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Identification of Clinically and Pathophysiologically Relevant Rheumatoid Factor Epitopes by Engineered IgG Targets

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Objective. Rheumatoid factors (RFs), which are anti-IgG autoantibodies strongly associated with rheumatoid arthritis (RA), are also found in other diseases and in healthy individuals. RFs bind to various epitopes in the constant (Fc-) domain of IgG. Therefore, disease-specific reactivity patterns may exist. This study was undertaken in order to develop a new approach to dissecting RF epitope binding patterns across different diseases.

Methods. We analyzed RF reactivity patterns in serum from patients with seropositive arthralgia, patients with RA, and patients with primary Sjögren's syndrome (SS) using bioengineered, natively folded IgG-Fc targets that demonstrated selective RF binding toward several distinct regions of the IgG-Fc domain.

Results. Rheumatoid factor responses primarily bound the Fc Elbow region, with a smaller number of RFs binding the Fc Tail region, while the Fc receptor binding region was hardly targeted. A restricted reactivity against the IgG-Fc Tail region was associated with less positivity for anti–citrullinated protein antibodies (ACPAs) and less arthritis development in arthralgia, whereas combined reactivity toward IgG-Fc Tail and Elbow regions was associated with more arthritis development. Reactivity toward the IgG-Fc Tail region was observed far more frequently in RA than in primary SS.

Conclusion. Bioengineered IgG targets enable serologic characterization of RF reactivity patterns, and use of this approach appears to reveal patterns associated with ACPA detection and arthritis development in patients with arthralgia. These patterns are able to distinguish RA patients from primary SS patients. This new methodology improves the clinical value of RFs and our understanding of their pathophysiologic processes.

INTRODUCTION

Rheumatoid factors (RFs) are the first autoantibody response identified and associated with pathologic changes in autoimmune diseases. RF antibodies were discovered in 1937 by Erik Waaler, who observed that red blood cells from sheep sensitized with rabbit anti-sheep serum agglutinated after adding serum from a patient with rheumatoid arthritis (RA) (1,2). RFs were later identified as autoantibodies that bind to other antibodies, specifically the constant domain (or Fc domain) of IgG (3,4) and can be of any isotype (5). The most extensively studied isotype is IgM RF, and it is present in ~70% of RA patients, with prevalence varying widely between studies (6). However, IgM RFs are also found in other autoimmune conditions, including primary Sjögren's syndrome (SS), as well as in Waldenström's macroglobulinemia, during chronic infections such as viral hepatitis, and even at low frequencies (increasing with age) in the healthy population (5,7–9). Testing for the presence of RFs is standard practice in the diagnostic

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evaluation of individuals suspected of having RA and is included in the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for RA (10), as is testing for anti–citrullinated protein antibodies (ACPAs), the other major class of autoantibodies in RA. Establishing RF status is not just important for diagnosing RA, but also for predicting the development of the disease in individuals at risk for developing RA and predicting disease course in RA patients (11–15).

Surprisingly, despite its strong link to RA, a mechanism for how RF contributes to pathologic changes has thus far not been established. A current hypothesis is that RFs in the joint enhance complement activation and production of proinflammatory cytokines by macrophages through complex formation with ACPA IgGs and non-ACPA IgG molecules (16,17). In healthy individuals, RFs may play a physiologic role in binding and clearing immune complexes (ICs). These physiologic RFs are thought to arise from so-called "natural antibodies," ill-defined low-affinity IgM antibodies that supposedly act as a first line of defense against various pathogens and reduce inflammation by clearing debris created by dead cells (18,19). Whether RFs that contribute to pathologic changes in the preclinical and clinical stages of RA arise from this pool of natural antibody–like RFs, or separately through an antigen-driven immune response induced by ACPA ICs, is currently unknown.

To measure RFs, commercially available RF assays used in clinical laboratories quantify agglutination of IgG-coated particles by RF or detect IgM RFs binding to immobilized IgG with isotype-specific antibodies. However, it is known that even in an individual patient, RFs are a heterogeneous pool of autoantibodies binding to multiple epitopes on the Fc region of human IgG (20–22). Studies have identified different RF reactivity patterns by, for example, testing reactivity against the 4 IgG subclasses, which differ slightly in their Fc region at the amino acid level (23,24). Specifically, IgG3-reactive RF responses were suggested to represent more pathogenic responses in RA (25,26). From analyzing single B cell clones isolated from RA patients, as well as cells from healthy immunized donors and individuals with Waldenström's macroglobulinemia, it was found that RFs derived from RA patients more often showed "pan" reactivity, i.e., reactivity toward all 4 subclasses (20,21). Furthermore, monoclonal RFs with similar overall binding characteristics were found to display subtly different fine specificities. In other words, these studies indicate more heterogeneity in reactivity of RA-derived RFs compared to RFs in healthy donors or in individuals with other diseases. If the RF response could be dissected into multiple individual reactivities, disease-specific RFs or RF reactivity patterns might be identified. Despite its great potential, characterization of RF reactivity patterns directly in serum has thus far not been explored as a method to improve the clinical value of RF assays.

A major obstacle in assessing RF reactivity patterns in sera is the requirement of target molecules to which a single type of RF would bind at a time. Earlier studies have attempted epitope mapping using linear peptide fragments (27,28), but such an approach cannot identify discontinuous epitopes, which require correctly folded proteins and form the overwhelming majority of protein epitopes (29). For the analysis of RF repertoires directly in sera, individual IgG targets with correctly folded epitopes, to which only a specific portion of the RFs will bind, are required.

In detailed studies on monoclonal RF binding, several individual molecular determinants have been identified as being important for RF binding. The "Ga" reactivity pattern, the first reactivity pattern described (30), is characterized by binding of RF to IgG1, IgG2, and IgG4, with low or absent binding to IgG3. It was shown that the histidine (H) amino acid residue located at position 435 (H435) in IgG1, IgG2, and IgG4 is a crucial determinant in the epitopes bound by many (monoclonal) RFs with Ga reactivity (21). Other amino acids in the CH_2 and/or CH_3 domains of IgG were also found to be important for the binding of monoclonal RFs showing a Ga binding pattern, and the contributing amino acids can differ between RFs (21). However, knowledge of a few determinants that are important for RF binding is in itself insufficient to arrive at a method that could identify clinically relevant RF reactivity patterns.

