimensional Health Assessment Questionnaire (MD-HAQ) score (range 0–3) (20), patient's and provider's global assessments (range 0–100), and treatments. A comorbidity count (range 0–9) was calculated

for each patient, using administrative codes (21). Because we were investigating the association of autoantibodies with followup clinical parameters, patients were excluded if autoantibody or DAS28 data were unavailable, if only a single clinical observation was recorded, or if the total followup duration was  $< 6$  months.

**Multiplex cytokine analysis.** Multiplex analysis of cytokines and chemokines in human serum was performed using a Bio-Plex Pro Human Cytokine 17-plex Assay (Bio-Rad) run on a Luminex 200 system according to the manufacturer's instructions, with the exception that a proprietary Bio-Rad assay dilution buffer was modified to contain reagents demonstrated to reduce the effects of heterophilic antibodies in multiplex immunoassays, as previously described (21). Data processing was performed by using Bio-Plex Manager 5.0 software, and analyte concentrations (picograms per milliliter) were interpolated from standard curves.

**Multiplex autoantigen arrays.** Antibodies targeting 37 putative RA-associated autoantigens were measured using a custom bead-based immunoassay on a Bio-Plex platform, as previously described (22,23). Of the 37 antigens, 30 are citrullinated and 7 are native (native histone 2A, histone 2B, apolipoprotein A-I [Apo A-I], filaggrin 48–65 peptide, vimentin, fibrinogen, and Apo-AI 231–248 peptide). Briefly, serum was diluted and mixed with spectrally distinct fluorescent beads conjugated with putative RA-associated autoantigens, followed by incubation with anti-human phycoerythrin-labeled antibody and analysis on a Luminex 200 instrument.

**In vitro ACPA immune complex (IC) stimulation assays.** ACPA IC stimulation of human macrophages was performed as previously described (12). Polyclonal rabbit IgG antibodies against fibrinogen (Pierce) or human IgG derived from patients with ACPA-positive RA (RA IgG) was used to generate plate-bound ICs containing citrullinated fibrinogen ICs. RA IgG derived from 3 pooled plasma samples that were shown by ELISA to contain high levels of anti-citrullinated fibrinogen antibodies was purified by affinity chromatography on protein G columns, according to the instructions of the manufacturer (Pierce).

Monoclonal IgM-RF derived from a lymphoblastoid cell line generated from a patient with RA (24) was a generous gift from Drs. Michael Steinitz and Reuven Laskov (Hebrew University, Jerusalem, Israel). IgM-RF was isolated from cell culture supernatant by ammonium sulfate precipitation, washed, and dialyzed against phosphate buffered saline (PBS). The resultant product was shown to contain primarily IgM, with no contamination by human IgG. This antibody has been demonstrated to bind human and rabbit IgG but not IgM and, when bound to ICs, fails to bind complement (25). Additional monoclonal IgM antibodies with *in vitro* RF activity that were isolated from patients with mixed cryoglobulinemia were generously provided by Dr. Mariana Newkirk (McGill University, Montreal, Quebec, Canada).

The purified RA IgG and monoclonal RF were separately concentrated by centrifugation over a 100-kd molecular weight filter column with buffer exchange to PBS (Amicon Ultra; Millipore) and were depleted of endotoxin by filtration through a polymyxin B column (Detoxi-Gel; Pierce). RA IgG and IgM-RF concentrations were estimated according to optical density at 280 nm, aliquoted, and stored at  $-80^{\circ}\text{C}$ . For generation of citrullinated fibrinogen ICs, flat-bottomed

