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Identification of Novel, Immunogenic HLA-DR-Presented *Prevotella copri* Peptides in Patients with Rheumatoid Arthritis

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Abstract

Objectives.—We previously identified HLA-DR-presented epitopes from a 27-kD protein of *Prevotella copri* (*Pc*) obtained from the PBMC of one RA patient. Herein, we sought to identify other HLA-DR-presented *Pc* peptides and source proteins from the PBMC of additional patients to better understand *Pc* immune responses and RA disease pathogenesis.

Methods.—Using tandem mass spectrometry, we searched for HLA-DR-presented *Pc* peptides in PBMC from RA and Lyme arthritis (LA) patients. The identified peptides and source proteins were tested for reactivity in RA patients, those with other arthritides, or the general population; the results were correlated with clinical findings.

Results.—Including *Pc*-p27, we have identified 5 HLA-DR-presented *Pc* peptides, each derived from a different *Pc* protein, in 3 of 4 RA patients, but none in 2 LA patients. When tested in our RA cohort, 14 of 19 patients (74%) had T cell responses and 47 of 89 patients (53%) had IgG or IgA responses with ≥1 of the 5 *Pc* peptides or proteins, most commonly IgA reactivity with *Pc*-p27. Additionally, 74% of RA patients with IgA antibodies to ≥1 *Pc* protein had anti-citrullinated protein antibodies (ACPA) compared with 49% of patients who lacked IgA *Pc* antibody responses (P=0.05), and IgA *Pc* antibody levels correlated with ACPA values.

Conclusions.—The majority of our RA patients had *Pc* immune responses. The correlation of IgA *Pc* antibodies, particularly to *Pc*-p27, with ACPA supports the hypothesis that specific

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AUTHOR CONTRIBUTION

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. Steere had full access to all of the data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design. Pianta, Strle, Costello, Steere.

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microbial antigens in the mucosa have a role in shaping or amplifying immune responses in RA joints.

Keywords

Rheumatoid arthritis; *Prevotella copri* ; T cell epitope; mass spectrometry

There is increasing evidence that mucosal immune responses to microbial agents in the periodontium, lung, or intestine may shape immune responses in the joints of patients with rheumatoid arthritis (RA) (1, 2). However, identification of microbial agents and immune responses that may connect mucosal and joint immunity remain incomplete. In a seminal study of the gut microbiota in RA patients, Scher et al reported an overabundance or *Prevotella spp.*, particularly *Prevotella copri* (*Pc*), in stool samples from patients with new-onset RA (NORA) (3), which was the stimulus for our *Pc* immune response studies.

We developed a novel technique to identify HLA-DR-presented microbial or self-peptides from synovial tissue, synovial fluid mononuclear cells (SFMC), or peripheral blood mononuclear cells (PBMC) of arthritis patients using nanoUPLC-tandem mass spectrometry (nano-LC-MS/MS), followed by determination of the antigenicity of the peptides and their source proteins using patients' samples (4). With this technique, we first searched for *Pc* peptides and self-peptides in 9 such samples (2 from PBMC) from RA patients. From the PBMC of 1 of the 2 patients, one HLA-DR-presented *Pc* peptide was identified, which was derived from a 27-kD *Pc* protein (*Pc*-p27) (5). *Pc* peptides were not identified from synovia or SFMC. When testing was done in our entire cohort of RA patients, 42% of 40 patients had Th1 responses to the *Pc*-p27 peptide, and 24% of 127 patients had IgG or IgA antibodies to the *Pc*-p27 protein.

From the same patient in whom the *Pc* peptide was identified, 2 novel, immunogenic HLA-DR-presented self-peptides, one derived from *N*-acetylglucosamine-6-sulfatase (GNS) and the other from filamin A (FLNA), were identified from her synovial tissue (6). These 2 self-proteins have sequences homologous with *Prevotella* epitopes; and patients who had T cell reactivity with 1 or both self-peptides also had responses to the corresponding *Prevotella* peptides (6), implicating molecular mimicry between these microbial and self-proteins as a possible link between gut microbial immunity and autoimmunity in joints.

