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Basic science

Rheumatoid factor recognizes specific domains of the IgG heavy chain complexed with HLA class II molecules

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Abstract

Objective: We previously reported that RF recognized the IgG heavy chain (IgGH)/RA-susceptible HLA class II molecule complex. In the present study, we investigated the molecular mechanisms underlying HLA binding to and the RF recognition of IgGH.

Methods: We synthesized various types of IgGH segments, including V_H, C_H1, C_H2 and C_H3, and transfected them with or without HLA class II molecules into the Human Embryonic Kidney 293T cell line. IgGH single domains linked with the HLA-Cw3 peptide, which binds to the binding groove of the HLA class II molecule, were also synthesized. The expression of IgGH domains on the cell surface and their recognition by RF were examined using flow cytometry.

Results: Flag-tagged IgGH segments containing C_H1 (C_H1, V_H-C_H1, C_H1-C_H2, V_H-C_H1-C_H2, C_H1-C_H2-C_H3 and V_H-C_H1-C_H2-C_H3) were clearly presented on the cell surface by HLA-DR4, while segments without the C_H1 domain were expressed at a low level, and the C_H3 single domain was only weakly detected on the cell surface, even with HLA-DR4. We then transfected IgGH single domains linked to the Cw3 peptide together with HLA-DR4 and showed that RF-containing sera from RA patients only recognized the C_H3 domain and none of the other single domains. When various segments without the Cw3 peptide were transfected with HLA-DR4, only the C_H1-C_H2-C_H3 segment and full-length IgGH were detected by the sera of RA patients.

Conclusion: The C_H1 domain of IgGH binds to the RA-susceptible HLA-DR molecule and is expressed on the cell surface. RF specifically recognizes the C_H3 domain of the IgGH/HLA-DR4 complex.

Keywords: RA, RF, IgG heavy chain, autoantigens and autoantibodies, major histocompatibility complex

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Rheumatology key messages

- The C_H1 domain of the IgG heavy chain is important for the antigen-presenting process of IgG.
- The C_H3 domain of the IgG heavy chain complexed with HLA class II molecule is recognized by RF.

Introduction

RF is an autoantibody that is produced in 70–80% of patients with RA and is widely used to diagnose RA and assess RA disease activity [1, 2]. RF is a low-affinity polyclonal IgM-type autoantibody that binds to denatured IgG or the Fc fragments of IgG [3, 4]. Although RF is produced by IgM-type RF-expressing B cells, the mechanisms underlying its production remain unclear [5].

We previously reported that misfolded proteins were transported by disease-susceptible HLA class II molecules to the cell surface without processing misfolded proteins into peptides and the misfolded protein/HLA class II complex stimulated the corresponding autoreactive B cells [6, 7]. Regarding RF, we demonstrated that IgG heavy chain (IgGH) proteins (not peptides) were presented on cell surfaces by RA-susceptible HLA-DR containing HLA-DRB1*04:01, *04:04 and *01:01, and RF from RA patients recognized the IgGH/HLA-DR4 complex [8]. We also detected the IgGH/HLA-DR complex in the synovial tissues of RA patients using a proximity ligation assay, which suggested that the production of RF

originates in synovial tissues. However, the molecular mechanisms by which IgGH is presented by HLA class II molecules and RF recognizes the IgGH/HLA class II complex have not yet been elucidated. Therefore, we herein investigated the specific regions crucial for IgGH binding to HLA class II molecules as well as the regions recognized by RF.

Methods

Plasmids

The cDNAs for different HLA class II alleles were cloned from cDNA prepared from pooled human peripheral blood mononuclear cells (Osaka, Japan) or human cell lines as previously reported [8]. the cDNA sequences of HLA were based on information in the Immunogenetics/HLA Database (www.ebi.ac.uk/ipd/imgt/hla/index.html)Osaka, Japan

The pME18s-Flag-IgG heavy chain (Flag-tagged IgGH, Osaka, Japan) with the BM40 signal sequence was used for the expression of IgGH. The sequence of the IgGH gene was verified by the sense primer for the variable region (5′-GTCTTGTCCCAGGTCACCTTGAAGGAG-3′) and the anti-sense primer for the constant region of IgG (5′-TCATTTACCCGGAGACAGGGAG-3′). Various consecutive combinations of the four domains of IgGH (V_H, C_H1, C_H2 and C_H3) were cloned with a Flag-tag (DYKDDDDK) at the N terminus, as shown in Fig. 1A, in order to identify the properties of specific IgGH regions. Restriction enzymes, XhoI at the 5′-terminus and NotI at the 3′-terminus, were used to clone the genes for specific IgGH segments (Supplementary Table S1, available at *Rheumatology* online). Cloned IgGH contained one (No. 2–5), two (No. 6–8) and three (No. 9 and 10) consecutive IgGH domains. The hinge region was always connected to the C_H2 domain.