**Table 1.** Characteristics of the patients with RA at the time of study enrollment\*

Characteristic	Concordant seronegative (n = 206)	Anti-CCP+/RF- (n = 102)	Anti-CCP-/RF+ (n = 134)	Concordant seropositive (n = 1,046)
<b>Sociodemographics and comorbidity</b>				
Age, mean $\pm$ SD years	64.5 $\pm$ 12.2	61.2 $\pm$ 11.9	63.3 $\pm$ 11.9	63.0 $\pm$ 11.2
Male sex	89.3	88.2	91.8	91.1
<b>Race/ethnicity</b>				
White	79.6	75.5	76.9	76.7
African American	15.1	16.7	15.7	16.7
Other	5.3	7.8	7.7	6.6
High school education or higher	86.7	86.7	85.6	84.0
Comorbidity count, mean $\pm$ SD	2.6 $\pm$ 1.6	2.2 $\pm$ 1.5	2.4 $\pm$ 1.5	2.3 $\pm$ 1.6
<b>RA factors</b>				
Ever smoking <sup>†</sup>	73.2	73.4	73.9	82.7
HLA-DRB1 SE positive <sup>†</sup>	54.6	77.8	54.4	76.5
2 HLA-DRB1 alleles	8.7	23.2	6.5	24.7
HLA-DR3 <sup>†</sup>	25.6	14.1	32.6	12.8
Age at diagnosis, mean $\pm$ SD years <sup>†</sup>	55.9 $\pm$ 15.0	50.7 $\pm$ 13.7	53.8 $\pm$ 13.7	51.8 $\pm$ 13.6
Disease duration, mean $\pm$ SD years <sup>‡</sup>	8.7 $\pm$ 10.1	10.5 $\pm$ 10.7	9.5 $\pm$ 10.7	11.2 $\pm$ 11.4
Prednisone treatment	35.0	40.7	40.7	45.2
Methotrexate treatment <sup>‡</sup>	45.4	64.8	51.2	49.7
Biologic agent treatment	16.9	24.2	23.6	21.1
Nodules <sup>†</sup>	12.1	24.5	26.1	33.8

\* Except where indicated otherwise, values are the percent. RA = rheumatoid arthritis; anti-CCP = anti-cyclic citrullinated peptide; RF = rheumatoid factor; SE = shared epitope.

<sup>†</sup> Overall  $P < 0.001$ , by analysis of variance.

<sup>‡</sup> Overall  $P < 0.01$ , by analysis of variance.

96-well culture plates were coated overnight at 4°C with 50  $\mu$ l of citrullinated fibrinogen (20  $\mu$ g/ml), washed in PBS containing 0.05% Tween 20, and incubated for 2 hours at 4°C with 100  $\mu$ l of polyclonal rabbit antibodies against fibrinogen (50  $\mu$ g/ml), 100  $\mu$ l of anti-citrullinated fibrinogen-positive IgG (10 mg/ml), or 100  $\mu$ l of anti-citrullinated fibrinogen-positive IgG preincubated with monoclonal IgM-RF (stock concentration 5 mg/ml used at 1:20 or 1:100 dilution); PBS alone was used as a control. Wells were again washed in PBS containing 0.05% Tween 20, and macrophages (50,000/well) in 200  $\mu$ l of RPMI containing 5% fetal calf serum were then added to the wells and incubated for 16 hours, at which time levels of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) in culture supernatant were measured by ELISA (PeproTech). All in vitro cell stimulations were performed in triplicate and in at least 2 separate experiments. Levels of TNF $\alpha$  production from in vitro macrophage-stimulation assays were compared using Student's unpaired *t*-test.

**Statistical analysis.** Patients were categorized into the following groups: double-negative (anti-CCP-/RF-;  $n = 206$ ), anti-CCP+/RF- ( $n = 102$ ), anti-CCP-/RF+ ( $n = 134$ ), and double-positive (anti-CCP+/RF+;  $n = 1,046$ ). Comparisons of patient characteristics were examined according to autoantibody subgroup, using chi-square tests or analysis of variance (ANOVA). Unadjusted comparisons of the 8 continuous disease activity measures assessed at enrollment were examined using one-way ANOVA with Tukey's post hoc test to compare each of the 4 subgroups. Levels of each cytokine as well as hsCRP were compared between the double-negative, anti-CCP+/RF-, anti-CCP-/RF+, and double-positive subgroups, using the Kruskal-Wallis test with Dunn's post hoc test.

The ESR was log-transformed prior to analysis to render a more normal distribution. Given the skewed distributions, joint counts were dichotomized into 0 tender/swollen joints and  $\geq 1$  tender/swollen joints, with comparisons examining the probability of having a joint count of  $>0$ . Continuous joint counts were reported for descriptive purposes.

We examined whether the associations observed between autoantibody status and disease activity at enrollment were independent of other covariates, and whether these associations were apparent over an extended period of observation. For multivariable analyses, double-negative cases served as the referent population. Generalized linear mixed models were used to evaluate multivariable associations of autoantibody group with disease activity assessed during followup. The generalized linear mixed models adjusted for the random effects from sites and correlations between the outcomes from the same patient over time via a compound symmetry correlation structure. Analyses were completed using Stata version 12, SAS version 9.3, and GraphPad Prism version 5.