In this work, we present a novel approach to elucidating the RF response and its epitopes in more detail, in order to identify clinically relevant RF binding patterns in different patient cohorts. For this goal, we designed a human IgG molecule with minimal residual RF binding, which formed the basis for a set of targets wherein RF binding was essentially confined to a specific region of the Fc domain. These IgG targets were used to directly evaluate the repertoire of polyclonal RF responses in sera from patients with RA, seropositive individuals with arthralgia at risk of developing RA, and individuals with primary SS. Our results illustrate that this approach is feasible and that certain patterns of reactivity are associated with RA and with the detection of ACPAs and the development of arthritis in individuals with arthralgia. The improved characterization of the RF response using our new methodology may enhance our understanding of the pathophysiologic roles of RF in various diseases (for example, by assessing IC formation). Furthermore, our findings can be used to improve RF assays by refining the ability to select clinically relevant RFs.

PATIENTS AND METHODS

Clinical settings and patients. Serum samples were collected from 4 different patient groups. In the first patient group, 639 baseline serum samples were obtained from patients with seropositive arthralgia in the Reade cohort, which has been enrolling patients since 2004 who have a history of arthralgia and who have tested positively for IgM RFs and/or ACPA IgGs (see Supplementary Table 1 for demographic characteristics, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract). These patients did not have arthritis at the time of first physical examination and had

never been diagnosed as having arthritis. Patients were followed up for 5 years or until they were diagnosed as having arthritis, with yearly clinical evaluations and additional visits for patients in whom arthritis was suspected. Presence of at least 1 swollen joint on physical examination of 44 joints by a trained medical doctor was defined as evidence of arthritis. In the second patient group, baseline serum samples were obtained from 97 RA patients just before starting therapy with the TNF blocker adalimumab (with 62% of these patients being RF positive using a conventional RF assay). In the third patient group, baseline serum samples were obtained from 200 patients with RA of recent onset (31). In the fourth patient group, serum samples were obtained from 62 individuals diagnosed as having primary SS that were originally included in the Register Sjögren UMCG Longitudinal (RESULT) cohort at the University Medical Center Groningen (UMCG) (with 68% of these individuals being RF positive using a conventional RF assay). All patients with RA and all patients with primary SS fulfilled the ACR/EULAR classification criteria for their respective diseases.

To obtain RF-positive healthy control samples, 488 serum samples obtained from in-house volunteers and residual samples from donors who frequently received booster injections of tetanus toxoid were tested for RF reactivity against human IgG. Based on an RF reactivity level of >2 arbitrary units (AU)/ml, 103 healthy individuals who were positive for RFs were selected as control subjects in the present study.

Arthralgia and RA studies were approved by the Ethics Committee of Slotervaart Hospital (Amsterdam, The Netherlands), and primary SS studies were approved by the Medical Research Ethics Committee of the UMCG. Written informed consent was obtained from all study participants and in-house volunteers.

Production of recombinant IgG targets. To characterize the binding of RF to predicted IgG epitopes, 7 different IgG molecules were produced to use as targets in RF assays. Six different human IgG1–based constant heavy chain (CH) constructs were designed: 1 coding for the human wild-type [WT] amino acid sequence of IgG1–CH (WT IgG) and 5 with nucleotide mutations resulting in replacement of predetermined human IgG1–CH amino acid sequences with their mouse IgG2b–CH analogs (See Supplementary Figure 1 for details on amino acid sequences, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract). The nucleotide replacements were selected by comparing the structure of human IgG and rabbit IgG, to which most RFs bind, with mouse IgG, to which almost no RFs bind, or were based on data from previous binding studies on monoclonal RFs and studies on crystal structures of monoclonal RFs complexed with IgG (20,21,32,33).

The mutated IgG targets were designated as follows: IgG-Bare, with 16 amino acid replacements divided over 3 clusters, with 1 in the CH2 domain (3 amino acid replacements), 1 in the CH2–CH3 Elbow region (9 replacements), and 1 at the tail end of the CH3 domain (4 replacements); IgG-CH2, with the nonmutated CH2 cluster; IgG-ER, with the nonmutated Elbow region cluster; IgG-Tail, with the nonmutated CH3 cluster; IgG-H435R, identical to IgG-ER, but with an additional H435R replacement; IgG-2b, with fully mouse IgG2b CH domains (Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract).

The IgG targets were produced as full recombinant chimeric IgG antibodies, all specific for biotin, by cloning synthetic constructs coding for anti-biotin variable domains (34,35) into a pcDNA3.1 expression vector, together with 1 of the 6 designed IgG1 constant heavy-chain constructs, a mouse IgG2b constant heavy-chain construct, or human κ constant domains. All antibodies were produced under serum-free conditions in FreeStyle 293 expression medium (Invitrogen) by cotransfecting relevant heavy-chain– and light-chain–expressing vectors in HEK 293F cells using 293fectin transfection reagent, according to the manufacturer's instructions (Invitrogen). The cells were cultured at a temperature of 37°C in an atmosphere of 8% CO_2, with shaking at 125 revolutions per minute. On day 5 of transfection, the cultures were centrifuged, and the supernatant was harvested, filtered over a Whatman Puradisc 30 syringe filter with a pore size of 0.20 μm (Sigma-Aldrich), and loaded on a HiTrap α-kappa column (ÄKTAprime). IgGs were eluted with 0.1*M* glycine pH 2.5–3. The eluate was immediately neutralized with 2*M* Tris HCl, pH 9, dialyzed and concentrated by multiple rounds of spinning down the sample using an Amicon Utra-4 Centrifugal Filter Unit 10-kd spin column, and resuspended in phosphate buffered saline (PBS). The concentration of the purified IgG was determined by measuring absorbance at 280 nm using a NanoDrop 1000 spectrophotometer (ThermoFisher), and the samples were aliquoted and stored at –20°C.

Enzyme-linked immunosorbent assays (ELISAs). RF reactivity against the individual IgG targets was analyzed by ELISA. All target antibodies were diluted in PBS to 1 μg/ml and coated overnight at 4°C on Nunc MaxiSorp 96-well flat-bottomed plates (ThermoFisher Scientific). Plates were washed 5 times with 0.02% PBS–Tween 20, and 100 μl of serum from patients, control serum, or a reference serum, diluted in 0.1% PBS–Tween 20, was added to the wells, followed by incubation for 60 minutes with shaking and at room temperature. After washing, IgM RF was detected by incubating the wells for 30 minutes with 100 μl horseradish peroxidase (HRP)–conjugated mouse monoclonal anti-human IgM (μ-chain–specific) antibodies (0.5 mg/ml, MH-25; Sanquin) at a dilution of 1:1,500 and visualized with 100 μg/ml of 3,3′,5,5′-tetramethylbenzidine in 0.11*M* acetate buffer, pH 5.5, containing 0.003% H_2O_2 (Merck). The reaction was stopped with 2*M* of H_2SO_4, and optical density was read at 450 nm and 540 nm for background correction using a BioTek microtiter plate reader. Levels of IgM RF were calculated using a calibrator curve of a national reference serum normally used in the standard IgM RF ELISA ("RELARES"). This reference serum has a defined IgM RF

level of 200 IU/ml (36). We arbitrarily defined the reference serum as containing 200 AU/ml of anti-IgG1–reactive IgM RF and calculated the levels of reactivity against the recombinant IgG targets on the linear part of the anti–WT IgG1 reactivity curve of the reference serum at a dilution of 1:6,400–1:409,600 in 2-fold dilution steps. This calculation included evaluation of reactivity toward the IgG-Bare target, which showed 2% reactivity based on the anti–WT IgG1 curve of the reference serum, as shown in Figure 1C.

