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Disordered Antigens and Epitope Overlap Between Anti–Citrullinated Protein Antibodies and Rheumatoid Factor in Rheumatoid Arthritis

Zihao Zheng,[1] Aisha M. Mergaert,[1] Lauren M. Fahmy,[1] Mandar Bawadekar,[1] Caitlyn L. Holmes,[1] Irene M. Ong,[2] Alan J. Bridges,[3] Michael A. Newton,[1] and Miriam A. Shelef[3]

Objective. Anti–citrullinated protein antibodies (ACPAs) and rheumatoid factor (RF) are commonly present in rheumatoid arthritis (RA) without a clear rationale for their coexistence. Moreover, autoantibodies develop against proteins with different posttranslational modifications and native proteins without obvious unifying characteristics of the antigens. We undertook this study to broadly evaluate autoantibody binding in seronegative and seropositive RA to identify novel features of reactivity.

Methods. An array was created using a total of 172,828 native peptides, citrulline-containing peptides, and homocitrulline-containing peptides derived primarily from proteins citrullinated in the rheumatoid joint. IgG and IgM binding to peptides were compared between cyclic citrullinated peptide (CCP)–positive RF+, CCP+RF–, CCP–RF+, and CCP–RF– serum from RA patients (n = 48) and controls (n = 12). IgG-bound and endogenously citrullinated peptides were analyzed for amino acid patterns and predictors of intrinsic disorder, i.e., unstable 3-dimensional structure. Binding to IgG-derived peptides was specifically evaluated. Enzyme-linked immunosorbent assay confirmed key results.

Results. Broadly, CCP+RF+ patients had high citrulline-specific IgG binding to array peptides and CCP+RF– and CCP–RF+ patients had modest citrulline-specific IgG binding (median Z scores 3.02, 1.42, and 0.75, respectively; $P < 0.0001$). All RA groups had low homocitrulline-specific binding. CCP+RF+ patients had moderate IgG binding to native peptides (median Z score 2.38; $P < 0.0001$). The highest IgG binding was to citrulline-containing peptides, irrespective of protein identity, especially if citrulline was adjacent to glycine or serine, motifs also seen in endogenous citrullination in the rheumatoid joint. Highly bound peptides had multiple features predictive of disorder. IgG from CCP+RF+ patients targeted citrulline-containing IgG-derived peptides.

Conclusion. Disordered antigens, which are frequently citrullinated, and common epitopes for ACPAs and RF are potentially unifying features for RA autoantibodies.

INTRODUCTION

In rheumatoid arthritis (RA), autoantibodies are both pathologic (1–3) and diagnostic (4). Patients with RA produce a variety of anti–citrullinated protein antibodies (ACPAs) with overlapping reactivity (5–8) that underlie the diagnostic anti–cyclic citrullinated peptide (anti-CCP) antibody tests. They also generate rheumatoid factor (RF), antibodies of any isotype that bind to the Fc portion of IgG, which is also used for diagnosis. In RA, autoantibodies that target homocitrulline, known as anti-homocitrullinated

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[1]Zihao Zheng, BS, Aisha M. Mergaert, BA, Lauren M. Fahmy, BS, Mandar Bawadekar, PhD (current address: Invenra, Inc., Madison, Wisconsin), Caitlyn L. Holmes, BS, Michael A. Newton, PhD: University of Wisconsin–Madison; [2]Irene M. Ong, PhD: University of Wisconsin–Madison and University of Wisconsin Carbone Comprehensive Cancer Center; [3]Alan J. Bridges, MD, Miriam A. Shelef, MD, PhD: University of Wisconsin–Madison and William S. Middleton Memorial Veterans Hospital.

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Address correspondence to Miriam A. Shelef, MD, PhD, 4130 Medical Foundation Centennial Building, 1685 Highland Avenue, Madison, WI 53705. E-mail: mshelef@medicine.wisc.edu.

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protein antibodies (AHCPAs) or anti–carbamylated protein antibodies, are produced (9). There appears to be some cross-reactivity between AHCPAs and ACPAs (7, 10–12), but this issue has not been completely resolved. Moreover, RA patients produce autoantibodies against malondialdehyde-acetaldehyde adducted proteins (13) and acetylated proteins (14), suggesting that autoantibodies in RA may primarily bind posttranslationally modified proteins (15). However, native proteins can also be targeted in RA (16–18), and autoantibodies against posttranslationally modified proteins often coexist with RF. It remains unclear why these seemingly unrelated antigens are targeted in RA.

Although the majority of patients with RA generate ACPAs and RF, ~25% are seronegative for both CCP and RF (19). RA patients who are seronegative may lack autoantibodies in general or common autoantibodies for this subset simply may not have been discovered yet. Additionally, some patients are seropositive for only RF or CCP. Little is known about autoantibody reactivity in patients who are positive for one but not both of these antibodies. However, an understanding of autoantibodies in these groups could shed light on the spectrum of disease in RA. In this study, we used a high-density peptide array to evaluate autoantibodies against citrulline-containing peptides, homocitrulline-containing peptides, and native peptides in seropositive and seronegative patients in order to identify unifying and novel features of autoantibody reactivity in RA.

MATERIALS AND METHODS

Human subjects. Human subject research was carried out in compliance with the Helsinki Declaration and was approved by the University of Wisconsin Institutional Review Board. Serum from age- and sex-matched controls and RA patients were selected from the University of Wisconsin (UW) Rheumatology Biorepository as described previously (20,21). Briefly, RA patients were identified based on having ≥2 outpatient visits within 24 months at which RA-associated International Classification of Diseases codes were used (22), or 1 visit and a positive CCP test result. RA diagnosis was confirmed by manual review of the 3 most recent progress notes from a rheumatologist. Anti-CCP was assessed by generation II anti-CCP or anti-CCP3 enzyme-linked immunosorbent assay (ELISA) (Inova), and RF was assessed by latex or polystyrene agglutination in the UW clinical laboratory. RA patients were included in the following groups if CCP and/or RF titers were negative or were >2 times the upper limit of normal: CCP+RF+, CCP–RF+, CCP+RF–, and CCP–RF–. Controls were excluded if they had any of the following as determined by verbal screening and manual review of the medical record: RA, lupus, Sjögren's syndrome, scleroderma, multiple sclerosis, type 1 diabetes mellitus, psoriasis, spondyloarthropathy, inflammatory bowel disease, or hematologic malignancy. A total of 48 RA patients and 12 controls were included in array studies, and 40 CCP+RF+ RA patients and 40 controls were included in confirmatory ELISAs.