Additional comparisons were performed on multiplex cytokines as well as multiplex ACPAs, using SAM (Significance Analysis of Microarrays) version 3.08 (24). Output was sorted based on false discovery rates (FDRs) in order to identify the antigens with the greatest differences in autoantibody reactivity between serologic subgroups. The use of FDRs obviates the need to adjust for multiple comparisons. Hierarchical clustering was performed using Cluster 3.0 to arrange the SAM results according to similarities among autoantibody specificities, and the results were displayed using Java Tree-View (version 1.1.3).

## RESULTS

**ACPAs and RF in the VARA cohort.** The characteristics of the 1,488 patients are shown in Table 1. The mean  $\pm$  SD followup was  $3.6 \pm 2.8$  years, with 16,822 encounters and 5,284 patient-years of observation. A majority of the patients ( $\sim 90\%$ ) were male, with a mean age of  $\sim 63$  years. Most of the patients (70%) were anti-CCP+/RF+, 14% were anti-CCP-/RF-, 9% were anti-CCP-/RF+, and 6.9% were anti-CCP+/RF-. There were significant differences across the autoantibody groups for age at diagnosis, disease duration, methotrexate treatment, and presence of nodules. Compared with the other patients, those who were anti-CCP-/RF- were older at the time of diagnosis, had a shorter disease duration, were less likely to be receiving methotrexate, and had a lower prevalence of nodules.

Ever smoking was more common among anti-CCP+/RF+ patients (83%) compared with patients in the other subgroups (73–74%;  $P < 0.001$ ). HLA-DRB1 SE positivity was higher in groups characterized by anti-CCP positivity ( $P < 0.001$ ), irrespective of RF status. HLA-DR3 positivity was less common among patients positive for anti-CCP antibodies ( $P < 0.001$  across groups). The average anti-CCP-2 titer was nearly identical in the CCP+/RF- and CCP+/RF+ groups (275.85 AU versus 273.64 AU), and similarly, the average RF titer in the CCP-/RF+ group was nearly identical to that in the CCP+/RF+ group (329.03 IU versus 328.12 IU).

**Relationship between the concurrent presence of ACPAs and RF and increased RA disease activity.** The double-positive group exhibited significantly higher

levels of RA disease activity, with a mean  $\pm$  SD baseline DAS28 of  $4.2 \pm 1.6$  compared with  $3.7 \pm 1.6$  in the double-negative group,  $3.4 \pm 1.6$  in the anti-CCP+/RF- group, and  $3.9 \pm 1.7$  in the anti-CCP-/RF+ group (overall  $P < 0.001$ , by ANOVA) (Table 2). After adjusting for multiple comparisons across the 4 groups, the ESR, and hsCRP and DAS28 values at the time of enrollment were higher in the double-positive group compared with the other groups. Post-test comparisons between each pair of groups demonstrated significantly higher ESRs and CRP levels among the double-positive group compared with the double-negative, anti-CCP+/RF-, and anti-CCP-/RF+ groups, and the DAS28 was significantly higher in the double-positive group compared with the double-negative and anti-CCP+/RF- subgroups, with a nonsignificant trend compared with the anti-CCP-/RF+ subgroup.

Similarly, in multivariable models, concurrent anti-CCP-positive and RF-positive autoantibody status was associated with longitudinal clinical and laboratory measures of disease activity, including the swollen joint count, ESR, and DAS28 when compared with the double-negative or anti-CCP+/RF- subgroups, while only a similar trend was noted when compared with the anti-CCP-/RF+ subgroup (Table 3).

**Concurrent presence of ACPAs and RF is predictive of RA-associated inflammation.** Compared with the double-negative group, the double-positive group exhibited significantly higher ESRs and hsCRP levels as well as higher levels of multiple circulating inflammatory cytokines, including TNF $\alpha$ , interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6,

**Table 2.** Measures of disease activity in patients with rheumatoid arthritis at the time of study enrollment, according to autoantibody status\*