To determine a cutoff value for the different assays, we analyzed a panel of 31 randomly selected healthy individuals. The median values used to determine positive signal reactivity for IgG2b, IgG-Bare, and WT IgG in the serum of these healthy individuals were as follows: 0.40 AU/ml (interquartile range [IQR] 0.25–0.55) for IgG2b, 0.38 AU/ml (IQR 0.30–0.59) for IgG-Bare, and 0.46 AU/ml (IQR 0.33–1.34) for WT IgG. For both IgG2b and IgG-Bare, but not for WT IgG, signals were log-normally distributed, and no correlation was found between IgG2b and IgG-Bare. Based on these results, we chose a conservatively low cutoff value for the IgG2b target, defined as the mean + 2SD of the log-transformed signals from all targets (i.e., a cutoff of

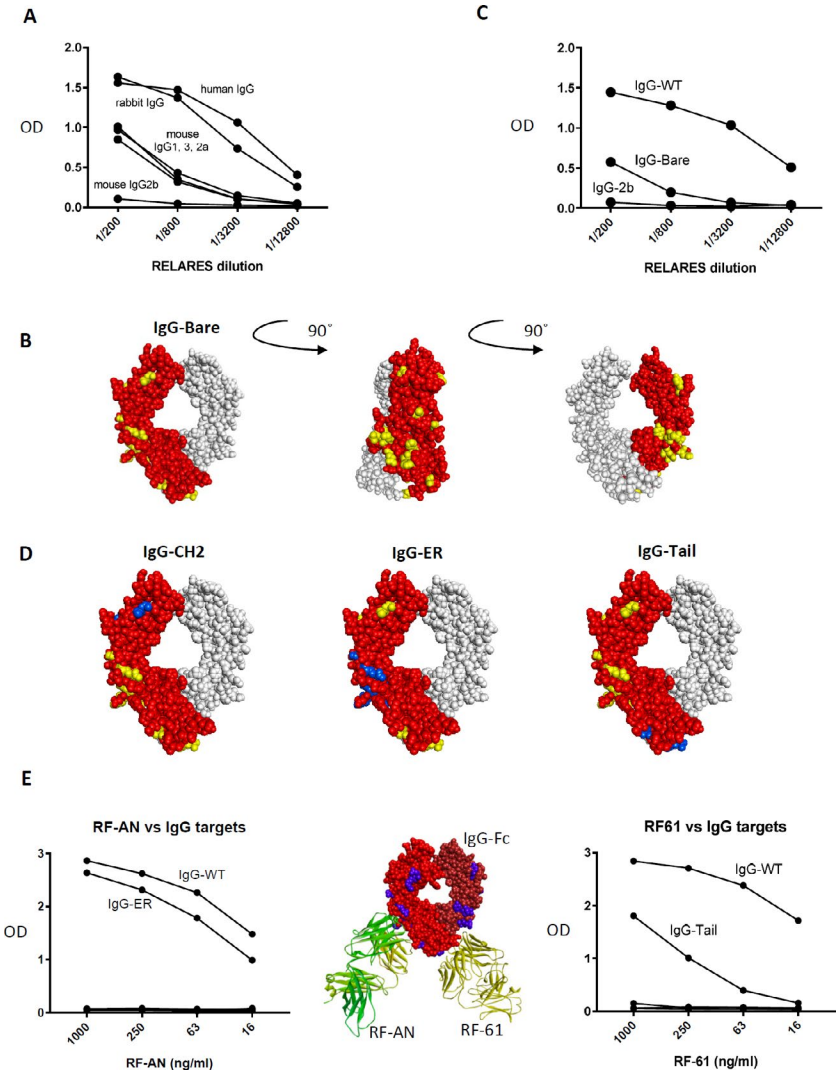

Figure 1. Development of recombinant IgG targets. **A**, IgM rheumatoid factor (RF) reactivity of a pooled RF standard reference serum (RELARES) against human IgG, rabbit IgG, and all 4 mouse IgG subclasses. **B**, Front, side, and rear views of the "parent" IgG target, designated IgG-Bare, with 15 amino acid replacements of human IgG1 to mouse IgG2b in the Fc domain (See Supplementary Figure 1 for details on amino acid replacements, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract), as well as an additional P445L mutation, all indicated in yellow. Note that the molecule is symmetric, and that in reality, the mutations are present in both chains. **C**, IgM RF reactivity against the IgG-Bare target, and control wild-type (WT) IgG and mouse IgG2b, tested with the pooled RF standard reference serum. **D**, Front views of 3 additional recombinant IgG targets, based on IgG-Bare, with different clusters of human amino acids reintroduced, indicated in blue. Cluster "IgG-CH2" is located in the CH2 domain, cluster "IgG–Elbow region (ER)" at the CH2–CH3 Elbow region, and cluster "IgG-Tail" at the tip of the CH3 domain. **E**, Binding properties of the IgG targets analyzed using 2 monoclonal IgM RFs: RFAN and RF-61. Middle panel shows binding of these RFs with IgG-Fc, based on previously published crystal structures 2J6E and 1ADQ (32,33) obtained from the RSCB Protein Data Bank (45) and created with Discovery Studio software version 4.5.

1.14 AU/ml), which was rounded to 1 AU/ml. Based on this established cutoff, the frequencies of positive signals among the 31 serum samples tested were as follows: 1 positive for IgG2b, 3 positive for IgG-Bare, and 10 positive for WT IgG.

To determine cross-reactivity between the IgG–Elbow region (ER) and IgG-Tail targets, samples were preabsorbed for 120 minutes on plates coated with 1 μg/ml of target antibody. The samples were then transferred to new target antibody–coated plates and incubated for 60 minutes, with the IgM RF levels of both sets of plates determined as described above.