High-density peptide array. Twelve–amino acid peptides from 224 UniProt sequences (see Supplementary Table 1, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41074/abstract) for 122 unique proteins (including variants) were tiled at a 1–amino acid interval to generate an array (Roche NimbleGen), as previously described (23). The majority of selected proteins were previously found to contain ≥1 citrulline in the rheumatoid joint (24–26), with some family members of the proteins included as well as a few known targets of ACPAs (3,8,27). Peptides containing arginine or lysine were included as native peptides and as peptides with all arginines replaced with citrullines or all lysines replaced with homocitrullines. Redundant peptides (found in >1 protein) were included once. Peptides from 5 proteins were included in duplicate. Each chip contained 12 copies of the array, which included 172,828 peptides: 35,459 citrulline-containing peptides, 41,608 homocitrulline-containing peptides, and 95,761 native peptides. Sera were sent to Roche NimbleGen for processing in order to apply to the array for analysis to detect IgA, IgM, and IgG that bound to each peptide (23), with sera diluted 1:100 in binding buffer (0.01*M* Tris HCl [pH 7.4], 1% alkali-soluble casein, 0.05% Tween 20). Six serum samples were run in duplicate on different chips. Roche NimbleGen provided raw signal intensities and a key for placing redundant peptides into the appropriate proteins.

ELISA. High-binding ELISA plates (Costar 3590; Corning) were coated with 5 μg/ml of streptavidin (Thermo Scientific) in phosphate buffered saline (PBS), incubated overnight at 4°C, washed 3 times with 0.2% Tween 20 in PBS (wash buffer), and incubated overnight at 4°C with 20 μg/ml of C-terminal biotinylated peptides (Peptide 2.0) in PBS. Peptides were EIFDSRGNPTVEK-biotin and EIFDScitGNPTVEK-biotin derived from α-enolase, and VFPLAPCSRSTSK-biotin, VFPLAPCScitSTSK-biotin, KPREEQYNSTYRK-biotin, KPcitEEQYNSTYcitK-biotin, FLYSRLTVDKSRK-biotin, and FLYScitLTVDKScitK-biotin derived from IgG heavy chain. Next, at room temperature, plates were washed 3 times, blocked with 5% nonfat dry milk for 2 hours, incubated for 2 hours with serum diluted 1:100 in wash buffer with 5% milk, washed 4 times, incubated for 2 hours with horseradish peroxidase–conjugated goat anti-human IgG (SouthernBiotech) diluted 1:5,000 in wash buffer with 5% milk, washed 3 times, developed with 3,3′,5,5′-tetramethylbenzidine, and read at 450 nm with 540 nm correction. Absorbance values were normalized to blank wells and sample-specific uncoated wells. Positive results were in the linear range.

Statistical analysis. Computations on peptide array data were performed using built-in functions and custom scripts within the R system for statistical computing (28). Raw intensity levels were transformed using a double logarithm (log[log(x)]) to stabilize variation and improve sensitivity (29).

Comparisons between RA patients and control subjects were conducted using a novel distribution analysis that considers all quantitative scores and does not threshold to identify "positive" peptides. First, for the signal intensity of each peptide or peptide-matched pair (i.e., signal from citrulline-containing peptide minus signal from corresponding arginine-containing peptide), we computed the Student's *t* statistic to measure differential abundance between an RA group and controls. We converted each *t* statistic to a Z score, which is essentially the number of standard deviations for the RA group from controls, using the probability integral transform, and then presented each set of Z scores as an empirical cumulative distribution function (ecdf).

Statistical significance was determined by sample-label permutation followed by repeated regeneration of Z score ecdfs; a measure of distributional distance was computed each time between the synthetic ecdf and the null integrated bell curve (>10,000 permuted data sets). The same inference results were obtained for different distance measures: mean difference, Kullback-Leibler divergence, and Hellinger distance. Using the Monte Carlo testing on sets of peptides, we could infer large-scale differential antibody abundance between RA and control groups, while respecting potential dependencies between abundance measures in the same subject without heavy multiple-comparison penalties associated with single-peptide testing. Comparisons between ELISA data and array data or between the proportion of arginine or lysine and average binding were made using distance covariance (30). ELISA data were compared between groups using a Mann-Whitney test or Wilcoxon's matched pairs signed rank test, with *P* values less than 0.05 considered significant.

RESULTS

Broad evaluation of autoantibody binding in RA. A peptide array was designed with overlapping peptides primarily from proteins with ≥1 citrulline detected in the rheumatoid joint (24–26), including native peptides, peptides with all arginines replaced by citrullines, and peptides with all lysines replaced by homocitrullines. Sera from controls and RA patients, categorized into CCP+RF+, CCP+RF–, CCP–RF+, and CCP–RF– groups, were subjected to the array in order to quantify IgM and IgG binding to each peptide. As shown in Supplementary Table 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.41074/abstract), subjects were similar across groups. Additionally, the array methodology was accurate and reproducible as determined by cluster analysis, duplicate sample analysis, duplicate peptide analysis, and confirmatory ELISA (Supplementary Figures 1 and 2, http://onlinelibrary.wiley.com/doi/10.1002/art.41074/abstract).