Measure	Concordant seronegative (n = 206)	Anti-CCP+/RF- (n = 102)	Anti-CCP-/RF+ (n = 134)	Concordant seropositive (n = 1,046)
Pain score (range 0–10)	5.1 $\pm$ 2.8	4.3 $\pm$ 2.7	4.8 $\pm$ 2.7	4.8 $\pm$ 2.8
MD-HAQ (range 0–3)	0.94 $\pm$ 0.63	0.81 $\pm$ 0.62	0.96 $\pm$ 0.58	0.98 $\pm$ 0.60
Tender 28 joint count	6.0 $\pm$ 7.5	4.5 $\pm$ 7.0	5.5 $\pm$ 6.7	6.3 $\pm$ 7.3
Swollen 28 joint count	4.1 $\pm$ 5.4	4.0 $\pm$ 5.7	5.3 $\pm$ 6.6	5.8 $\pm$ 6.4
Patient's global assessment (range 0–100)	45.1 $\pm$ 27.0	35.8 $\pm$ 23.2	43.4 $\pm$ 26.1	43.8 $\pm$ 25.8
Provider's global assessment (range 0–10)	39.9 $\pm$ 24.5	29.1 $\pm$ 19.8	32.0 $\pm$ 25.2	37.8 $\pm$ 23.3
Log-transformed ESR, mm/hour $\dagger$	19.4 $\pm$ 19.6	21.2 $\pm$ 21.9	24.8 $\pm$ 24.7	30.2 $\pm$ 24.0
CRP, mg/liter $\ddagger$	0.92 $\pm$ 1.78	0.92 $\pm$ 0.74	1.20 $\pm$ 1.78	1.36 $\pm$ 2.13
DAS28 $\S$	3.7 $\pm$ 1.6	3.4 $\pm$ 1.6	3.9 $\pm$ 1.7	4.2 $\pm$ 1.6

\* Joint counts were dichotomized as 0 versus  $\geq 1$ . Statistical significance, as determined by analysis of variance, was defined as  $P < 0.00625$ . Values are the mean  $\pm$  SD. Anti-CCP = anti-cyclic citrullinated peptide; RF = rheumatoid factor; MD-HAQ = Multidimensional Health Assessment Questionnaire; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = Disease Activity Score in 28 joints.

$\dagger P < 0.001$ , overall and concordant seropositive versus concordant seronegative;  $P = 0.007$ , concordant seropositive versus anti-CCP-/RF+;  $P = 0.001$ , concordant seropositive versus anti-CCP+/RF-.

$\ddagger P < 0.001$ , overall, concordant seropositive versus concordant seronegative and versus anti-CCP+/RF-;  $P = 0.01$ , concordant seropositive versus anti-CCP-/RF+.

$\S P < 0.001$  overall;  $P = 0.001$ , concordant seropositive versus concordant seronegative and versus anti-CCP+/RF-.

**Table 3.** Multivariable associations of autoantibody status with measures of disease activity in patients with RA during followup\*

Variable	Discordant					
	Anti-CCP+/RF-		Anti-CCP-/RF+		Concordant, anti-CCP+/RF+	
	$\beta$ coefficient	<i>P</i>	$\beta$ coefficient	<i>P</i>	$\beta$ coefficient	<i>P</i>
Pain score (range 0–10)	-0.489	0.094	-0.008	0.972	-0.288	0.097
MD-HAQ (range 0–3)	-0.161	0.034	-0.000	0.996	-0.015	0.725
Tender joint count (0 vs. >0)	-0.251	0.181	0.326	0.042	0.052	0.624
Swollen joint count (0 vs. >0)†	0.100	0.562	0.619	<0.001	0.507	<0.001
Patient's global assessment (range 0–100 mm)	-6.088	0.015	-0.212	0.925	-1.588	0.315
Provider's global assessment (range 0–100 mm)	-3.804	0.069	2.597	0.191	2.418	0.069
Log-transformed ESR, mm/hour‡	0.444	<0.001	0.410	<0.001	0.652	<0.001
DAS28§	-0.003	0.987	0.387	0.005	0.418	<0.001

\* The models were adjusted for age, sex, disease duration, comorbidity score, race/ethnicity, and treatment, including methotrexate, prednisone, and biologic agents. The concordant seronegative group served as the referent population. RA = rheumatoid arthritis; anti-CCP = anti-cyclic citrullinated peptide; RF = rheumatoid factor; MD-HAQ = Multidimensional Health Assessment Questionnaire; ESR = erythrocyte sedimentation rate; DAS28 = Disease Activity Score in 28 joints.