HLA-Cw3 peptide-linker-IgGH domains were designed by attaching the HLA-Cw3 peptide (GSHSMRYFYTAVSRPGR) and linker (GSGSGS) sequences at the N-terminus of the specific IgGH domains in the Flag-tagged pME18s vector. To generate the cDNA of Cw3-linker-IgGH domains, PCR was performed using the three primers listed in Supplementary Table S2, available at *Rheumatology* online. Newly cloned plasmids were expected to express peptides in the order of the Flag-tag, Cw3 peptide and linker followed by the IgGH single domain, and this was confirmed by a DNA sequence analysis and the sizes of the peptides.

Human samples

The collection and use of human samples were approved by the certified review board of Kyoto University and written informed consent was obtained from all patients according to the guidelines of the certified review board. All patients were diagnosed with RA based on the ACR/EULAR classification criteria for RA in 2010 [9].

Cells and transfection

Human Embryonic Kidney 293T cells (HEK293T, RIKEN Cell Bank, Tsukuba, Japan) were regularly tested for mycoplasma contamination. Expression vectors encoding green fluorescent protein (GFP), HLA-DRα, HLA-DRβ and each IgGH domain were co-transfected into cells using Polyethylenimine Max (Polysciences, Warrington, PA, USA). Cells were collected 48 h after transfection and cell surface

expression and the RF recognition of IgGH domains were both examined by flow cytometry.

Flow cytometry

DNA-transfected cells were primarily stained by anti-HLA-DRα (L243, Santa Cruz Biotechnology, TX, USA) mAb, anti-Flag mAb (L5, BioLegend, San Diego, CA, USA) and the sera of RA patients, followed by allophycocyanin (APC)-conjugated anti-mouse-IgG (H+L), anti-rat-IgG (H+L) and anti-human IgM Ab (all from Jackson ImmunoResearch, West Grove, PA, USA). Stained cells were assessed by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) and data were analysed by FlowJo (Becton Dickinson, Franklin Lakes, NJ, USA). The mean fluorescence intensities of autoantibody binding to HLA-DR and IgGH-transfected cells were calculated by subtracting the mean fluorescence intensity of autoantibody binding to GFP-negative transfectants.

Results

Important IgGH segments for presentation on the cell surface by HLA class II molecules

Plasmids encoding 10 types of IgGH segments were prepared by combinations of the four domains of IgGH (V_H, C_H1, C_H2 and C_H3), which were linked to the Flag-tag at the N-terminus (Fig. 1A).

The plasmid containing single domains (#2–5 in Fig. 1A) was transfected into HEK293T cells with or without HLA-DRA1*01:01/HLA-DRB1*04:01 (HLA-DR4) and the surface expression of IgGH domains was detected by the anti-Flag antibody. As shown in Fig. 1B, none of the single IgGH domains was detected on the cell surface without the expression of HLA-DR4. However, with HLA-DR4 expression, the C_H1 domain was expressed on the cell surface at the highest level followed by the C_H2 and V_H domains, whereas the C_H3 domain was only weakly detected.

Each of the 10 types of IgGH segments (Fig. 1A) was co-transfected with HLA-DR4. Segments including the C_H1 domain (V_H-C_H1-C_H2-C_H3, V_H-C_H1-C_H2, C_H1-C_H2-C_H3, V_H-C_H1 and C_H1-C_H2) were constantly expressed on the cell surface at high levels (Fig. 1C), whereas those without the C_H1 domain (C_H2-C_H3) were expressed at low levels.