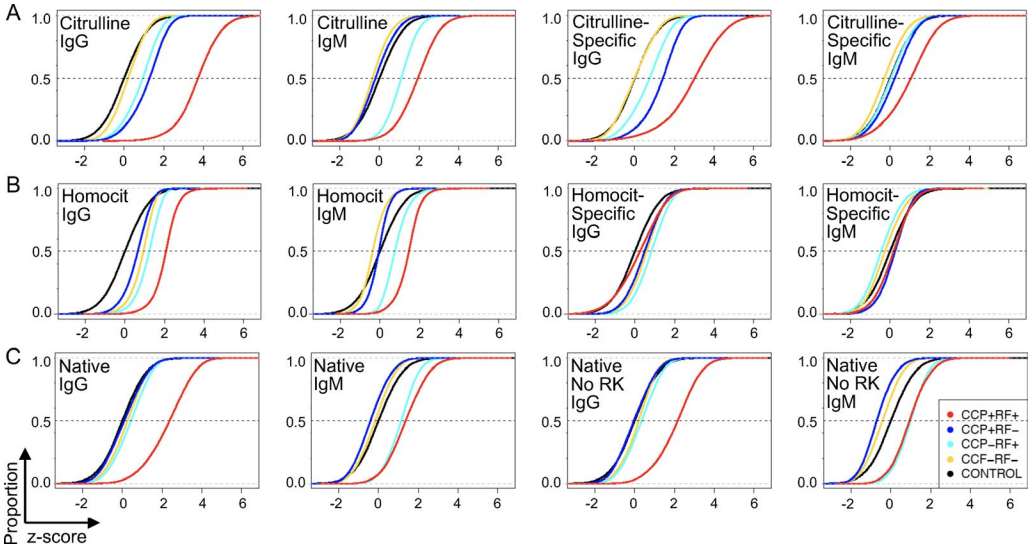

Figure 1. Broad patterns of antibody reactivity against peptides in rheumatoid arthritis (RA). IgG and IgM binding were measured in all samples for each peptide and the Z score for each peptide calculated for each RA group (cyclic citrullinated peptide–positive [CCP+] rheumatoid factor–positive [RF+], CCP+RF–, CCP–RF+, and CCP–RF–), compared to controls. The proportion of Z scores less than the specific Z value was graphed as an empirical cumulative distribution function (ecdf) plot. The median Z score for each RA group is where its ecdf curve crosses the dotted line at 0.5. Statistical significance was assessed by permutation analysis. **A**, Citrulline-containing peptides and citrulline-specific binding (binding values for citrulline-containing peptides minus corresponding arginine-containing peptides) (n = 35,459 peptides or peptide pairs). **B**, Homocitrulline (homocit)–containing peptides and homocitrulline-specific binding (binding values for homocitrulline-containing peptides minus corresponding lysine-containing peptides) (n = 41,608 peptides or peptide pairs). **C**, All native peptides and native peptides excluding peptides with arginine or lysine (n = 95,761 native peptides, n = 18,694 native peptides without arginine or lysine). For all panels, n = 12 samples per group; $P < 0.0001$ for all comparisons despite some small visual differences (except citrulline-specific IgM binding for CCP–RF+ versus control, which was not significant).

Following these confirmatory tests, we evaluated general antibody binding patterns in the 4 RA groups, first focusing on citrulline. As expected, CCP+RF+ patients had very high IgG binding to citrulline-containing peptides (Figure 1A). CCP+RF– and CCP–RF+ groups showed modest IgG binding. For IgM, the CCP+RF+ group had high binding to citrulline-containing peptides, with modest binding in the CCP–RF+ group. We next quantified citrulline-specific binding (binding to citrulline-containing peptides minus corresponding arginine-containing peptides) and found very high citrulline-specific IgG binding in the CCP+RF+ patients and modest citrulline-specific IgG binding in CCP–RF+ and CCP+RF– groups. For IgM, there was modest citrulline-specific binding in the CCP+RF+ group. We also performed similar analyses of IgA binding (Supplementary Figure 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41074/abstract). Although there appeared to be a small increase in citrulline-specific IgA binding in CCP+RF+ subjects, we could detect only low IgA binding in general, limiting our analyses. Notably, similar to most studies evaluating antibody binding in RA, in CCP+RF+ patients we could not determine how much detected binding was direct binding of Ig to citrulline-containing peptides versus indirect binding of RF to IgG that was bound to citrulline-containing peptides.

We next evaluated reactivity with homocitrulline-containing peptides (Figure 1B). CCP+RF+ subjects showed increased IgG binding to homocitrulline-containing peptides, with small increases in binding for the other 3 groups. For IgM binding, the CCP+RF+ group had an increase in binding, with a small increase in the CCP–RF+ group. Overall, there was low homocitrulline-specific IgM and IgG binding in all RA groups (i.e., binding to homocitrulline-containing peptides minus corresponding lysine-containing peptides).

Finally, we assessed binding to native peptides (Figure 1C). Moderate binding of IgG to native peptides in the CCP+RF+ group was observed, as well as modest IgM binding to native peptides in both RF+ groups. To reduce the possibility of cross-reactivity with citrulline- and homocitrulline-containing peptides, antibody binding to native peptides that did not contain arginine or lysine was assessed. Again, we observed moderate IgG binding to native peptides only in the CCP+RF+ group and modest IgM binding in both RF+ groups.

Collectively, these data suggest that citrulline is a major driver of autoantibody reactivity in our RA patients who were positive for both RF and CCP, as expected, with little specific reactivity against homocitrulline and moderate binding to native peptides. Moreover, seronegative subjects had little antibody binding in general, and RA patients with seropositivity for only RF or only CCP showed intermediate binding, primarily with citrulline.

A dominant target of IgG in RA is citrulline, irrespective of protein identity, particularly if citrulline is adjacent to glycine or serine. We next evaluated whether peptides from specific proteins were preferentially targeted in RA. We identified peptides bound by IgG at a level 10 times