† *P* = 0.426, anti-CCP+/RF+ versus anti-CCP-/RF+; *P* = 0.008, anti-CCP+/RF+ versus anti-CCP+/RF-.

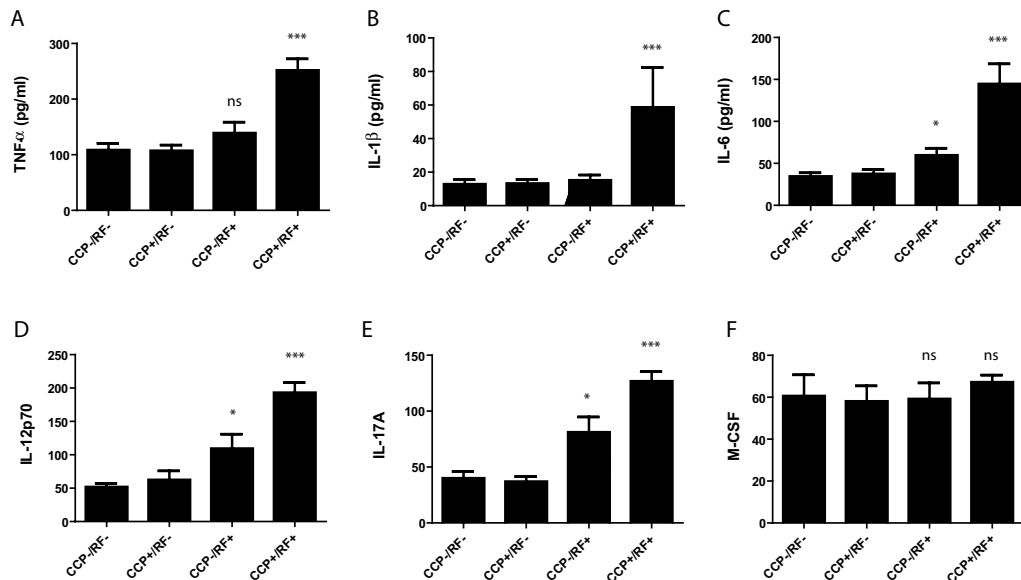
‡ *P* = 0.005, concordant seropositive versus anti-CCP-/RF+; *P* = 0.036, concordant seropositive versus anti-CCP+/RF-.

§ *P* = 0.786, concordant seropositive versus anti-CCP-/RF+; *P* = 0.002, concordant seropositive versus anti-CCP+/RF-.

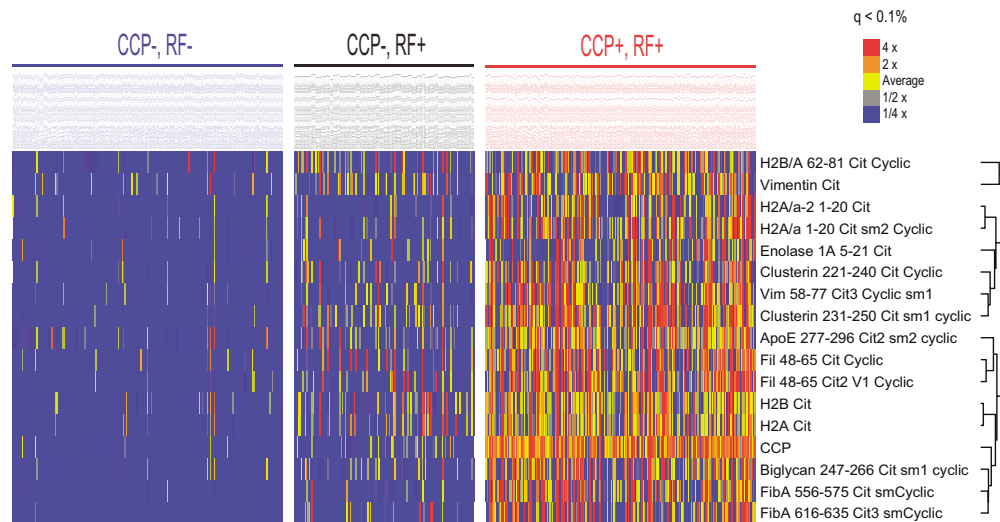
IL-12p70, and IL-17A (Figure 1) (all *P* < 0.001 by ANOVA). Although it is likely that many cytokines, including those most up-regulated in the double-positive group, are highly related and thus not subject to a high risk of Type I error in multiplex cytokine analysis, stringent implementation of Bonferroni's correction for

17 potentially independent cytokines would require a *P* value of 0.0029 for significance, and this was achieved for all of the cytokines shown in Figure 1.