Complement deposition ELISA. Plates were coated overnight at 4°C with 10 μg/ml of biotinylated human serum albumin (HSA; Sanquin) in PBS. After the plates were washed, 100 μl of the different IgG targets, diluted in high-performance ELISA buffer (HPE), was added to the wells, followed by incubation for 60 minutes. The plates were then washed again, and 100 μl of veronal buffer (3 m*M* barbital, 1.8 m*M* sodium barbital, and 145 m*M* NaCl, pH 7.4) was added along with 10% normal human serum, 10 m*M* $CaCl_2$, and 2 m*M* $MgCl_2$. Thereafter, the plates were incubated for 60 minutes and washed, and then deposition of C3b was detected by incubation of the supernatant with 100 μl of HRP-conjugated anti-C3.19 in HPE for 45 minutes. The results were visualized with tetramethylbenzidine. The reaction was stopped with 2*M* H_2SO_4, and absorbance was measured at 450 nm and 540 nm.

Additional target antibodies and monoclonal RFs. Polyclonal human IgG was obtained from intravenous immunoglobulin (Nanogam; Sanquin). Polyclonal rabbit IgG was purified from rabbit plasma using protein G affinity chromatography (HiTrap Prot G HP; GE Healthcare Life Sciences). Purified WT mouse subclass IgG antibodies were purchased from BD Biosciences. Two monoclonal IgM RFs, RF61 and RF-AN, were produced as described previously (37).

Statistical analysis. Logistic regression analyses were performed using log-transformed values for antibody levels as the continuous variable and development of arthritis within 2 years as the categorical response variable. As an additional input variable in some of the analyses, we used the skewedness of the data toward either mostly ER reactivity or mostly Tail reactivity, with results expressed as a normalized ratio. Using Spearman's correlation analysis, we found that the normalized Tail:ER (TE) ratio showed no correlation with ER reactivity ($r = 0.01$, $P = 0.008$). Logistic regression analysis was carried out using R version 3.4.3. Additional statistical analyses were carried out using GraphPad Prism 7.

RESULTS

IgG targets. In order to develop RF assays that could classify RF responses according to their specificities for different IgG Fc epitopes, several recombinant IgG1 molecules with various amino acid replacements in the Fc domain were engineered. First, a "parent" target was designed, which was an IgG1 molecule with most RF reactivity removed. Our starting point was the observation that RFs are cross-reactive with different animal IgGs in vastly different degrees. In particular, cross-reactivity toward rabbit IgG is high, whereas cross-reactivity toward mouse IgG is low (38), and cross-reactivity toward mouse IgG2b is particularly low (Figure 1A). Comparing the sequences of human IgG1, rabbit IgG, and mouse IgG2b yielded multiple positions shared by human IgG1 and rabbit IgG, but not mouse IgG2b. We selected a subset of these based on solvent exposure, among other considerations, and a human IgG construct was designed and produced with 15 amino acid replacements in the Fc domain wherein the "human amino acid" was replaced with the "mouse amino acid" as well as an additional P445L mutation (Figure 1B) (see Supplementary Figure 1 for details on amino acid sequences, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract). In the pooled RF serum standard, RF reactivity toward this partially "murinized" IgG, designated IgG-Bare, was indeed found to be greatly reduced, with ~2% RF reactivity remaining compared to the reactivity toward WT IgG1, which corresponds to results typically seen on a conventional RF assay (Figure 1C).

Next, with IgG-Bare as the starting point, 3 additional recombinant IgG targets were produced in which we reintroduced human amino acids in 3 different clusters, to determine if specific RF reactivity toward these individual clusters could be evaluated. These clusters included "IgG-CH2" located in the CH2 domain, "IgG-ER" at the CH2–CH3 Elbow region, and "IgG-Tail" in the tail region of the CH3 domain (Figure 1D and Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract). All targets were able to induce activation of complement component C3b to a similar degree (Supplementary Figure 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract), indicating that all targets were correctly folded.

Binding properties of the IgG targets were first tested with 2 monoclonal IgM RFs: RF61 and RF-AN. The IgG Fc epitopes bound by RF61 and RF-AN have previously been determined in crystal structure studies (32,33), which showed that RF-AN binds epitopes in cluster IgG-ER and RF61 in cluster IgG-Tail. Indeed, while reactivity was lower than against WT IgG, RF61 bound IgG-Tail, but not IgG-ER, and vice versa for RF-AN (Figure 1E), demonstrating differential recognition of specific epitopes on the different targets.

Reactivity against engineered IgG targets in seropositive arthralgia patients. To test whether the IgG targets could be used to identify distinct RF responses, IgM RF reactivity against these targets was assessed in 639 patients from the Reade seropositive arthralgia cohort (Supplementary Table 1,

available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract). In this cohort, the development of arthritis is strongly linked to ACPA positivity (11). Of the 639 serum samples from this cohort, 214 were RF-positive, 179 were ACPA-positive, and 187 were positive for both RFs and ACPAs. Fifty-nine patients had ambiguous antibody status, with antibody levels around the cutoff value in the conventional assays or inconsistent results from multiple measurements. For analyses regarding ACPA status, these samples were considered ACPA-negative (see below).

RF levels in the arthralgia cohort were highest against the WT IgG1 target (Figure 2A). Minimal reactivity against the control mouse IgG2b target (designated IgG-2b) was seen (see results expressed on the logarithmic scale in Figure 2A). Compared to the reactivity against WT IgG, reactivity against IgG-Bare was also low, indicating that the most important hot spots for RF binding on IgG Fc were successfully disrupted with the 16 amino acid mutations, and that the number of RF epitopes on IgG Fc is limited. Nevertheless, there was more residual reactivity against IgG-Bare than against IgG-2b on a group level, and a small number of patients exhibited substantially more anti–IgG-Bare than anti–IgG-2b reactivity, primarily in samples with high levels of RF (anti–WT IgG–reactive samples) (Supplementary Figure 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract).