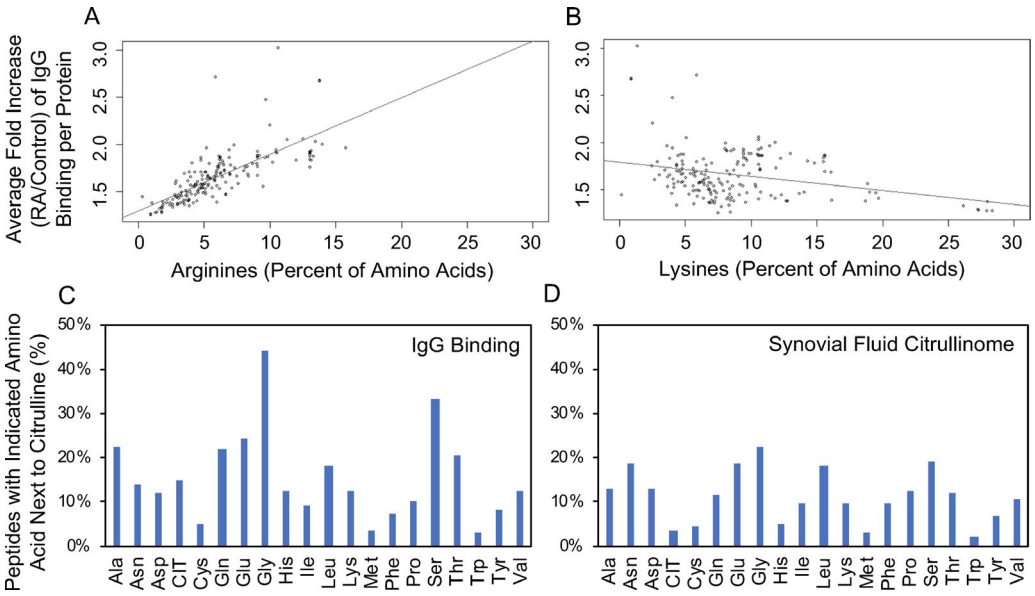

Figure 2. Amino acids that correlated with increased IgG binding in rheumatoid arthritis (RA). **A**, The average fold increase in IgG binding for RA versus control sera was determined for each peptide (including native, citrulline-containing, and homocitrulline-containing peptides) and was then averaged for each protein to generate the average fold increase of IgG binding per protein. The percentage of amino acids in each protein encoded as arginine was then compared to the average fold increase of IgG binding per protein ($r = 0.74$, $P < 0.0001$, by Pearson's correlation). **B**, The average fold increase of IgG binding per protein was compared to the percentage of amino acids in each protein encoded as lysine. **C**, The percentage of peptides that contain each indicated amino acid next to citrulline was determined for all citrulline-containing peptides for which there was >10 times more IgG binding in RA compared to control. **D**, The percentage of peptides that contain each indicated amino acid next to citrulline was determined for citrulline-containing peptides reported in the rheumatoid joint. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41074/abstract.

greater in RA than in controls (Supplementary Table 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41074/abstract) and found that every protein whose peptides were included in the array had ≥1 peptide that met this criterion. Moreover, every peptide with >10 times increased binding in RA (9,098 unique peptides) contained citrulline except for 2 native peptides from fibrinogen, which lacked arginine. Some proteins had more peptides highly bound by IgG than others. Given the high reactivity with citrulline overall in our subjects (Figure 1), we determined whether the amount of arginine in a protein (citrullines in our array) correlated with the level of IgG binding for its peptides. We assessed the fold increase in IgG binding for each peptide in the RA group versus controls. We then averaged the fold increase in IgG binding for all peptides in each protein and compared this average to the percent of amino acids in each protein encoded as arginine. There was a strong correlation between the proportion of encoded arginines and the binding of serum IgG in RA (Figure 2A). In contrast, there was no positive correlation between the proportion of encoded lysines and the binding of IgG in RA (Figure 2B). Taken together, these data suggest that a major determinant of IgG binding in RA is citrulline, not protein identity, similar to findings on monoclonal antibodies derived from patients with RA (7).

We then determined whether there was a motif related to high IgG binding in our RA sera. Using MEME software (31) and the highly bound citrulline-containing peptides mentioned above, no strong motifs could be identified. Rather, certain amino acids appeared frequently in multiple minimal motifs. Thus, we verified how often each amino acid was adjacent to citrulline in the highly bound peptides. Some amino acids, most strikingly glycine and serine, were frequently next to citrulline in highly bound peptides (Figure 2C), suggesting either a preference for antibody development against citrullines next to these amino acids or preferred citrullination of arginines next to these amino acids. Of note, these amino acids were not simply more commonly encoded next to arginine, since the lowest bound citrulline-containing peptides by IgG did not have a high frequency of glycine or serine next to citrulline.

To differentiate between antibody development and citrullination preference, we evaluated 451 citrulline-containing peptides detected in rheumatoid synovial fluid (24–26) and determined the percentage of peptides that contained each amino acid next to citrulline. The citrullinome peptides differed

Table 1. Native peptides (without arginine or lysine) with >2 times higher IgG binding in rheumatoid arthritis sera than in control sera

Protein	Peptides (distance from disorder, no. of amino acids)	Disordered amino acids/total amino acids (%)
Apolipoprotein B-100	IPDFDVDLGTIL (23), VGINGEANLDFL (8), GINGEANLDFLN (9), INGEANLDFLNI (10), NGEANLDFLNIP (11)	371/4,563 (8)
Ceruloplasmin	SSTVTPTLPGET (0)	183/1,065 (17)
Collagen α2 (I) chain	SGGGYDFGYDGD (0)	471/1,366 (34)
Complement C4a/b	PMPQAPALWIET (1), EANEDYEDYEYD (0), QLNDFLQEYGTQ (0), LNDFLQEYGTQG (0)	219/1,744 (13)
Complement C5	DLGCGAGGGLNN (0), LGCGAGGGLNNA (0), GCGAGGGLNNAN (0), CGAGGGLNNANV (0)	217/1,676 (13)
Complement C7	INNDFNYEFYNS (3), NNDFNYEFYNST (2), INNNPEFLQLAE (16)	88/843 (10)
Complement C8 α chain	DAQSVYDASYYG (24), GISSEFYDNAND (3), ISSEFYDNANDL (2), SSEFYDNANDLL (1)	115/584 (20)
Complement factor B	ETIEGVDAEDGH (0)	24/764 (3)
Coronin-1A	YPPTAGPDPALT (22)	52/461 (11)
Fibrinogen γ chain	CEIDGSGNGWTV (67), GDAFDGFDFGDD (98), DAFDGFDFGDDP (97), AFDGFDFGDDPS (96), FDGFDFGDDPSD (95)	63/453 (14)
Fibronectin	TSLSAQWTPPNV (15)	487/2,386 (20)
High mobility group protein B1	EDEEDEEDEEEE (0), EDEEDEEEEEDE (0), EDEEEEEDEEDE (0), EDEEDEDEEEDD (0)	74/215 (34)
High mobility group protein B2	EDEDEEEEDEDE (0)	70/209 (33)
Histone H2A	GGVLPNIHPELL (4)	87/372 (23)
IgM heavy chain	SILTVSEEEWNT (8)	54/453 (12)
Inter-α trypsin inhibitor heavy chain H4	APPATSNPDPAV (0)	268/930 (29)
Myeloid cell nuclear differentiation antigen	SSVSDFNQNFEV (52)	143/407 (35)
Plasminogen	QGEPLDDYVNTQ (13)	35/810 (4)
Tenascin	VEWDPLDIAFET (9), LDGPSGLVTANI (2)	486/2,201 (22)
Vinculin	DDYEPELLLMPS (0)	294/1,134 (26)
Zinc α2–glycoprotein	DIVEYYNDSNGS (36), VVVAVPPQDTAP (16), VVAVPPQDTAPY (15), VAVPPQDTAPYS (14), AVPPQDTAPYSC (13), VPPQDTAPYSCH (12)	17/298 (6)

from the array peptides in that the citrullinome included all peptides with detected endogenous citrullines, whereas the highly bound array peptides were derived from a subset of proteins identified in the citrullinome with all arginines replaced by citrullines. We found that the overall pattern of amino acids frequently found next to endogenous citrulline was roughly similar to the pattern of amino acids frequently found next to citrulline in highly bound peptides (Figure 2D). Again, both glycine and serine were commonly next to citrulline. Taken together, these data suggest that arginine next to serine or glycine is frequently citrullinated and frequently targeted by IgG in RA.