We also examined the expression of IgGH with different HLA alleles [10, 11]. When RA-susceptible alleles of HLA-DR (HLA-DRB1*01:01 and HLA-DRB1*04:04) were expressed on the cell surface, the results of the expression of IgGH domains were similar to those with HLA-DRB1*04:01 (Supplementary Fig. S1A, available at *Rheumatology* online). When RA-resistant alleles (HLA-DRB1*11:01, HLA-DRB1*13:01 and HLA-DRB1*15:01) were expressed, the expression levels of IgGH segments were markedly lower than those with HLA-DR of RA-susceptible alleles; however, expression patterns were similar (Supplementary Fig. S1B, available at *Rheumatology* online).

Important IgGH segments for RF recognition

We investigated whether RF recognized a specific IgGH domain (V_H, C_H1, C_H2 and C_H3) using the HLA-Cw3 peptide-linked IgGH domain (Cw3-IgGH domain), as shown in Fig. 2A. Since the HLA-Cw3 peptide strongly binds to the peptide-binding groove of HLA class II molecules, we used it as an anchor to HLA-DR in order to identify the IgGH domain recognized by RF [12].
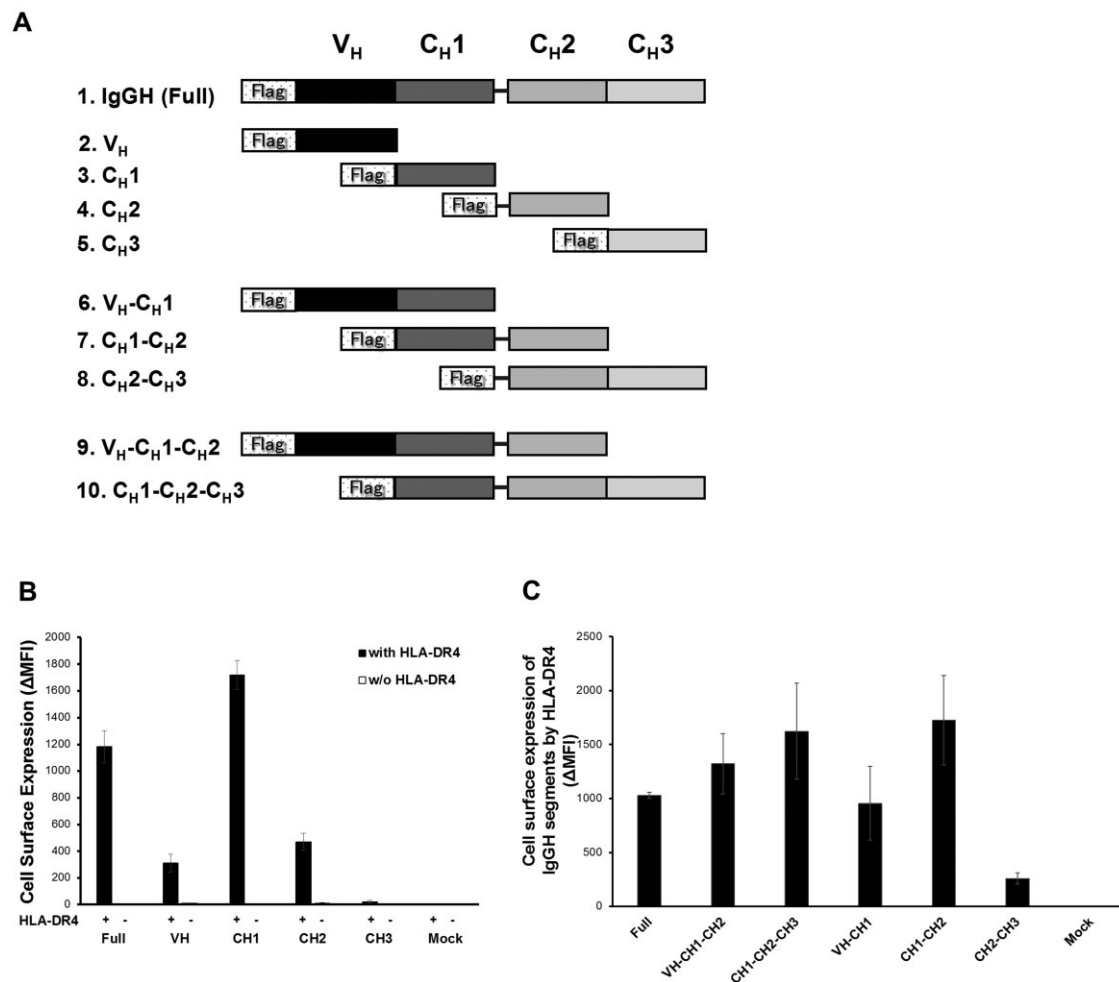
Figure 1. Cell surface expression of each IgGH segment/HLA-DR4 complex. (**A**) Schematic diagram of different IgGH segments. (**B**) Cell surface expression of each IgGH segment when transfected into HEK293T cells with (solid bars) or without (open bars) HLA-DR4. (**C**) Cell surface expression of other IgGH segments when transfected with HLA-DR4. (B and C) Cell surface expression of Flag-tagged IgGH segments detected by an anti-Flag mAb followed by APC-conjugated anti-rat IgG (H+L) using FACSCalibur. Data are shown as the mean (S.D.) of triplicate experiments. IgGH: IgG heavy chain; HEK: Human Embryonic Kidney; APC: allophycocyanin