In the current study, we searched for HLA-DR-presented *Pc* peptides from the PBMC of 2 new RA patients and, for comparison, from 2 Lyme arthritis (LA) patients. We report here the identification of 4 new HLA-DR-presented *Pc* peptides (T cell epitopes) from the 2 new RA patients. When samples from our recent RA cohort were tested for reactivity with these 4 *Pc* proteins and the previously identified *Pc*-p27, the majority of patients had T and/or B cell responses to ≥1 of these 5 *Pc* antigens. Moreover, the correlation between IgA responses to *Pc* proteins and ACPA support the hypothesis that specific microbial antigens in the mucosa may shape immune responses in RA.

PATIENTS AND METHODS

Patients.

The study was approved by the Human Investigations Committee at Massachusetts General Hospital (MGH); all subjects gave written informed consent. All RA patients met the 2010 American College of Rheumatology/ European League Against Rheumatism criteria for RA (7). HLA-DR typing was performed at the American Red Cross in Dedham, Massachusetts.

Isolation and identification of HLA-DR presented peptides.

We have previously published methods for immunoprecipitation of HLA-DR molecules from patient samples, followed by the elution and identification of HLA-DR-presented peptides using nano-LC-MS/MS (4). Here, only PBMC were analyzed, as we did not previously identify *Pc* proteins from synovia or SFMC. Spectra-to-peptide assignments were made by searching each patient's MS/MS dataset against a UniProt *Pc* database (assembled in-house) using 3 search engines, Mascot, OMSSA, and X!Tandem. A consensus match among ≥2 programs was required for identification of a peptide sequence, with a Mascot score ≥20, OMSSA e-value ≤0.01, and X!Tandem ≤10. To rule out erroneous assignment of a human sequence as a *Pc* sequence, each microbial sequence was screened against the most recent version of the UniProt human database.

Enzyme-linked immunospot (ELISpot) T cell assay.

Each HLA-DR-presented candidate microbial antigen was synthesized and HPLC-purified in the Core Proteomics Laboratory at MGH. Each peptide was used first to stimulate the matching patient's PBMC in an IFNγ ELISpot assay. Immunogenic peptides were then tested in larger numbers of patients, as previously described (5). A positive T cell response was defined as 3 standard deviations (SD) above the mean value of healthy subjects.

Determinations of *Pc* antibodies.

Recombinant preparations of the 5 *Pc* proteins were made by GenScript using an *E. coli* expression vector (pET30a). Target protein purity was estimated to be about 90% based on densitometric analysis using SDS-PAGE gels.

ELISA assays were performed, with modifications of previously described methods (5). After coating overnight at 4°C with each recombinant *Pc* protein (1 μg/ml), the plates were incubated at room temperature with blocking buffer (PBST, 5% milk) for 1 hour. Depending on the *Pc* protein, each patient's serum sample (diluted 1:50 or 1:100) was added for 2 hours, followed by horseradish-peroxidase (HRP)-conjugated goat anti-human IgG or IgA (Dako) (diluted 1:2,000 or 1:3,000) for 1.5 hours and then TMB substrate (BD) for 10 to 15 minutes. A positive antibody response was defined as 2 standard deviations (SD) above the mean value of the general population.

Statistical analysis.

Quantitative data were analyzed using the Mann-Whitney test or t test with Welch correction, categorical data using Fisher's exact test, and correlations using Spearman's

correlation test. All analyses were performed using GraphPad Prism 8. All *P* values were two-tailed. P values ≤ 0.05 were considered statistically significant.

RESULTS

Identification of naturally presented HLA-DR *Pc* peptides.

When HLA-DR-presented peptides were eluted from PBMC from 2 new RA patients and analyzed by nano-LC-MS/MS, 4 new *Pc* peptides were identified. When combined with the 2 previously reported RA patients (5), 3 of the 4 RA patients tested had 1-to-3 HLA-DR-presented *Pc* peptides, and each of these 3 patients had *Pc* IgG or IgA antibodies. *Pc* peptides or antibodies were not identified in the remaining RA patient or in the 2 LA patients. Clinical data for all 6 RA and LA patients is presented in suppl table 1.