Multiple features of intrinsic structural disorder in peptides bound by IgG in RA. Given the moderate binding of native peptides observed in our RA subjects, we further evaluated these peptides. We identified native peptides that were >2 times more highly bound by IgG in RA patients as compared to controls, excluding peptides with arginine or lysine to reduce potential effects from cross-reactivity with citrulline- or homocitrulline-containing peptides. As shown in Table 1, a total of 49 peptides met these criteria. Interestingly, several of the proteins from which the peptides were derived were related to complement. Additionally, many of the peptides contained repeated amino acids or repeated short motifs, hallmarks of intrinsically disordered regions of proteins, i.e., regions that lack stable 3-dimensional structure. We were intrigued by this observation, since arginine-glycine and arginine-serine, which we determined were minimal motifs for both citrullination and autoantibody binding in RA (Figure 2), are found in intrinsically disordered regions of proteins (32,33). Thus, we verified whether bound native peptides were located in disordered regions by identifying regions of predicted disorder in the parent proteins, using the Protein DisOrder prediction System (PrDOS) (34). Thirty-five percent of the native peptides that were bound >2 times more in RA patients than in controls were predicted to be within disordered regions. In contrast, only 18% of the 49 native peptides with the least binding in RA were in disordered regions.

We also calculated the median distance from disorder for the 49 highest binding and lowest binding native peptides, resulting in 4 and 32 amino acids, respectively ($P = 0.0003$). Finally, PrDOS predicted that 67% of the 100 highest bound citrulline-containing peptides were in regions of disorder compared to only 38% of the lowest bound citrulline-containing peptides, with median distances from disorder of 0 and 12 amino acids, respectively ($P < 0.0001$).

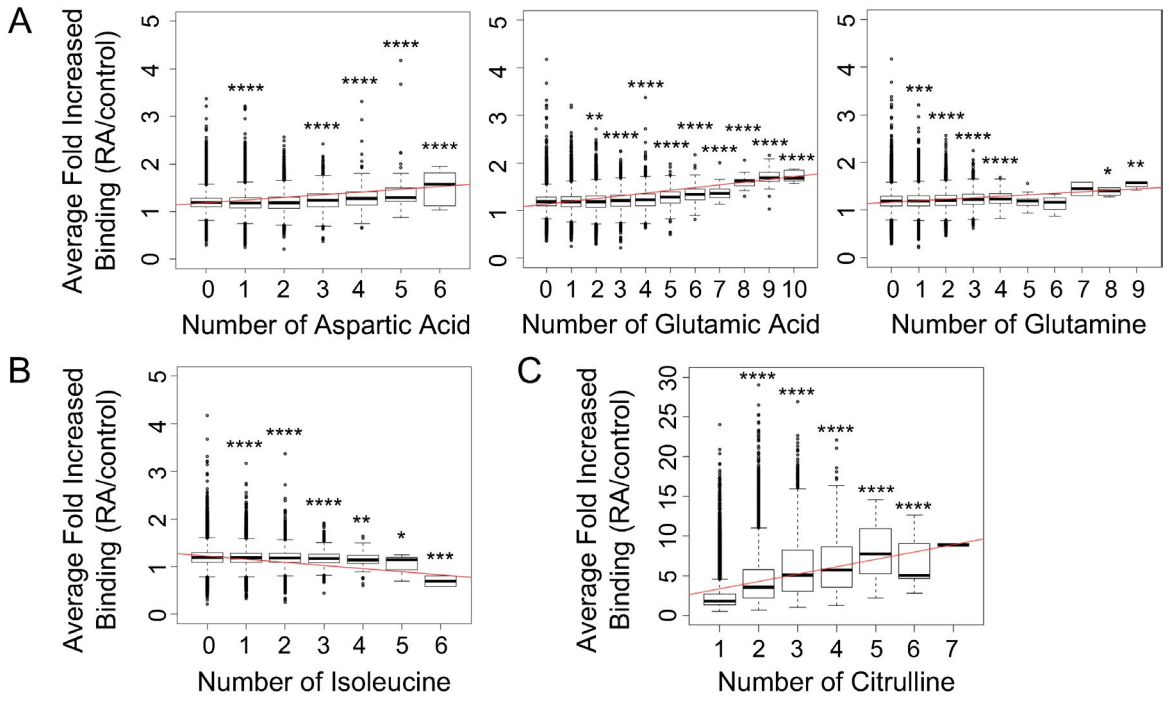

Figure 3. IgG in rheumatoid arthritis (RA) targets peptides with amino acids associated with intrinsic disorder. Peptides were grouped by the number of specific amino acids present, and the average fold increase in binding for all RA patients compared to controls was plotted for each peptide group. Results are shown for amino acids in native peptides (n = 95,761) that demonstrated a real increase (**A**) or reduction (**B**) in IgG binding or a real increase in IgG binding for citrulline-containing peptides (**C**) (n = 35,459) with an increasing number of that amino acid, in RA patients (n = 48) compared to controls (n = 12). The difference in binding for each peptide group was compared to peptides in which that amino acid was not present (i.e., the 0 group), or in the case of citrulline-containing peptides, compared to a single citrulline present (i.e., the 1 group). Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$, by *t*-test. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41074/abstract.

Intrinsically disordered regions also tend to contain arginine, glycine, lysine, glutamine, serine, glutamic acid, aspartic acid, and proline (32,35). To evaluate whether specific amino acids in peptides correlated with IgG binding in RA, we quantified the average binding of IgG in RA patients versus controls for native peptides with different amounts of each amino acid. We found that with increased representation of most amino acids in peptides, there was no real change in IgG binding in RA. However, higher IgG binding to peptides in RA was observed with increasing numbers of aspartic acid, glutamic acid, and glutamine (Figure 3A). Smaller increases in IgG binding in RA were seen with increasing numbers of glycine or proline (data not shown). Interestingly, increasing representation of isoleucine, which is associated with ordered protein regions (35), was associated with reduced IgG binding in RA (Figure 3B). We performed a similar analysis with citrulline-containing peptides. We found a dramatic increase in IgG binding in RA patients, compared to controls, with increasing citrulline content (Figure 3C), which vastly overshadowed the effects of any other amino acid. Considered together, these data suggest that peptides in regions of intrinsic structural disorder are targeted in RA.