Reactivity against cluster IgG-CH2, which overlaps with the region where most Fc receptors bind to IgG, was low in most patients, and as there was a close correlation between reactivity against IgG-CH2 and reactivity against IgG-Bare, it may be concluded that the Fc receptor binding region is rarely specifically targeted by RF responses (Figure 2B). The largest part of the overall RF binding was reactivity toward cluster IgG-ER, which is expected given that this is the largest cluster. Reactivity toward cluster IgG-Tail was variable, and its correlation with the

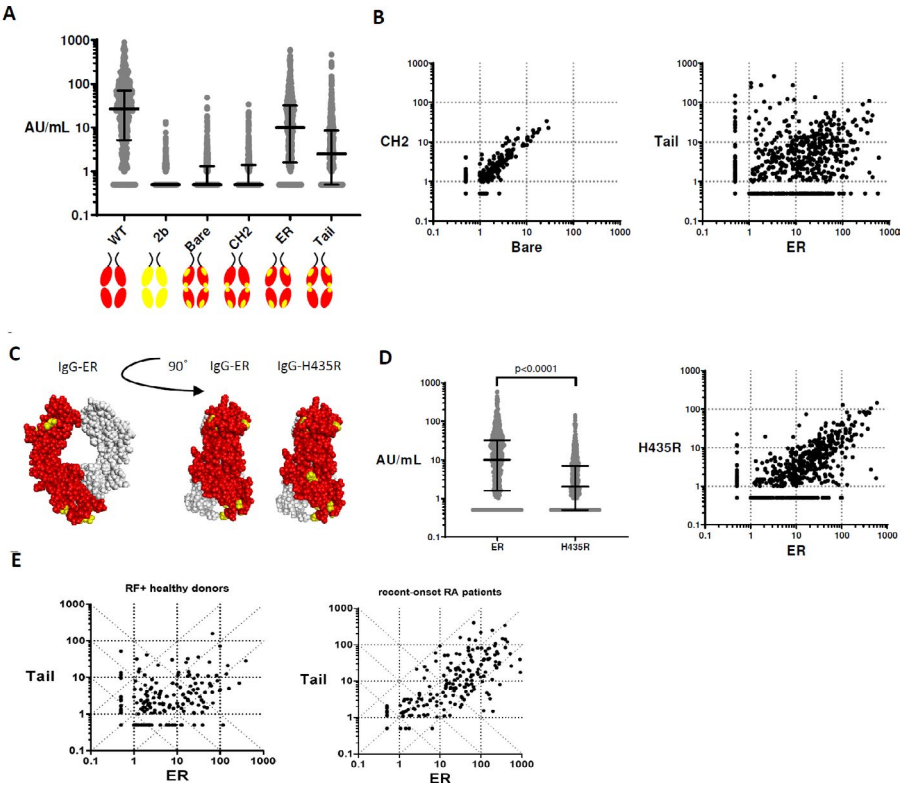

Figure 2. Reactivity against IgG targets in patients with seropositive arthralgia. **A**, Levels of IgM RF reactivity against 6 recombinant IgG targets, measured in 639 patients with seropositive arthralgia (whose serum was positive for RF and/or for anti–citrullinated protein antibodies) (top panel). Illustrations in the bottom panel show the Fc domains of each target, with areas of mouse IgG2b amino acids indicated in yellow. Bars show the median and interquartile range. A cutoff point for positive reactivity was set at 1 AU/ml. **B**, Scatterplots depicting a close correlation between levels of anti–IgG-CH2 reactivity and levels of anti–IgG-Bare reactivity (left) and comparison of RF reactivity against the IgG-Tail and IgG-ER regions (right). While many samples show reactivity against both targets, some specifically react with either IgG-Tail or IgG-ER. **C**, Design of the recombinant IgG target "IgG-H435R," which is identical to IgG-ER, except for a mutation of histidine being replaced by arginine at position 435. **D**, Comparison of RF reactivity against targets IgG-ER and IgG-H435R in patients with seropositive arthralgia (left) and scatterplots depicting the correlations (right). Bars show the median and interquartile range. *P* value was calculated by Wilcoxon's matched pairs signed rank test. **E**, Comparison of RF reactivity against IgG-Tail and IgG-ER targets in the serum of RF-positive healthy donors (individuals without rheumatoid arthritis [RA] who were assessed as having an RF reactivity level of >2 AU/ml) and the serum of patients with recent-onset RA. 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.41430/abstract.

overall reactivity toward WT IgG was much weaker than what was observed between the reactivity toward cluster IgG-ER and overall reactivity toward WT IgG (Supplementary Figure 3, available at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract). Since cluster IgG-ER and cluster IgG-Tail are spatially separated from each other, we hypothesized that we would be able to find separate RF responses against these 2 clusters. Indeed, when anti–IgG-ER and anti–IgG-Tail reactivities were compared, we found that, among the samples showing RF reactivity, the reactivity was either skewed toward 1 of the targets or skewed toward both targets (Figure 2B). Furthermore, absorption experiments indicated that RFs binding to the Elbow and Tail are different subsets to a substantial degree (Supplementary Figure 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract).

Cluster IgG-ER is situated at the CH2–CH3 Elbow region and has been suggested to correspond to those parts of the Fc region that are important for Ga reactivities. To be able to determine how much of the reactivity against cluster IgG-ER corresponds to classic (i.e., H435-dependent) Ga reactivity, an additional IgG target (IgG-H435R) was produced that differs from IgG-ER only at position 435, with an arginine, which is present in WT IgG3 at position 435, replacing the histidine (Figure 2C). For almost all patients with arthralgia and anti–IgG-ER reactivity (i.e., anti–IgG-ER levels of ≥1), this reactivity was at least partly dependent on the presence of H435 in the Fc domain (Figure 2D), with a median loss of reactivity of 76% when H435 was mutated compared to IgG-ER without the H435 mutation. These data suggest that classic Ga reactivity is an important part of the RF response in almost all patients. Taken together, these results demonstrate the feasibility of dissecting reactivity patterns of RF responses using our new methodology.

Association of RF reactivity pattern with ACPA status and arthritis. Next, we investigated whether in the patients with arthralgia, RF reactivity patterns that are associated with ACPA status and clinical outcome could be identified. First, we analyzed whether levels of reactivity against the individual targets correlated with ACPA (i.e., anti-CCP2) levels. A weak correlation was found between RF reactivity against WT IgG and ACPA levels ($r = 0.12$, $P = 0.002$), whereas a stronger correlation was observed between reactivity against the IgG-ER and IgG-Tail targets and ACPA levels (for correlation with IgG-ER reactivity, $r = 0.16$, $P < 0.0001$; for correlation with IgG-Tail reactivity, $r = 0.24$, $P < 0.0001$) (Supplementary Figure 5A, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract).

As mentioned, earlier studies have suggested that a broader RF response indicates a more pathogenic, RA-associated RF response (20–23). Therefore, we analyzed IgG-ER and IgG-Tail responses in RF-positive healthy donors as well as in patients with recent-onset RA (Supplementary Table 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract). In RA patients, a generally broader anti-IgG response was observed, with substantial reactivity against both IgG-ER and IgG-Tail observed in many RA patients ($r = 0.77$, $P < 0.0001$) as compared to a more often skewed response, in which reactivity was against primarily one of these targets, as opposed to both targets, in many healthy donors ($r = 0.45$, $P < 0.0001$) (Figure 2E) or in patients with seropositive arthralgia ($r = 0.41$, $P < 0.0001$) (Figure 2B).