The 5 HLA-DR-presented *Pc* peptides identified, to date, were each derived from a different *Pc* protein: 27-kD protein (*Pc*-p27), ribonuclease HII protein (*Pc*-ribo), DNA binding protein (DNAbind), glutamate-5-kinase protein (*Pc*-glut), and type III restriction endonuclease protein (*Pc*-endo) (suppl Figure 1). Peptide length ranged from 10-to-19 amino acids, which is typical of HLA-DR-class II-presented peptides. Each peptide had 100% sequence homology with the corresponding *Pc* protein, but had limited sequence homology with any human peptide, suggesting that they were not human peptides erroneously assigned with a microbial database.

T cell reactivity to *Pc* peptides.

As reported previously, when PBMC from 40 NORA patients were stimulated with the peptide sequence from *Pc*-p27, 17 (42%) secreted IFN-γ levels that were >3 SD above the mean value of healthy controls (P=0.0002) (Figure 1). To conserve cells, samples from these patients were not retested for *Pc*-p27 responses. However, the 4 new *Pc* peptides were tested for this study using PBMC from 20 of the 40 NORA patients in whom enough cells remained.

Of 20 NORA patients, 40% responded to the promiscuous binding *Pc*-ribo and 45% reacted with the *Pc*-DNAbind peptide (Figure 1). A smaller percentage of patients (20%) responded to the 2 peptides with more restricted HLA-DR binding profiles (*Pc*-glut and *Pc*-endo). Of the 19 patients in whom testing was done with all 5 proteins, 14 (74%) had T cell reactivity with ≥1 of the 5 *Pc* peptides.

B cell reactivity to *Pc* proteins.

IgG and IgA antibody responses to the 5 *Pc* proteins were determined in 89 RA patients, including 54 NORA and 35 chronic RA (CRA) patients (Figure 2). The 89 patients included 17 of the 20 patients in whom T cell testing was done and 72 patients in our new RA cohort enrolled during the past 2 years. Because the results were similar in NORA and CRA patients, these data were combined for presentation here. For comparison, serum samples were tested from 37 patients with other chronic inflammatory arthritides (IA), including spondyloarthropathies, psoriatic arthritis, or sarcoidosis; from 80 patients with

Lyme arthritis (LA), and from 45 individuals in the general population (GP), including hospital personnel and blood donors.

Of the 89 RA patients, 24 (27%) had IgA antibody responses to *Pc*-p27, which were >2 SD above the mean value in the general population (Figure 2). The number of patients with positive IgA responses to *Pc*-p27 and quantitative *Pc*-p27 values were significantly greater than those in comparison groups. Although only small numbers of patients had positive IgA responses to the 4 new *Pc* proteins, quantitative values were frequently greater in the RA cohort than in other groups.

Among the 89 RA patients, 12% had positive IgG *Pc*-p27 responses, 10% had IgG antibodies to *Pc*-ribo, and 10% had IgG *Pc*-endo antibodies, which tended to be higher than that in the other groups, but the number of patients who had positive values were not significantly different among the groups. However, quantitative values for each *Pc* protein, except for *Pc*-DNAbind, were significantly greater in RA patients than in the IA group. Surprisingly, 13 of the 80 LA patients (16%) had elevated IgG responses to *Pc*-DNA binding protein, which was a higher percentage than that in RA patients, raising the question of whether *Pc*-DNAbind has a cross-reactive antibody epitope with a spirochetal protein. Unlike RA patients, only a small number of LA patients had IgA responses to *Pc*-DNAbind or other *Pc* proteins.

Most patients in RA or comparison groups had IgG or IgA responses to only a single *Pc* protein. Only 8 RA patients had positive IgG or IgA responses to >1 *Pc* protein, but none had both IgG and IgA responses to the same protein. Altogether, 29 RA patients (33%) had IgA responses to ≥1 of the 5 *Pc* proteins compared with 18 of 161 patients (11%) in all other groups ($P<0.0001$), and 26 of the 89 RA patients (29%) had IgG antibody responses to ≥1 of the 5 *Pc* proteins compared with 39 of 161 patients (24%) in the other groups ($P=0.45$). A total of 47 of the 89 RA patients (53%) had IgG or IgA responses to ≥1 of the 5 *Pc* proteins.