Additionally, all cytokine levels were compared between the double-negative group, the anti-CCP-/RF+ group, and a set of double-positive patients



**Figure 1.** Higher serum levels of rheumatoid arthritis (RA)-associated cytokines in RA patients who were double-positive for anti-cyclic citrullinated peptide (anti-CCP) and rheumatoid factor (RF). Patients were categorized as being double-negative (anti-CCP-/RF-; n = 204), anti-CCP+/RF- (n = 96), anti-CCP-/RF+ (n = 135), or double-positive (anti-CCP+/RF+; n = 1,031). Between-group comparisons were performed using the Kruskal-Wallis test with Tukey's multiple comparison post hoc test. \* = *P* < 0.01; \*\*\* = *P* < 0.001 versus anti-CCP-/RF-. TNF $\alpha$  = tumor necrosis factor  $\alpha$ ; NS = not significant; IL-1 $\beta$  = interleukin-1 $\beta$ ; M-CSF = macrophage colony-stimulating factor.



**Figure 2.** Heatmap showing elevated levels of anti-citrullinated protein antibodies in the anti-CCP<sup>-</sup>/RF<sup>+</sup> group compared with the double-negative group. Levels of autoantibodies against 37 putative targets of the RA immune response were compared between the anti-CCP<sup>+</sup>/RF<sup>-</sup> group (n = 96) and the anti-CCP<sup>-</sup>/RF<sup>+</sup> group (n = 135) using a multiplex antigen microarray. SAM software was used to sort output based on false discovery rates in order to identify antigens with the greatest differences in autoantibody reactivity between serologic subgroups. Labels on the color key are the fluorescence intensity relative to the average values in the evaluated cohort. Cit = citrullinated; Vim = vimentin; ApoE = apolipoprotein E; Fil = filaggrin; FibA = fibrinogen A (see Figure 1 for other definitions).

matched for age, sex, and disease duration. Three-way group comparisons were performed using SAM version 3.08 (23), and output was sorted based on FDRs. Notably, the use of FDRs obviates the need to adjust for multiple comparisons. Supplementary Figure 1 (available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.38307/abstract>) demonstrates significant up-regulation of 10 of 17 cytokines in the double-positive group compared with the double-negative group and both the anti-CCP<sup>+</sup>/RF<sup>-</sup> and anti-CCP<sup>-</sup>/RF<sup>+</sup> groups.

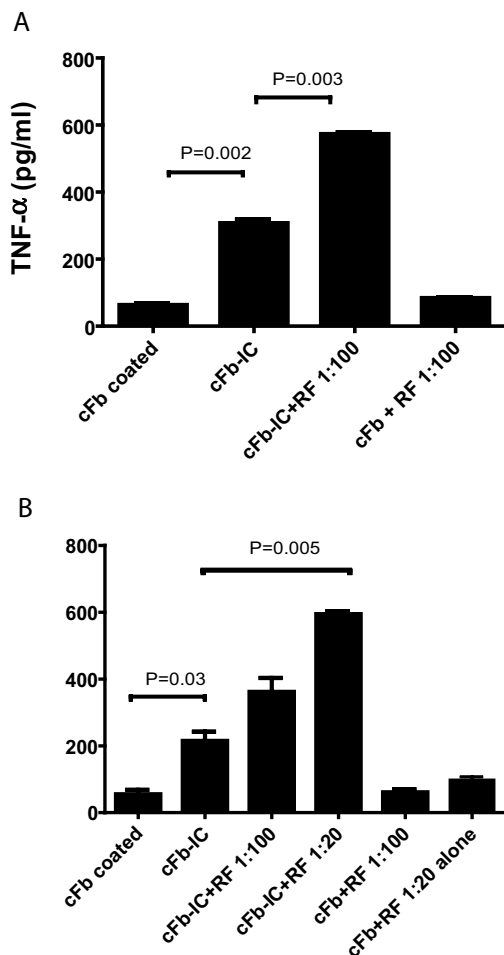
A smaller but, in many cases, statistically significant elevation of cytokine levels (as well as some clinical measures of disease activity) was noted in the anti-CCP<sup>-</sup>/RF<sup>+</sup> group compared with the double-negative and anti-CCP<sup>+</sup>/RF<sup>-</sup> groups. We hypothesized that such elevations in the anti-CCP<sup>-</sup>/RF<sup>+</sup> group may reflect the presence in some patients of physiologic levels of ACPAs not detected by or below the range of the commercial anti-CCP-2 ELISA. To support this hypothesis, we used a multiplex antigen array to assess ACPA subspecificities that might be present in the anti-CCP<sup>-</sup>/RF<sup>+</sup> group. Figure 2 shows that the levels of 11 of 30 ACPAs were elevated in the anti-CCP<sup>-</sup>/RF<sup>+</sup> group compared with the double-negative group. Thus, we hypothesized that low levels of ACPAs that are not