We initially assessed the expression of Cw3-pep-IgGH single domains complexed with HLA-DR4 on the cell surface by detecting the Flag-tag. In comparisons with IgGH single domains without the Cw3 peptide, the V_H, C_H2 and C_H3 domains with the Cw3 peptide were detected on the cell surface with HLA-DR4, particularly the C_H3 domain (Fig. 2B).

The reactions of RF with the Cw3-IgGH domain expressed with HLA-DR4 on the cell surface were then examined using serum from an RA patient (RF titre: 1152.5 IU/ml). Since the Cw3 peptide bound to the site of the binding groove in HLA-DR4, all of the single Cw3-pep-IgGH domains were exposed to RF in sera by their expression with HLA-DR4. Therefore, RA serum only recognized the C_H3 domain expressed with HLA-DR4 (Fig. 2C). Furthermore, similar results were obtained for RF binding to IgGH domains using sera from 20 other RA patients (Fig. 2D). These results strongly suggest that the epitopes of IgGH recognized by RF were located in the C_H3 domain.

RF recognition of specific IgGH segments complexed with HLA-DR

Based on the present results, we hypothesized that the C_H1 domain of IgGH bound to the binding groove of HLA-DR

and RF recognized the C_H3 domain of the IgGH/HLA-DR complex, as shown in Fig. 3A. To confirm this hypothesis, 10 types of IgGH segments (Fig. 1A) were transfected into HEK293T cells with HLA-DR4, and their recognition by RF was analysed using RF-positive sera from three RA patients. As shown in Fig. 3B, only the C_H1-C_H2-C_H3 segments besides intact IgGH (V_H-C_H1-C_H2-C_H3) complexed with HLA-DR4 were recognized by RF-containing sera from RA patients. Although the expression of the segments V_H-C_H1, V_H-C_H1-C_H2 and C_H1-C_H2 was expected on the cell surface, they were not recognized by RA sera. Moreover, the C_H2-C_H3 segment was not detected by RA sera despite its expression being expected on the cell surface to some extent by HLA-DR4 (Figs 1C and 3B). These results confirmed that the C_H1 domain bound to HLA-DR4 and RF recognized epitopes in the C_H3 domain.