Clinical correlations.

Among the 89 RA patients, the majority (60%) had NORA, the sex ratio was 4 to 1 in favor of women; 60% had ACPA, 38% had rheumatoid factor (RF), and 62% had ACPA or RF, percentages typical of early RA cohorts (8). Among patients with IgA *Pc*-p27 antibodies, 75% had ACPA compared with 55% of those without IgA *Pc*-p27 antibodies ($P=0.1$), and there was a similar trend for RF (Table 1). Moreover, IgA antibody responses to *Pc*-p27, *Pc*-ribo, or *Pc*-glut correlated directly with ACPA levels; and IgA antibodies to *Pc*-p27, *Pc*-ribo, *Pc*-glut or *Pc*-endo correlated with RF levels (Table 2). Overall, ACPA were found in 74% of those who had IgA responses to ≥1 of 5 *Pc* proteins compared with 49% of those who lacked such responses ($P=0.05$). Conversely, among the 34 patients who lacked ACPA or RF, 6 (18%) had IgA and 3 (8%) had IgG *Pc*-p27 antibodies. Finally, patients with *Pc* tended to have higher levels of inflammatory markers and a higher frequency of shared epitope HLA-DRB1 alleles than patients without *Pc* antibodies, but the differences were not statistically significant.

DISCUSSION

Using a novel approach in which HLA-DR-presented peptides were identified directly from patient samples, we have now identified 5 immunogenic HLA-DR-presented *Pc* peptides from PBMC in 3 of 4 RA patients tested. The large sample volumes and the complexity of the technique precluded evaluation of large numbers of patients. However, when serum samples from our current RA cohort and comparison groups were tested for IgG or IgA reactivity with each of the 5 *Pc* proteins, the most robust difference between the groups was IgA *Pc*-p27 reactivity. Of the 89 RA patients, 24 (27%) had IgA responses to this protein, a significantly higher percentage than that in the other groups, making it an attractive diagnostic target. However, in contrast with our initial study (5), the number of patients with positive IgG *Pc* responses was not significantly greater in RA patients, though quantitative values were often higher in the RA group than in the other groups.

Because of the importance of anti-citrullinated protein antibodies (ACPA) in RA (9, 10), we searched our MS/MS spectra carefully for evidence of peptides with the one-Dalton gain that could indicate an arginine-to-citrulline conversion. Of the 5 *Pc* T cell epitopes studied here, only *Pc*-p27 contained an arginine, and that peptide was not citrullinated. However, ACPA correlated significantly with *Pc*-p27 antibodies, suggesting that another portion of the protein may become citrullinated. Moreover, when we previously citrullinated 2 autoantigens, GNS and FLNA, which had T cell epitopes with sequence similarity with *Prevotella* sp., RA patients had higher antibody responses to citrullinated GNS than to its non-citrullinated proteoform (6), suggesting that the GNS self-protein may be citrullinated *in vivo*.

In the current study, the correlation of IgA *Pc* responses with ACPA values support a central hypothesis in RA pathogenesis that specific microbial antigens in the mucosa, which may cross-react with like self-proteins (6), may shape immune responses in RA joints (1). ACPA appear to be beneficial in controlling microbes in the mucosa but may become detrimental in joints (1). In addition, we previously found *Prevotella* DNA in joint fluid in 3 of 5 patients with IgG *Pc* antibody responses (5), suggesting that *Pc* or their products may sometimes reach joints where they may further amplify inflammatory responses. There is a provocative, emerging literature about distant spread of commensal organisms resulting not only in autoimmunity, but also in malignancies and adverse treatment outcomes (11, 12).

Limitations of this study include the small number of patients in whom it was possible to test for HLA-DR-presented *Pc* peptides. However, this initial assessment shows that nano-LC-MS/MS is now sensitive enough to identify immunogenic *in vivo*-HLA-DR-presented microbial peptides directly from PBMC, which can then be tested in large numbers of patients. Second, the reasons for gut dysbiosis and mucosal *Pc* immunoreactivity are not yet defined in RA patients. However, in a recent analysis of ileal biopsies in 50 HLA-B27-positive patients with ankylosing spondylitis, adherent, invading rod-shaped bacteria, identified primarily as *E. coli* or *Prevotella sp.*, were often seen in the epithelial layer of the gut mucosa along with significant down-regulation of tight junction proteins, resulting in a loosening of the epithelial and gut vascular barriers (13). A similar process may occur in RA patients.