strongly represented by the anti-CCP-2 assay may synergize with RF to induce RA-associated inflammation.

**Augmentation of the stimulatory capacity of ACPA ICs by monoclonal RF.** We previously demonstrated the ability of ACPA ICs to stimulate macrophage cytokine production via costimulation of Fc $\gamma$  receptor and the innate immune receptor Toll-like receptor 4 (12). To evaluate the effect of RF on ACPAs, we preincubated ACPA-containing RA IgG with monoclonal IgM-RF before formation of ACPA ICs. As previously described, in vitro stimulation of monocyte-derived macrophages by ACPA ICs demonstrated increased cytokine production ( $P = 0.002$  versus citrullinated fibrinogen only), and the addition of monoclonal IgM-RF resulted in a further significant increase in macrophage TNF $\alpha$  production as compared with ACPA ICs alone ( $P = 0.003$ ) (Figure 3A). Notably, we additionally tested 2 monoclonal IgM antibodies with in vitro RF activity, which were isolated from patients with mixed cryoglobulinemia. However, the addition of these IgM antibodies at 20  $\mu$ g/ml, 100  $\mu$ g/ml, and 200  $\mu$ g/ml failed to augment macrophage TNF secretion in response to ACPA ICs (data not shown).

Given the potential inclusion of IgG-RF (or IgM-RF) in our ACPA-positive IgG preparation, we further investigated the ability of monoclonal IgM-RF to





**Figure 3.** IgM-RF augments macrophage activation by anti-citrullinated protein antibody (ACPA) immune complexes (ICs) in vitro. Monocyte-derived macrophages were added to plates precoated with human citrullinated fibrinogen (cFb) ICs (A) or with citrullinated fibrinogen ICs formed using polyclonal rabbit IgG antibodies against fibrinogen (B), in the presence or absence of monoclonal IgM-RF. Macrophage stimulation was assessed by measuring the level of TNF $\alpha$  in harvested cell culture supernatants. Results are representative of experiments performed at least twice. Bars show the mean  $\pm$  SEM of triplicate cultures. See Figure 1 for other definitions.

enhance the stimulatory activity of anti-citrullinated fibrinogen ICs, this time formed with a polyclonal rabbit antibody against fibrinogen previously demonstrated to bind native and citrullinated fibrinogen (12) and which could be targeted by our monoclonal IgM-RF by ELISA and Western blotting (data not shown). In results analogous to those of previous studies, we observed the ability of anti-citrullinated fibrinogen ICs to stimulate macrophage cytokine production and, as with human RA-derived IgG preparations, the ability of monoclonal

IgM-RF to augment anti-citrullinated fibrinogen IC-induced macrophage activation, as evidenced by increased cytokine production (Figure 3B).

## DISCUSSION

Although several previous studies have demonstrated increased disease activity in the presence of either anti-CCP or RF, to our knowledge, this study is the first to identify a synergistic role for ACPAs and RF in mediating RA-associated inflammation and disease activity. We demonstrated that baseline autoantibody status was associated with select measures of disease activity, both at presentation and over time. Specifically, joint swelling and higher baseline DAS28 values were more commonly observed in patients with RA who were positive for both anti-CCP and RF and, to a lesser extent in those positive for RF regardless of anti-CCP status. Interestingly, ESRs were similarly increased in all autoantibody-positive groups (both double-positive and single-positive) compared with seronegative patients, even after accounting for the numerically higher frequency of RA-related treatments used in seropositive cases; however, values were further increased in the double-positive group compared with each single-positive group. The observed increases in disease activity and the levels of clinical markers of inflammation were paralleled by a nearly identical pattern of elevation among several inflammatory cytokines previously associated with RA pathophysiology (26), including those that we and other investigators have previously demonstrated to be produced in response to macrophage stimulation by ACPA ICs (12,13).