IgG binding of citrulline-containing peptides derived from IgG in patients with CCP+RF+ RA. One of the most well-known antigens in RA is the Fc portion of IgG, the target of RF. Native peptides derived from IgG heavy chain were not identified as bound by IgG >2 times more in RA patients than in controls (Table 1), potentially because known RF epitopes are discontinuous (36) and not included in our array, or because bound epitopes contain arginine or lysine, which were excluded from the analysis. Therefore, we examined the recognition of peptides derived from IgG more closely. RF+ patients showed modest IgM binding to all peptide types derived from the constant region of IgG1 heavy chain (Figure 4A), with similar binding to peptides derived

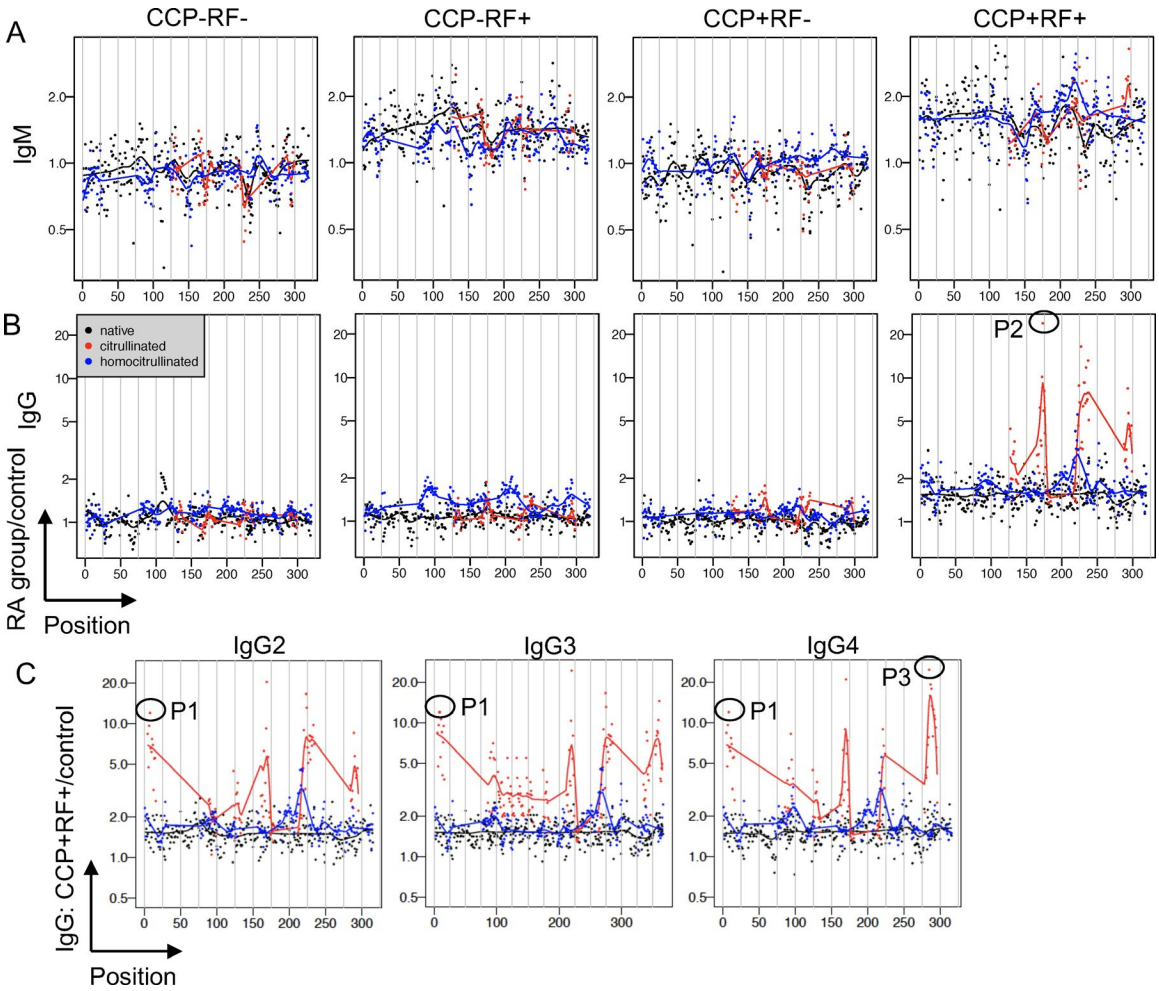

Figure 4. In RA, IgM binds IgG-derived peptides at a low level, and IgG binds primarily citrulline-containing IgG peptides at a high level. **A** and **B**, Binding of IgM (**A**) and IgG (**B**) in RA patients, in relation to controls, is shown for each peptide according to its position in the constant region of IgG1. RA patients were categorized as CCP–RF–, CCP–RF+, CCP+RF–, and CCP+RF+ (n = 12 per group). **C**, IgG binding in CCP+RF+ RA patients, in relation to controls, is shown for each peptide of the constant regions of IgG2, IgG3, and IgG4 (n = 12). Peptides (native and citrulline-containing versions) circled in **B** and **C** were evaluated by enzyme-linked immunosorbent assay as described in Figure 5. See Figure 1 for definitions.