Greater understanding of the interactions between gut commensals and joint autoimmunity will likely influence the diagnosis and treatment of RA. In addition to DMARDs, adjunctive treatment aimed at the control of gut "pathobionts", such as targeted non-absorbable antibiotic therapy, probiotic strategies, dietary changes, or fecal matter transplants, may prove to be effective and safe. Moreover, the identification of T cell epitopes to microbial and related self-proteins may lead to therapies with blocking peptides. Animal models have shown that blocking peptides may ameliorate pathogenic responses (14), and reestablishment of tight junctions in the gut restores gut homeostasis, which may reverse autoimmune processes (15). In the future, biomarkers, such as those identified here, may contribute to the diagnosis and treatment of patients with gut-associated RA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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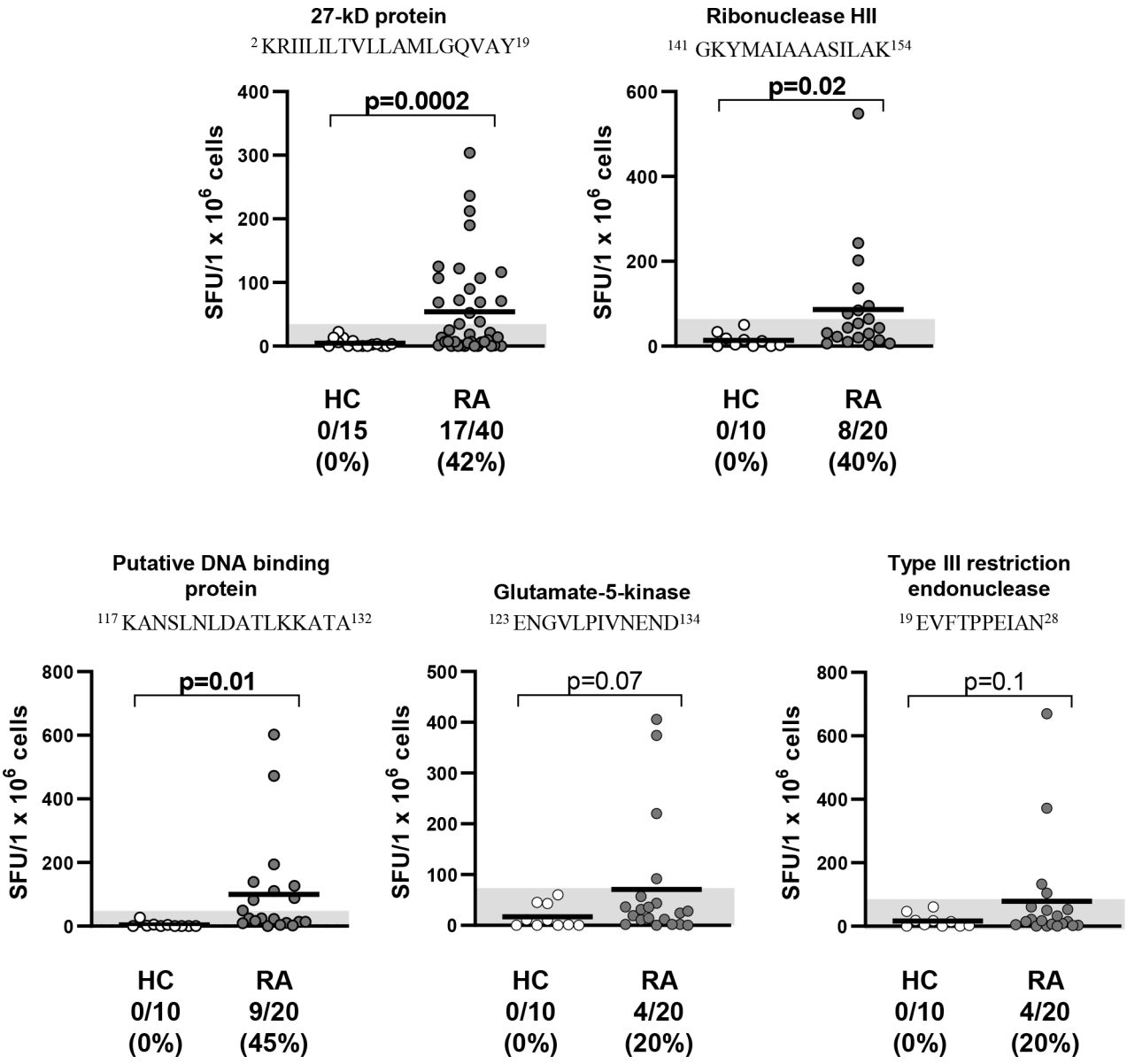

