IgM and IgA Rheumatoid Factors Purified from Rheumatoid Arthritis Sera Boost the Fc Receptor – and Complement-Dependent Effector Functions of the Disease-Specific Anti–Citrullinated Protein Autoantibodies

Florence Anquetil, Cyril Clavel, Géraldine Offer, Guy Serre and Mireille Sebbag

*J Immunol* published online 13 March 2015

http://www.jimmunol.org/content/early/2015/03/13/jimmunol.1402334

Supplementary Material

http://www.jimmunol.org/content/suppl/2015/03/13/jimmunol.1402334.4.DCSupplemental

Why *The JI*?

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2015 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
IgM and IgA Rheumatoid Factors Purified from Rheumatoid Arthritis Sera Boost the Fc Receptor– and Complement-Dependent Effector Functions of the Disease-Specific Anti–Citrullinated Protein Autoantibodies

Florence Anquetil,*†‡§ I Cyril Clavel,*†‡§ Géraldine Offer,*†‡§ Guy Serre,*†‡§ and Mireille Sebbag*†‡§

Rheumatoid factors (RF) and the disease-specific anti–citrullinated protein autoantibodies (ACPA) coexist in the joints of rheumatoid arthritis (RA) patients where they probably contribute to synovitis. We investigated the influence of IgM and IgA RF on the FcR- and complement-dependent effects of ACPA immune complexes (ACPA-IC). When stimulated by ACPA-IC formed in the presence of IgM RF or IgA RF fractions purified from RA serum pools, M-CSF-generated macrophages skewed their cytokine response toward inflammation, with increases in the TNF-α/IL-10 ratio and in IL-6 and IL-8 secretion, and decreases in the IL-1Ra/IL-1β ratio. In the IgM RF-mediated amplification of the inflammatory response of macrophages, the participation of an IgM receptor was excluded, notably by showing that they did not express any established receptor for IgM. Rather, this amplification depended on the IgM RF-mediated recruitment of more IgG into the ACPA-IC. However, the macrophages expressed FcγRI and blocking its interaction with IgA inhibited the IgA RF-mediated amplification of TNF-α secretion induced by ACPA-IC, showing its major implication in the effects of RF of the IgA class. LPS further amplified the TNF-α response of macrophages to RF-containing ACPA-IC. Lastly, the presence of IgM or IgA RF increased the capacity of ACPA-IC to activate the complement cascade. Therefore, specifically using autoantibodies from RA patients, the strong FcR-mediated or complement-dependent pathogenic potential of IC including both ACPA and IgM or IgA RF was established. Simultaneous FcR triggering by these RF-containing ACPA-IC and TLR4 ligation possibly makes a major contribution to RA synovitis. The Journal of Immunology, 2015, 194: 000–000.

Tumor necrosis factor-α is a master cytokine of rheumatoid arthritis (RA) synovitis, driving infiltration by immune cells and production of other proinflammatory cytokines and cartilage-destructive enzymes, and contributing to differentiation of bone-erosive osteoclasts (1). Lining and intimal synovial tissue macrophages are its dominant cell source (2).

Abbreviations used in this article: ACPA, anti–citrullinated protein autoantibody; C-FBG, citrullinated fibrinogen; D-PBS, Dulbecco’s PBS without calcium and magnesium; HAGG, human heat-aggregated γ-globulin; IC, immune complex; RA, rheumatoid arthritis; RF, rheumatoid factor.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/525/00-05

Stimulation by RA-associated cytokines such as IL-17 or IL-26, TLR engagement, and contact with Ag- or cytokine-activated T cells have been proposed as stimuli for its production (3–7). However, recognition that RA is associated with the production of rheumatoid factor (RF), an autoantibody recognizing the Fc portion of IgG, had long ago led to consider immune complexes (IC) containing this autoantibody as triggers for synovial tissue inflammation. Although it had been marginalized due to the lack of specific association between RF and RA, the concept of RA as an IC-mediated autoimmune disease has regained interest following more recent investigations involving anti–citrullinated protein autoantibodies (ACPA). Although they differ from RF by their highly specific association to RA, similar to RF, ACPA correspond to very prevalent autoantibodies that develop before RA onset and predict an aggressive disease course (8, 9). Moreover, in RA joints, ACPA IgGs are synthesized and concentrated (10, 11) and citrullinated autoantigens are generated (12–19), among which citrullinated fibrin was identified as a prominent ACPA target (13). Therefore, the formation of highly disease-specific ACPA-containing IC (ACPA-IC) in rheumatoid joints most probably occurs. Recently, the effector pathways they trigger have been explored. Their capacity to activate the classical and alternative pathways of complement was demonstrated (20). We recently investigated the capacity of IC formed following ACPA interaction with citrullinated fibrin to stimulate FcγR-mediated proinflammatory responses of synovial tissue macrophages. An in vitro model was developed in which citrullinated fibrinogen (C-FBG) selectively captures ACPA from the IgG fraction of RA serum pools (ACPA IgG). Stimulation of human monocyte-derived macrophages by such ACPA-IC without exogenous complement...
components allowed establishing their capacity to trigger TNF-α secretion by macrophages following engagement of stimulatory Fcγ receptors, in particular FcγRIIa (21). These data were subsequently confirmed, together with the participation of this receptor (22).