Discussion

The present study confirmed that intracellular IgGH was transported to the cell surface by RA-susceptible HLA class II molecules via its C_H1 domain. We also demonstrated that RF recognized the IgGH/HLA-DR complex mainly by the C_H3 domain, which was
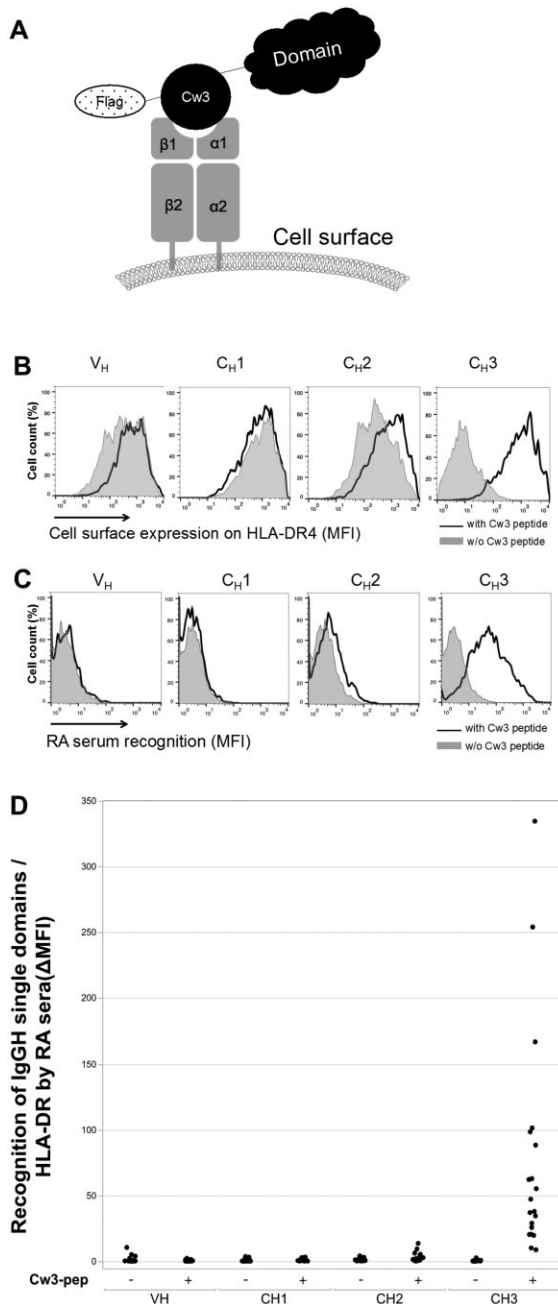
Figure 2. Cell surface expression and RF recognition of the IgGH single segment/HLA-DR4 complex. (**A**) An image of the HLA-DR4/Cw3-peptide-IgGH single domain complex expressed on HEK293T cells. (**B**) The cell surface expression of Flag-tagged IgGH single domains constructed with (solid line) or without (shaded) the Cw3 peptide when they were transfected with HLA-DR4. Expression was detected by an anti-Flag mAb followed by APC-conjugated anti-mouse IgG (H+L). (**C**) RF binding to Flag-tagged IgGH single domains linked with (solid line) or without (shaded) the Cw3 peptide/HLA-DR4 complex. (**D**) Sera from 20 RF-positive RA patients that reacted with HLA-DR4/IgGH single domains with or without the Cw3 peptide. The RF titers of the samples ranged between 10.7 and 1152.5 IU/ml. (B and C) MFIs were obtained by gating GFP-positive cells. (C and D) Expression was detected by sera followed by APC-conjugated anti-human IgM. IgGH: IgG heavy chain; HEK: Human Embryonic Kidney; APC: allophycocyanin; MFI: mean fluorescence intensity; GFP: green fluorescent protein

located outside of the binding groove of HLA class II molecules. These results strongly suggest that IgGH is presented by HLA class II molecules but is recognized by RF via different domains as the speculated structure shown in Fig. 3A.

In the present study, IgGH acted as an autoantigen, not an antibody. To become an autoantigen, it needs to be presented on HLA class II molecules, and the results obtained herein indicate that the C_H1 and C_H3 domains are both critical parts in the RF recognition process. Since the C_H1 domain binds to the HLA class II molecule, it may be important that the IgG light chain is not bound to IgGH at the C_H1 domain, which forms IgGH as a misfolded protein. By fixing the C_H1 domain at the HLA class II molecule, the conformation of IgGH appears to be changed such that RF recognizes cryptic epitopes in the C_H3 domain. RF does not bind to the native form of IgG, which is folded at the hinge region between C_H1 and C_H2, thereby masking epitopes, and heat-denatured IgG forms an open structure that enables RF to access these epitopes [13]. Therefore, the presentation of IgGH by the HLA class II molecule appears to render IgGH to a form that is similar to the structure of heat-denatured IgGH.

RF generally detects the Fc region of IgGH [1, 4, 14]; however, we herein demonstrated that RF did not react with the C_H2-C_H3/HLA-DR4 complex. Although the C_H2 domain exhibited the ability to load on HLA-DR4 in order to present C_H3 outside of the peptide-binding groove, the results obtained showed that RF did not detect the epitope on this segment.