Figure 1. T cell responses to *P. copri* HLA-DR-presented peptides in RA patients.

Five peptides derived from 5 *P. copri* (*Pc*) proteins were synthesized and used to stimulate PBMC from RA patients by IFN-γ ELISpot assays. Using TEPITOPE, the *Pc*-p27, *Pc*-ribonuclease HII, and *Pc*-DNAbinding peptides were predicted to be promiscuous binders of ≥20 of the 25 HLA-DR molecules modeled in the program. The predicted binding of the *Pc*-glutamate-5-kinase and *Pc*-type III restriction endonuclease peptides was restricted primarily to DRB1*04 molecules. The superscript numbers around each peptide sequence show the location of the amino acids within the source protein. The horizontal bar represents the mean value, and the grey area shows >3SD above the mean value in healthy control subjects (hospital personnel). The groups were compared using unpaired t test with Welch correction. SFU = spot forming units per 1×10^6 cells. RA = rheumatoid arthritis, and HC = healthy controls.

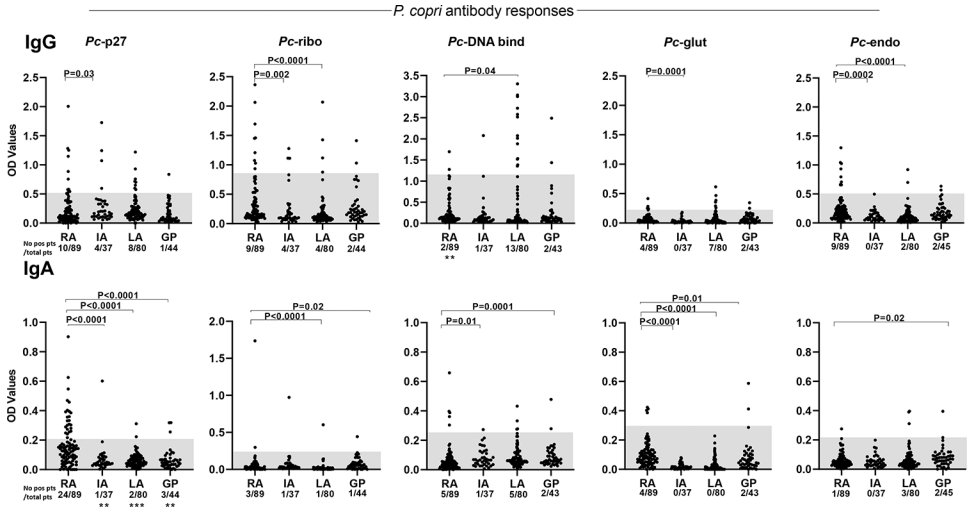

Figure 2. Antibody responses to *P. copri* proteins in RA patients, those with other forms of arthritis, and those in the general population.

IgG and IgA antibody responses to the 5 *Pc* proteins are shown. The shaded areas represent 2 SD above the mean value in the general population. Quantitative values were compared between RA patients and those in each of the other groups using Mann-Whitney test; the P values for these comparisons are shown above the data points. The number of individuals in each group with positive responses were compared between RA patients and those in each of the other groups by Fisher's exact test; P values for these comparisons are shown at the bottom of each panel (**=0.001 and ***=0.0001). Only significant P values are shown. OD = optical density, RA = rheumatoid arthritis, IA = other chronic inflammatory arthritides, LA = Lyme arthritis, and GP = general population.