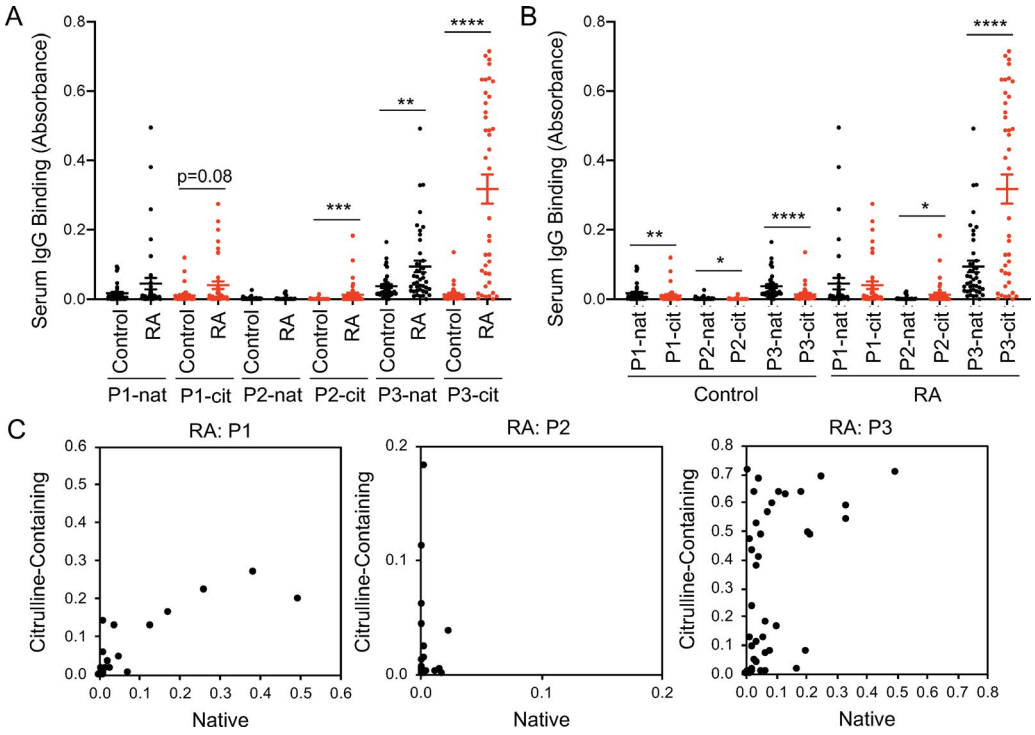

Figure 5. IgG binds to citrulline-containing peptides of IgG in RA. Peptides (native [nat] and citrulline-containing [cit] versions) circled in Figure 4 were used in an enzyme-linked immunosorbent assay to detect IgG binding in CCP+RF+ RA patient sera (n = 40) and control sera (n = 40). **A**, Results in RA patients versus controls were compared. **B**, Results for native versus citrulline-containing peptides were compared. **C**, For each subject, IgG binding is shown for the citrulline-containing peptide versus the native peptide. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$, by Mann-Whitney test (**A**) or Wilcoxon's matched pairs signed rank test (**B**). See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41074/abstract.

from IgG2, IgG3, and IgG4 (data not shown). IgG binding in CCP–RF+ RA patients compared to controls was modestly greater for homocitrulline-containing than citrulline-containing or native peptides derived from IgG1 ($P < 0.0001$; Figure 4B), similar to IgG2, IgG3, and IgG4 (data not shown). More dramatically, the CCP+RF+ group demonstrated very high IgG binding predominantly to citrulline-containing peptides in the constant region of IgG1 (Figure 4B), with similar results for peptides derived from IgG2, IgG3, and IgG4 (Figure 4C).

Next, we selected 3 highly bound IgG-derived peptides with strong sequence homology across IgG isoforms and quantified IgG binding to those peptides by ELISA using a larger number of subjects. For 1 native and 2 citrulline-containing peptides, CCP+RF+ sera showed increased IgG binding compared to controls (Figure 5A). For the 1 native peptide with increased IgG binding in CCP+RF+ sera, binding to the citrulline-containing peptide was >3 times greater than the native version in RA (Figure 5B). Additionally, for individual RA patients, IgG binding to 2 of the 3 IgG-derived peptides appeared to favor citrulline (Figure 5C). Interestingly, although there was overall low binding to IgG-derived peptides in controls, controls had slightly higher binding to native peptides than citrulline-containing peptides (Figure 5B). Collectively, these data suggest that IgG can target citrulline-containing peptides of IgG in RA, identifying epitopes that could be bound by an antibody defined as an ACPA or an RF.

DISCUSSION

Using a high-density peptide array and 4 serogroups of RA, we revealed interesting features of RA autoantibody reactivity. First, and not surprisingly, we found that citrulline was a dominant autoantibody target in the polyclonal repertoire of RA. The highest level of IgG binding in RA was to citrulline-containing peptides, and the encoded arginine content (citrullines on our array) directly correlated with average peptide IgG binding for each protein. Also, ≥1 citrulline-containing peptide from every protein on the array was bound by IgG. These findings are consistent with a similar lack of protein specificity reported for monoclonal autoantibodies in RA using a different set of peptide antigens (7), as well as with observations that ACPAs are cross-reactive (5–8) and recognize citrulline largely independently of surrounding peptides (37). Thus, our data support the theory that citrulline itself is a major driver of IgG reactivity in RA.

Our evaluation of homocitrulline reactivity revealed primarily nonspecific homocitrulline binding, and none of the peptides that were most highly bound by IgG contained homocitrulline. These

results suggest that homocitrulline may not be targeted to as great an extent as citrulline, consistent with the finding that AHCPAs have lower avidity than ACPAs (38). However, any homocitrulline-specific binding dependent on a nearby citrulline or other modified amino acid would not have been detected, since our peptides contained either citrulline or homocitrulline, but not both. Additionally, if homocitrulline-containing epitopes are primarily discontinuous, they would not have been detected using our array of linear peptides. However, homocitrulline-containing peptides were bound by rheumatoid monoclonal antibodies using a similar array (7). Also, homocitrullination increases structural disorder (39) and thus increases the likelihood of linear epitopes. Importantly, homocitrulline reactivity may change over time in subjects (7), causing variability among studies. Future experiments with additional subjects, modifications, and time points may clarify this issue.

We also observed moderate IgG binding to native peptides in CCP+RF+ patients. Unlike IgM binding, which was increased against native peptides in both RF+ groups, moderate IgG binding of native peptides was absent in CCP–RF+ patients and was thus unlikely due to promiscuity of RF. Furthermore, the IgG binding was unlikely due to cross-reactivity against citrulline- and homocitrulline-containing peptides, given a similar increase in IgG binding in CCP+RF+ patients when peptides with arginine or lysine were excluded. IgG binding to native peptides could be due to an unknown endogenous posttranslational modification resulting in cross-reactivity with native peptides in the array. For example, increased glutamic acid, which can mimic phosphoserine, was associated with increased IgG binding to native peptides in our array (Figure 3). If rheumatoid autoantibodies target phosphoserine in vivo, we might have simply detected cross-reactivity with glutamic acid with our array peptides. Alternatively, autoantibodies in RA may commonly target a host of native antigens as part of a polyclonal response and/or polyreactive autoantibodies.