We hypothesized that in our cohort of patients with seropositive arthralgia, a minimally skewed, "RA-like" RF reactivity pattern is associated with a high level of ACPA positivity and substantial arthritis development, whereas a highly skewed pattern correlated with a low level of ACPA positivity and little arthritis development. Associations between ACPA status and reactivity patterns were tested by plotting the TE ratio in order to visualize skewedness of RF reactivity toward either target versus WT and to make comparisons between ACPA-positive and ACPA-negative patients. Strong skewing of the RF response toward either the Tail or Elbow region was indeed associated with ACPA negativity (Figure 3A). For example, the subsets of patients with either a high (>2) or low (≤0.1) TE ratio were predominantly in the ACPA-negative group, whereas the majority (70%) of patients who have demonstrated reactivity against both targets were in the ACPA-positive group. Furthermore, we found significantly less ACPA positivity in patients with an RF reactivity pattern dominated by classic Ga reactivity (defined as an H435R:ER ratio of ≤0.1) compared to patients with an RF reactivity pattern that was not dominated by classic Ga reactivity (25 [27%] of 93 patients versus 299 [63%] of 474 patients showing ACPA positivity) (Supplementary Figure 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract). This is in accordance with earlier studies suggesting that RFs from RF-positive healthy donors mainly show classic Ga reactivity.

Next, we determined whether the RF reactivity pattern is associated with development of arthritis. We analyzed 465 arthralgia patients with a level of RF reactivity against WT IgG of ≥1 who had been followed up for 2 years. Of these, 139 patients (30%) developed arthritis within 2 years, with a median time to arthritis of 6.9 months. As seen in Figure 3B, an RF reactivity pattern skewed toward either the Tail region or Elbow region is associated with a lower risk of progression to arthritis. Using the same example as above (i.e., arbitrarily defining skewedness as a TE ratio of ≤0.1 or a TE ratio of >2), a skewed RF response was observed in 198 patients (43% of 465 patients assessed). Of these 198 patients, 39 (20%) developed arthritis within 2 years, versus 100 (37%) of 267 patients with a TE ratio between 0.1 and 2. Additionally, we analyzed the skewedness of the RF response in patients with recent-onset RA (Figure 3C), with less skewedness observed in this subgroup. Of the 177 patients in whom the RF level against WT IgG was >1, 138 (78%) had a TE ratio between 0.1 and 2.

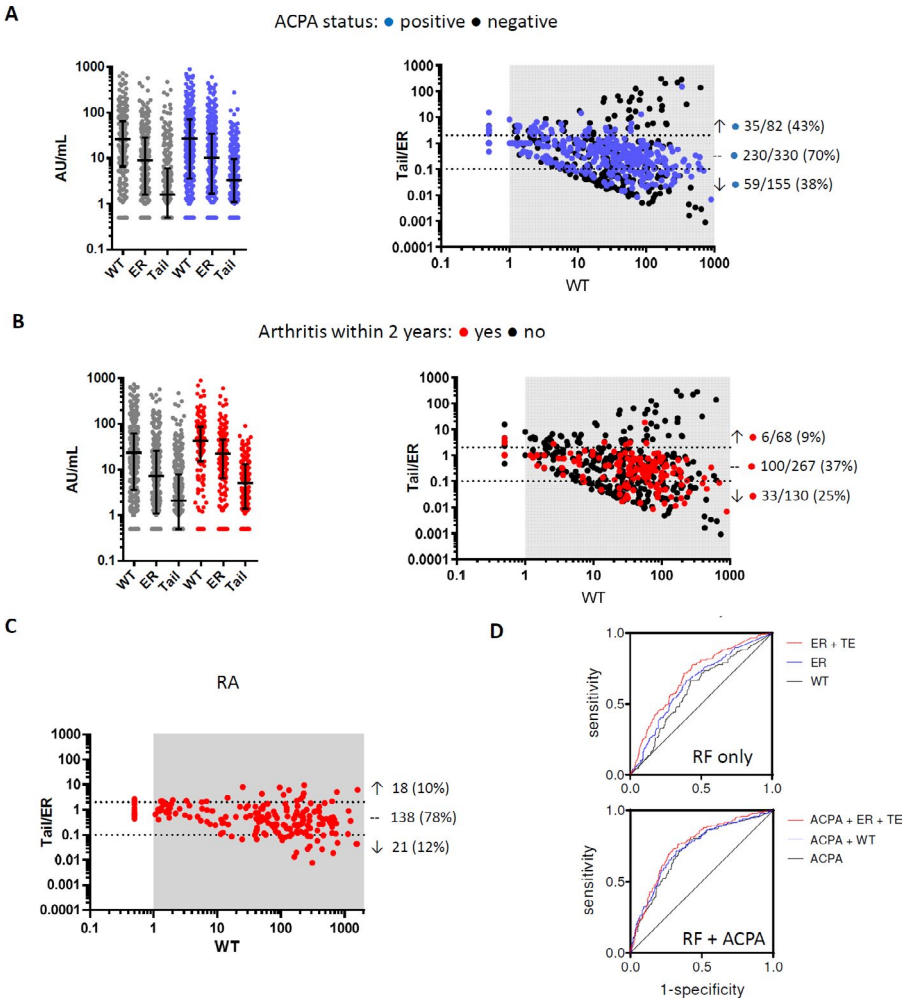

Figure 3. RF reactivity patterns and clinical outcomes in patients with arthralgia. **A** and **B**, Left, RF reactivity with WT IgG, IgG-ER, and IgG-Tail targets among patients with arthralgia who were either positive (blue) or negative (gray) for anti–citrullinated protein antibodies (ACPAs) (**A**) or among patients with arthralgia according to the development (red) or lack of development (black) of arthritis within 2 years (**B**). Bars show the median and interquartile range. Right, Skewing of the RF reactivity pattern toward IgG-Tail (Tail:ER [TE] ratio >2) or IgG-ER (TE ratio ≤0.1) versus RF reactivity toward WT IgG. In **A**, frequencies are based on data from 567 patients, using a cutoff value of >1 AU/ml for anti–WT IgG RF reactivity (shaded area). In **B**, frequencies are based on data from 465 patients with an RF anti–WT IgG reactivity level of >1 AU/ml who were followed up for 2 years. **C**, Skewedness toward IgG-ER or IgG-Tail versus anti–WT IgG titer for 200 patients with recent-onset rheumatoid arthritis (RA). In 177 patients with an anti–WT IgG reactivity level of >1 AU/ml, 138 (78%) had a TE ratio between 0.1 and 2. **D**, Receiver operating characteristic curve analyses showing the association between autoantibody status and development of arthritis within 2 years. For RF anti–WT IgG or IgG-ER, the area under the curve (AUC) was 0.603 and 0.646, respectively. Combining ER titer and a normalized TE ratio yielded an AUC of 0.697. Analysis of ACPA levels yielded an AUC of 0.718. The presence of ACPAs plus WT IgG resulted in an enhanced association with arthritis development (AUC 0.732), and the combination of ACPAs with IgG-ER and TE ratio had a greater enhanced association (AUC 0.753). 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.41430/abstract.