Table 1:

Correlation of Demographic and Clinical Parameters with *Prevotella copri* Antibodies

Characteristic	*Pc*-p27 IgG (N=10)	*Pc*-p27 IgA (N=24)	No *Pc*-p27 IgA or IgG (N=55)	Any *Pc* IgG (N=26)	Any *Pc* IgA (N=31)	No *Pc* IgG/IgA (N=41)
Demographics						
Age, median, (range)	52 (24–75)	46 (19–91)	51 (19–80)	53 (24–75)	49 (19–91)	52 (19–75)
Sex, female/ male	5/5 ***P=0.03***	20/4	46/9	17/9 *P=0.1*	25/6	34/7
Smoking, n. (%)						
Current	1 (10)	1 (4)	8 (15)	3 (12)	2 (7)	6 (15)
Former	2 (20)	5 (21)	16 (29)	5 (19)	7 (23)	14 (34)
Never	7 (70)	18 (75) *P=0.1*	31 (56)	18 (69)	22 (71) *P=0.1*	21 (51)
Autoantibodies						
RF, n. positive (%)	5 (50)	11 (46)	19 (35)	13(50)	14 (45)	13 (32)
ACPA, n. pos. (%)	7 (70)	18 (75) *P=0.1*	30 (55)	18(69) *P=0.1*	23 (74) ***P=0.05***	20 (49)
HLA-DRB1 Alleles						
SE, n. patients pos./n. tested (%)	4/7 (57)	12/19 (63)	21/38 (55)	12/18 (67)	15/24 (63)	16/29 (55)
Disease Activity						
ESR, mm/hr, median (range)	22 (2–67)	23 (4–107)	14 (2–60)	21 (2–107) *P=0.08*	23 (4–107) *P=0.07*	12 (2–60)
CRP, mg/L, median (range)	16 (0.5–92)	3.7(0.1–126)	4.7 (0.2–96)	9.6 (0.5–126) *P=0.09*	7.7 (0.1–126)	4.3 (0.2–96)
DAS-28-ESR	4.5 (2.0–7.0)	3.6 (1.3–8.2)	3.3 (1.0–7.0)	3.7 (1.6–8.2)	3.7 (1.3–8.2) *P=0.1*	3.17 (1.0–7.0)
DAS-28-CRP	3.8 (2–7)	3.1 (1.5–7.6)	3.2 (1.1–6.8)	3.5 (1.3–7.6)	3.5 (1.1–7.6)	3.17 (1.1–6.8)

SE, shared epitope, alleles *0101, 0102, 0401, 0404, 0405, 0408, 1001; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibodies; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS-28, disease activity score-28.P values shown are for comparison with neither *Pc*-p27 IgA/IgG antibodies or Neither *Pc* peptide IgG/IgA group respectively.

Table 2:

Correlation of RA Autoantibodies with *Prevotella copri* Antibodies

	ACPA	Rheumatoid Factor
***Pc* Antibody IgG**		
Pc-p27 IgG	r = 0.116, p = 0.3	r = 0.086, p = 0.4
Pc-ribo IgG	r = 0.172, p = 0.1	r = 0.135, p=0.2
Pc-DNA bind IgG	r= 0.098, p=0.4	r= 0.026, p=0.8
Pc-glut IgG	r= 0.051, p=0.6	r= −.0752, p=0.5
Pc-endo IgG	r= −0.137, p=0.2	r= −.0631, p=0.6
***Pc* Antibody IgA**		
Pc-p27 IGA	**r = 0.283, p = 0.008**	r = 0.194, p= 0.07
Pc-ribo IgA	**r = 0.251, p = 0.018**	**r = 0.245, p = 0.02**
Pc-DNA bind IgA	r = 0.006, p = 0.95	r = 0.087, p = 0.42
Pc-glut IgA	**r = 0.271, p = 0.01**	**r = 0.260, p = 0.014**
Pc-endo IgA	r = 0.112, p = 0.3	**r = 0.212, p = 0.047**

ACPA, anti-citrullinated protein antibodies. Correlations were performed using a Spearman Test. Values in bold reflect statistically significant correlations.