The increased inflammation and disease activity observed in the double-positive group is supportive of a potential role for these RA-associated autoantibodies in mediating the pathogenesis of RA. However, because association does not prove causality, we used an in vitro model of ACPA IC-mediated inflammation to identify a novel mechanism by which the interaction of ACPAs and RF may contribute to the pathophysiology of RA inflammation and disease activity. We thus demonstrate a unifying mechanism by which the 2 overtly distinct autoantibody types that characterize RA can interact to promote RA disease pathogenesis.

RF was first identified by the ability of RA serum to agglutinate IgG-coated sheep red blood cells, was subsequently defined as an immunoglobulin targeting the Fc portion of IgG (3), and is now considered to be characteristic of the presence of RA and has been associated with increased disease severity (9,11). How-

ever, the pathogenic role of RF has been questioned and, to date, poorly defined. In 1961, Ragan stated that “the significance of rheumatoid factor in the pathogenesis of rheumatoid arthritis remains conjectural” (3).

More than 50 years later, little progress has been made in our understanding. Previous studies have suggested the ability of RF to accelerate experimental models of IC-mediated vascular damage (27), and although there is evidence for the ability of RF to fix complement (28,29), other studies suggest that RF may in fact prevent complement activation by IgG ICs, thus leading to the speculation that the effects of RF are in fact mediated by attachment to the Fc region of the IgG molecule rather than complement activation (30). As such, this study is the first to suggest a mechanistic role of RF in RA disease propagation. Additionally, it has long been known that RF is present in the setting of non-RA immune activation, including most notably, viral and bacterial infection.

Prior studies have identified the ability of RF-expressing B cells to recognize ICs (but not monomeric IgG) and to present the retained antigen to T cells (31), thus enhancing the immune response to foreign antigen. Therefore, the generation of RF may have a teleological purpose in its potential ability to stabilize protective ICs. We propose that soluble RF may have developed to provide a protective evolutionary advantage against infectious pathogens. Because the immune response associated with RA and similar autoimmune conditions is also associated with the presence of RF, we hypothesize that inflammatory disease pathogenesis has co-opted the beneficial role of IC stabilization, thus enhancing the pathogenic capacity of disease-associated autoantibodies and resultant ICs.

The current study has several limitations. Given the large proportion of older patients examined (who are reflective of current VA beneficiaries), caution should be used before applying these results to a broader RA patient population. Also, given reports highlighting the pathogenic role that ACPAs might play in propagating RA-related joint damage (14), further analyses examining the relationship of autoantibody status with clinical and radiographic outcomes in RA are important.

Despite use of methods optimized to minimize the effects of heterophilic antibodies, our laboratory studies do not eliminate the potential of heterophilic antibodies such as RF to induce erroneously elevated signal in sandwich immunoassays (32,33). Thus, the risk remains that the observed elevation of cytokine levels in the double-positive group is in part a result of such bias. However, the relative lack of signal in the RF single-

positive group, as well as supporting clinical and ESR data, provide evidence against a significant contribution of such confounding in this study. Finally, the complexity and heterogeneity of IgM-RF remain great. Although most monoclonal RF characterized to date target the C $\gamma$ 2–C $\gamma$ 3 interface in the Fc region on the IgG molecule (34), the variations in exact binding sites as well as the role of various posttranslational modifications (35,36) to affect RF binding, as well as the typically polyclonal nature of RF in vivo, may limit the generalizability of our IC data generated with a single monoclonal IgM-RF.

In summary, our results showed that a significantly increased level of disease activity as well as increased levels of systemic inflammation markers were associated with the concordant presence of RF and ACPAs in a multivariable analysis accounting for a variety of other potentially associated clinical variables. Here, we demonstrate a mechanism by which the IgM-RF–ACPA interaction may directly contribute to RA disease pathogenesis. These results not only provide useful information for predicting the severity of RA but also, by identifying a mechanistic interaction between ACPAs and IgM-RF, advance our understanding of the role of RA-associated autoantibodies in mediating the pathogenesis of RA.

#### AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sokolove had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Analysis and interpretation of data.** Lahey, Wagner, Thiele, Sayles, Caplan, Cannon, Kerr, Mikuls, Robinson.

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