Logistic regression analysis was carried out to evaluate the association between autoantibody status and development of arthritis within 2 years in more detail. RF reactivity against WT IgG alone was significantly associated with arthritis development ($P < 0.0001$), and receiver operating characteristic analysis yielded an area under the curve (AUC) of 0.603 (Figure 3D). Individually, reactivity toward the Elbow and Tail regions was also significantly associated with arthritis development ($P < 0.0001$). Analysis of reactivity toward the Elbow region indicated an AUC of 0.646, suggesting that the association with arthritis development over 2 years was increased compared with reactivity toward WT IgG. Furthermore, the combination of reactivity toward both the Elbow and Tail regions showed an even stronger association with arthritis development within 2 years.

When we combined the titer of reactivity toward the ER and the normalized TE (as a measure of skewness toward 1 of these targets), an AUC of 0.697 was obtained, with both parameters being significantly correlated ($P < 0.001$). ACPA on its own yielded an AUC of 0.718 ($P < 0.0001$). The combination of ACPA levels and reactivity toward WT IgG had an enhanced association with

arthritis development over 2 years (AUC 0.732, $P < 0.001$); the combination of ACPA levels with ER reactivity and TE ratio resulted in a further enhanced association with arthritis development (AUC 0.753, $P < 0.001$ for ACPA with ER; $P = 0.016$ for ACPA with TE). The model combining ACPA levels with the TE ratio had significantly improved predictive power for arthritis development over 2 years when compared to the model combining ACPA levels with ER reactivity, as evaluated using Vuong's closeness test ($P = 0.007$). This translates to a true-positivity rate of 76% and a false-positivity rate of 33%. At equal false-positive rates, ACPA levels alone had a true-positive rate of 69%, and the combination of ACPA levels and reactivity with WT IgG had a true-positive rate of 72%, whereas WT IgG alone yielded a true-positive rate of 48%, and the combination of ER and the TE ratio yielded a true-positive rate of 61% for the development of arthritis within 2 years.

Although classic Ga reactivity (H435R:ER ratio of ≤0.1) was associated with low ACPA positivity (see above), this reactivity pattern was not associated with a lower rate of arthritis development within 2 years (Supplementary Figure 5B, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract).

Overall, these data suggest that a broader RF response, characterized by substantial reactivity against more epitopes, is associated with the presence of ACPAs and a higher probability of developing arthritis.

Differential RF patterns in RA and Sjögren's syndrome. To further determine the clinical potential of the engineered IgG targets, we looked for differences in RF reactivity pattern between the cohort of patients with established RA and the cohort of patients diagnosed as having primary SS. Our results showed that levels of RF reactivity against WT IgG, IgG-ER, and IgG-Tail were lower in primary SS compared to RA (Figure 4A). However, while the difference in median WT IgG levels was less than a factor of 2 and not statistically significant, anti–IgG-Tail levels were lower by a factor of >3.5 in patients with primary SS compared to patients with RA. Moreover, in the primary SS samples that did show reactivity against the Tail target, this reactivity was likely residual reactivity toward the IgG-Bare target rather than specific reactivity toward the IgG-Tail cluster. This became apparent when anti-Tail reactivity was corrected for anti-Bare reactivity by plotting the Tail:Bare ratio and was also apparent from the close correlation observed between IgG-Tail reactivity and IgG-Bare reactivity (Figure 4B). In short, there appeared to be a lack of RF reactivity against the Tail region epitopes in patients with primary SS.

DISCUSSION

The purpose of the present study was to characterize RF reactivity patterns on a molecular level and identify clinically relevant RF reactivity patterns in different patient groups. This study is

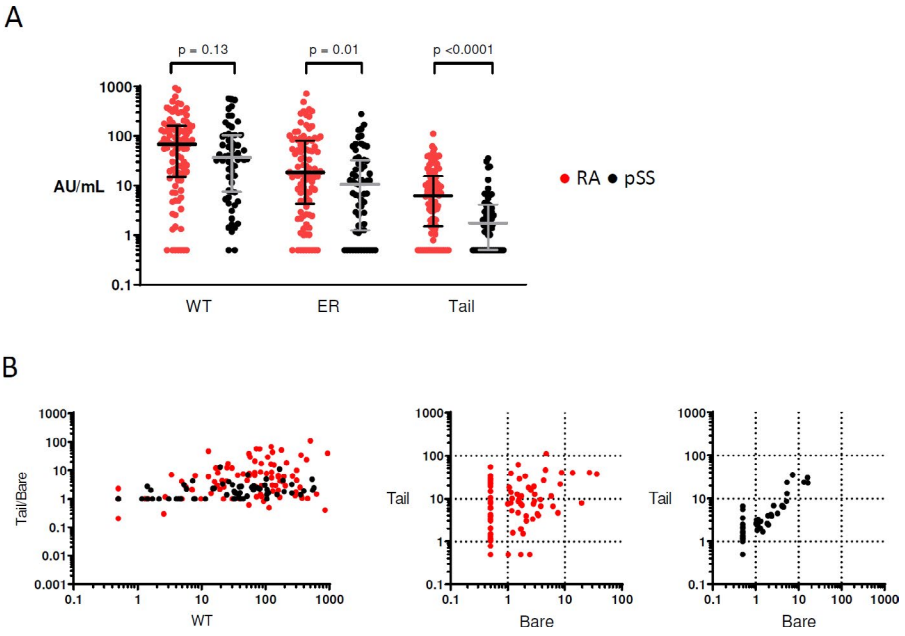

Figure 4. RF reactivity patterns in patients with rheumatoid arthritis (RA) and patients with primary Sjögren's syndrome (pSS). **A**, Levels of RF reactivity against WT IgG, IgG-ER, and IgG-Tail in a cohort of 97 patients with established RA (red) compared to samples from 62 patients diagnosed as having primary SS (black). Levels of anti-Tail reactivity were significantly lower in primary SS patients ($P < 0.0001$ by Mann-Whitney test). Of the samples obtained from the primary SS cohort, 60, 55, and 54 samples showed >1 AU/ml of reactivity toward WT, ER, and Tail, respectively. In the RA group, 90, 88, and 80 samples showed >1 AU/ml of reactivity toward WT, ER, and Tail, respectively. Bars show the median and interquartile range. **B**, Comparison of reactivity against IgG-Bare and IgG-Tail showing that for primary SS patients, most anti-Tail reactivity is likely residual reactivity toward the IgG-Bare target rather than specific reactivity toward the IgG-Tail target, as evidenced by a low Tail:Bare ratio and the close correlation between IgG-Tail and IgG-Bare reactivity. 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.41430/abstract.