Upon further characterization of citrulline reactivity in our experiments, we detected a preference for glycine or serine next to citrulline in highly bound peptides as well as next to endogenous citrullines in the rheumatoid joint, suggesting that these amino acid pairs may be commonly targeted by autoantibodies due to their common presence in antigens. There were some differences between the patterns of amino acids next to citrulline in the citrullinome and highly bound peptides that could reflect differences in the efficacy of major histocompatibility complex, B cell, or T cell receptor binding, other factors in generating an immune response, or simply sample size. However, our findings related to glycine are consistent with the findings of others. The presence of glycine next to citrulline has been observed in the citrullinome of 30 human tissues (40), in motifs targeted by the citrullinating peptidylarginine deiminases 2 and 4 (41–43), and in autoantibody binding in RA (7,44,45). The preference for citrullination of or IgG binding to peptides with citrulline next to serine has not been previously described. Perhaps the unique features of our subject groups, analyses, or array allowed for this novel finding. Interestingly, arginine-serine and arginine-glycine-glycine repeats are commonly found in disordered regions of proteins (32,33). Moreover, disordered regions of proteins are citrullinated to a higher degree than ordered regions, and citrullination increases disorder (39). Taken together, these observations suggest that disorder, citrullination, and the highest affinity autoantibodies in RA are profoundly linked.

We obtained additional evidence that autoantibodies in RA target regions of intrinsic disorder. Native and citrulline-containing peptides bound by IgG in RA were more likely to be found in regions of disorder than poorly bound peptides. Moreover, increased content of amino acids associated with intrinsic disorder (32,35) correlated with increased IgG binding in RA. Similarly, in lupus, approximately one-third of characteristic autoantigens contain stretches of charged amino acids (46), which is common in disordered regions, and nuclear autoantigens are often disordered proteins (47). Furthermore, although many examples of structured epitopes exist, epitopes predicted to be within regions of disorder were slightly more likely to be recognized by antibodies, compared to epitopes in ordered regions in a wide range of normal and pathologic conditions (48,49). We were unable to directly compare the binding in disordered versus ordered regions, since ordered epitopes are often discontinuous and thus were not present on our array. However, our findings provide a body of evidence that autoantibodies in RA bind intrinsically disordered regions, which are frequently citrullinated (39), a concept that has potential importance regarding to the basis of autoantibody development in RA. Of note, it has been hypothesized that repetition and charge lead to autoantibody reactivity (50). However, in RA, the charge may not be the driving factor as charge is reduced with citrullination. Perhaps disorder is the main driver due to the high degree of accessibility of intrinsically disordered regions of proteins (51) or due to bound DNA or RNA activating Toll-like receptors.

Since the binding of citrulline-containing and native peptides was predominantly seen in CCP+RF+ RA, many of our findings related to targeting disordered regions may predominantly apply to this group. However, our subject selection also allowed for an evaluation of antibody binding in seronegative RA and in RA patients positive for only CCP or RF. Despite our evaluation of 178,828 peptides, seronegative patients showed little binding to citrulline-containing or native peptides, suggesting minimal autoantibody presence. Interestingly, IgG binding to homocitrulline-containing peptides was greater in CCP–RF– patients than controls, suggesting that homocitrulline could be the basis for an improved diagnostic test for seronegative RA. However, despite using multiple models, we have not yet identified any peptides predictive of an RA diagnosis in seronegative patients. At least a subset of seronegative RA may be truly seronegative, lacking autoantibodies in general. An absence of autoantibodies in seronegative disease might suggest a different disease pathophysiology than seropositive disease, given evidence that RF and ACPAs are pathologic (1,2). Alternatively, RA may exist on a continuum.

In support of this idea, we observed that levels of citrulline-specific IgG in CCP+RF– and CCP–RF+ disease fell between levels in CCP+RF+ and CCP–RF– disease. Future studies with even larger arrays that are not limited by protein selection bias or by linear epitopes, or perhaps arrays incorporating additional posttranslational modifications, combinations of posttranslational modifications, or full-length proteins, may resolve these issues.

Our final observation was that IgG from CCP+RF+ sera targets citrulline-containing peptides of IgG. This finding raises the possibility that IgG-RF (IgG that binds IgG) could also be an ACPA, making citrullinated IgG, which has been reported in humans (24,26,52), a linchpin connecting ACPAs and RF in RA. IgM did not preferentially target citrulline-containing peptides of IgG. IgM-RF, which also occurs in healthy individuals, may arise independently of citrulline due to smoking or pathogens, whereas the development of IgG-RF, which requires a break in tolerance and T cell help, may be driven by targeting citrulline, like other ACPAs (53). However, it is not known whether the IgG we detected originally developed against citrullinated IgG, or whether the observed targeting of citrulline-containing IgG peptides was due to cross-reactivity of ACPAs. Also, not all IgG that bound IgG-derived peptides targeted citrulline (Figure 5), potentially indicating different mechanisms for the break in tolerance for generating IgG-RF (53). Finally, despite having a unique name for its autoantibody, IgG may be similar to other proteins in RA, with native epitopes targeted, but less so than citrulline-containing epitopes. It will be important for future work to clearly define the reactivity of RF in RA and diseases with RF that do not have a citrulline bias, in order to shed light on disease pathophysiology as well as to fully understand and optimize RF-based diagnostic tests.

In summary, using the 4 serogroups of RA and a high-density peptide array, we demonstrated very strong autoantibody targeting of citrulline, particularly if adjacent to glycine or serine, as well as a moderate binding to native peptides primarily in CCP+RF+ RA and very little antibody binding in seronegative disease. Furthermore, we identified disorder as a feature of IgG-targeted peptides and epitope overlap between ACPAs and RF.

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. Shelef 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.

Study conception and design. Bridges, Shelef.
Acquisition of data. Mergaert, Fahmy, Bawadekar, Shelef.
Analysis and interpretation of data. Zheng, Mergaert, Fahmy, Bawadekar, Holmes, Ong, Bridges, Newton, Shelef.

ADDITIONAL DISCLOSURES

After these experiments were completed, but before manuscript submission, author Bawadekar became employed by Invenra, Inc.

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