The epitopes of RF recognition sites have been extensively examined. Historically, the Fc region of IgGH is considered to be recognized by RF. A more detailed study was recently performed and identified C_H2 and C_H3 as epitopes [15]. In the present study, only C_H3 was recognized by RF. We screened the sera of 20 RF-positive RA patients, and none reacted with the Cw3 peptide-C_H2/HLA-DR4 complex. However, we obtained some preliminary data to show that RF reacted more strongly with Cw3 peptide-C_H2-C_H3/HLA-DR4 than with Cw3 peptide-C_H3/HLA-DR4 (data not shown), which suggests the presence of epitopes constructed by C_H2 and C_H3. These results are consistent with the findings reported by Falkenburg *et al.* [15]. Furthermore, the 3D structure of IgGH appears to be of importance because the region between C_H2 and C_H3 may also be a target of RF based on the exchange of specific amino acids across this region, indicating that not only the sequence of amino acids, but also the 3D structures of proteins affect the affinity of RF.

One of the limitations of the present study is that we only used one IgG clone. Other IgG classes or IgG isotypes may give different results. Another limitation is that we only used one cell line (HEK293T) for transfection. Since antigen-presenting cells *in vivo* need to be cells in the B cell lineage, HEK293T cells may not be the ideal cell line. Furthermore, 20 RA serum samples may not have been sufficient to conclude that C_H3 is the only epitope of RF.

As described in the Introduction, we previously detected the IgGH/HLA class II complex in the synovial tissues of RA patients and showed that IgGH was presented on the cell surface more by the HLA-DR molecules of RA-susceptible alleles than by those of RA-resistant alleles [8]. Based on these findings, we speculate that RF production in synovial tissues is initiated by a stimulus with the IgGH/HLA-DR complex against autoreactive naïve B cell lineage cells possessing RF specificities. However, the mechanisms underlying the production of RF remain unclear and a number of questions remain unresolved, such as why and how IgG becomes the autoantigen, why limited IgG regions become the epitopes for RF recognition, and why IgM-RF, but not IgG-RF, is still dominant, even in patients with chronic RA [1, 6].
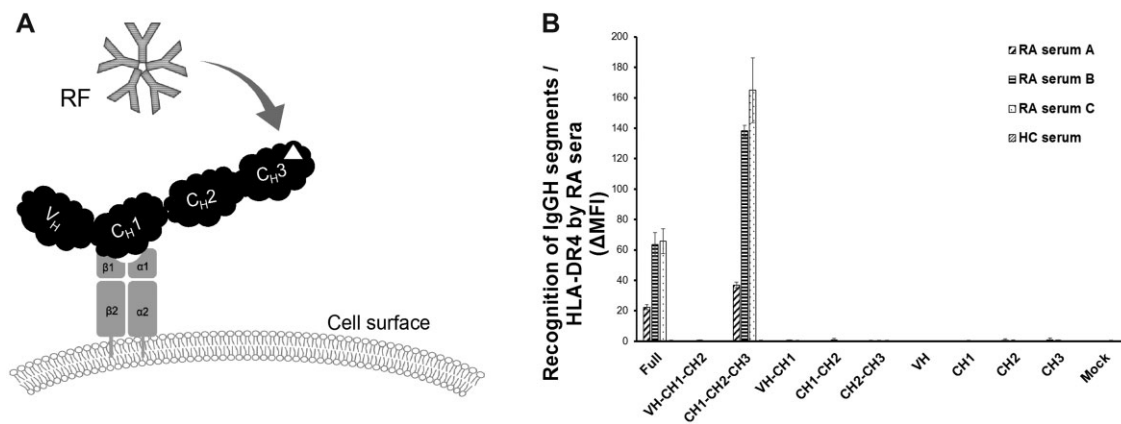
Figure 3. RF recognition of IgGH segments. (**A**) Hypothetical illustration of RF recognition of HLA-DR4 and the IgG heavy chain on the cell surface. (**B**) Sera from three RA patients reacted with the IgGH fragments bound to HLA-DR4. Serum A with an RF titre of 56.2 IU/ml, serum B 333.2 IU/ml and serum C 863.9 IU/ml. Binding was analysed using sera followed by APC-conjugated anti-human IgM. Data are shown as the mean (s.d.) of triplicate experiments. IgGH: IgG heavy chain; APC: allophycocyanin; HC: healthy control

Nevertheless, the present results provide important insights. V_H, C_H1 and C_H2, but not C_H3, may be presented by MHC class II, whereas only C_H3 becomes the epitope of RF, which is central to these questions.

Supplementary material

Supplementary material is available at *Rheumatology* online.

Data availability

All data relevant to the present study are included in the article or are uploaded as supplementary information.

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