the first to dissect RF reactivity patterns toward multiple regions of the IgG Fc domain in a targeted manner using appropriately engineered IgG molecules and, on a large scale, directly in patient sera, instead of characterizing a limited number of isolated RF clones. We showed that mutating 3 groups of predicted RF epitopes, comprising 16 amino acids in total, was sufficient to eliminate binding of a substantial part of the RF responses. Selective reversal of only a subset of these mutations generated a set of targets capable of identifying specific RF reactivity patterns in patients with arthralgia, RA, or primary SS. This new methodology ultimately allows discrimination of RF responses to many different epitopes.

Generally speaking, we find that a broader RF response appears to signal a more pathogenic RF response. Significant skewing of an RF response toward either the Tail region or ER epitopes is associated with a lower risk of arthritis development in patients with arthralgia and less ACPA positivity and is also a more dominant pattern in the subset of healthy individuals with a detectable RF response. "Epitope spreading" (a broadening of an antibody response over time) is seen as an important factor in sustaining and exacerbating autoimmune disease and has been well-documented in the context of ACPA reactivities among individuals considered at risk of developing RA (39–41). Our data suggest that epitope spreading may be of equal importance in the RF response. Interestingly, RF responses in patients with primary SS seem to lack reactivity against the Tail region epitope altogether. A recent study demonstrated the differential expression of disease-associated RFs in patients with primary SS (42). Our new methodology may provide additional insight into the pathophysiologic role of RFs in patients with primary SS.

Mechanistically, a broader RF response against multiple epitopes could have a greater potential to form large ICs, thereby triggering complement activation in the joint as well as inducing proinflammatory responses by macrophages because of increased IC-mediated signaling through Fcγ receptors. Furthermore, the relative absence of observable reactivity with the IgG–CH2 cluster, which overlaps with Fc receptor binding regions, suggests that when RFs bind to IgGs, the IgG targets may theoretically still be able to interact with Fcγ receptors. Indeed, RF binding to IgG appears to be only partially blocked by soluble Fcγ receptor IIa (43). Thus, our data support the hypothesis that RFs can increase the pathogenic potential of ACPA IgGs by forming RF–ACPA ICs that bind complement components through IgM RFs and engage Fcγ receptors through ACPA IgGs.

It is unknown why the CH2–CH3 Elbow region, which appears to capture the majority of the previously identified Ga reactivity, is the site most targeted by RFs, but it is a dominant epitope for RF responses in both RA patients and non-RA patients as well as in healthy donors. We further investigated these Ga reactivities using an H435R mutant of IgG–ER. The comparison of reactivity against the ER and H435R targets in the arthralgia cohort shows that not all RF responses binding in the CH2–CH3 Elbow region are the same and that a further dissection of this reactivity is feasible (Figure 2D). The association of the H435R:ER ratio with ACPA status suggests that such a dissection may provide additional clinical value.

The difference in reactivity against the target with the fully murinized Fc domain (IgG-2b) compared to IgG-Bare suggests that there are still minor RF specificities to be detected outside the ER and Tail region epitopes. Our method may need refinement (i.e., an improved version of the IgG-Bare target) to enhance resolution for measuring these reactivities. This was also illustrated by the primary SS data, wherein much of the reactivity measured against IgG-Tail appeared to actually represent reactivity against epitopes still present in IgG-Bare. Nonetheless, the available targets already demonstrate that combining RF binding data results in much more specific information about multiple RF reactivities.

Besides providing insight into the pathogenic potential of RF responses, our findings may also be of practical use for optimizing RF assays. Measuring RFs is important for diagnosing RA and predicting disease severity, but the assays currently used in clinical laboratories are not standardized and lack specificity (44). We showed in the present study that RF reactivity patterns are associated with clinical outcomes in patients with arthralgia. By using these novel IgG targets, instead of the current WT human IgGs or rabbit IgGs, clinically irrelevant RF responses can potentially be eliminated in RF assays. For example, by using IgG-ER as the target antibody, RF responses exclusively directed against the Tail epitopes would not be detected. Since these RF responses are almost exclusively restricted to arthralgia patients who do not develop arthritis, RF assays would gain in specificity. Based on the current data, the gain in specificity from replacing WT IgG with IgG-ER would not result in a lower sensitivity for diagnosing RA, as the sole patient who developed arthritis with a TE ratio of >4 had only 1 swollen joint and was diagnosed as having undifferentiated arthritis rather than RA (Figure 3B). IgG-ER may thus replace WT IgG as the primary target to measure RF, alone or in combination with IgG-Tail, which yields additional discriminatory potential for predicting development of arthritis (Figure 3D and Supplementary Figure 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41430/abstract) and for distinguishing response differences between RA and primary SS (Figure 4).

Currently, equal weight is given to RF and ACPA status and level in the RA classification criteria, despite the lower specificity of RF testing. Standardizing RF assays by using better defined and more specific RF targets could improve the clinical value of the classification criteria.

A limitation of the present study is that the inclusion of patients in the seropositive arthralgia cohort was based on positivity for RF and/or ACPAs in conventional assays. Conventional RF assays generally use a relatively high cutoff value for positivity to avoid picking up low levels of RF in individuals without RA and thus maintain a reasonable specificity. By specifically measuring RA-related RF reactivity patterns, our

new methodology is expected to achieve a lower cutoff value. This may enable detection of clinically relevant RF reactivity in individuals with RF levels that currently fall below the detection limit and are thus not included in the seropositive arthralgia cohort analyzed in the current study.

In conclusion, the present study demonstrates the validity of a new approach to molecularly dissect RF responses into individual specificities using novel engineered IgG targets. Our methodology of serologically characterizing RF reactivity patterns showed clinical value in different diseases and disease stages and provided pathophysiologic insights into this important autoantibody response.

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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. Falkenburg 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. Falkenburg, van Schaardenburg, Wolbink, Rispens.

Acquisition of data. Falkenburg, Oskam, Koers, Ooijevaar-de Heer, van Schaardenburg, Wolbink, Rispens.

Analysis and interpretation of data. Falkenburg, Oskam, Koers, van Boheemen, Verstappen, Bootsma, Kroese, van Schaardenburg, Wolbink, Rispens.

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