RF is also produced and concentrated in the rheumatoid synovial tissue (23–28). IgG1, the dominant ACPA isotype, is recognized by all of the so far described RA-derived RF (29). Moreover, RF seem to exhibit enhanced avidity toward agalactosylated forms of IgG (30), which are elevated in RA patients with high disease activity (31). In RA patients, it was reported that ACPA IgG1 tended to be more agalactosylated compared with total serum IgG1, and that, in the joints, ACPA IgG1 was more heavily agalactosylated than in the general circulation (32). Within RA joints, ACPA IgG and citrullinated autoantigenic peptides likely form IC. Taken together, these data strongly argue that, therein, such IC could constitute a (privileged) target of RF. We have previously demonstrated the ability of several monoclonal IgMs with RF activity from patients with mixed cryoglobulinaemia to skew the ACPA-IC–induced proinflammatory cytokine response of macrophages toward more inflammation (33). However, notably given the possible influence on this effect of the fine epitope specificity of RF clones, and, conceivably, of their potentially variable posttranslational modifications, it was necessary to assess whether IgM RF from RA patients also exhibited this property. Moreover, serum IgA RF, usually associated with IgM RF, can be detected in a high proportion of RA patients and is particularly associated with bone erosions and extra-articular manifestations (34, 35). In the present study we assessed the proinflammatory potential of ACPA-IC formed in the presence of IgM or IgA RF purified from RA serum pools, using the previously developed in vitro macrophage stimulation model. The influence of the RA-associated IgM or IgA RF on the capacity of ACPA-IC to mediate activation of the complement cascade was also evaluated.

Materials and Methods

Human sera, Igs, and other reagents

Sera were from informed and consenting patients attending the Rheumatology Center of Toulouse and have been declared and approved by the Comité de Protection des Personnes Sud Ouest et Outre-Mer II (Toulouse, France). The monoclonal IgM RF paraprotein HUL obtained from a patient with type II cryoglobulinaemia was provided by Jean-Louis Pasquali (Strasbourg, France) (36). Human IgM Fc fragments (IgM-Fcγ5) from a patient with type II cryoglobulinemia was provided by Jean-Louis Pasquali (Strasbourg, France) (36). Human IgM Fc fragments (IgM-Fcγ5) were used in the phage stimulation model. The influence of the RA-associated IgM or IgA RF on the capacity of ACPA-IC to mediate activation of the complement cascade was also evaluated.

Quantitative dot blot assay for IgM, IgA, or IgG

The tested fractions (serial dilutions in TBS [pH 8]) were dot-deposited on nitrocellulose membranes (Hybond-C Extra; GE Healthcare). After blocking in TBST containing 2.5% skimmed dry milk (TBSTM), they were probed with TBST-2 diluted HRP-conjugated goat F(ab’)2 Ab fragments directed to human IgM (Fcγ5 specific, 1/1,500,000), IgA (F(ab’)2 specific, 1/1,500,000), or IgG (Fcγ specific, 1/500,000), all from Jackson ImmunoResearch Laboratories, for the detection of IgM, IgA, or IgG, respectively. HRP activity was visualized using ECL reagents (GE Healthcare) and autoradiography. The scanned dot blot signals were quantified using ImageJ software (National Institutes of Health). In each assay, a quantitative standard curve was plotted using the signals given by serial dilutions of a relevant human Ig calibrator, that is, the myeloma-derived monoclonal IgM, the polyclonal IgA, or human serum IgG (Sigma-Aldrich). All samples were tested in two independent duplicates and resulting signals were averaged. The concentration of IgM, IgA, or IgG in the fractions was interpolated from the dilution giving the highest signal falling within the linear portion of the curve. Values taken into account correspond to the mean of at least two independent determinations.

Quantitative ELISAs for IgM RF or IgA RF

Microtiter plates (MaxiSorp; NUNC) were coated with human IgG Fc fragments (Athena Research and Technology) at 15 μg/ml in PBS (pH 7.4). After blocking with PBS containing 2% BSA (PBS-BSA) and washing

<table>
<thead>
<tr>
<th>Serum Pool*</th>
<th>No. of Sera</th>
<th>No. of RA Patients</th>
<th>Volume (ml)</th>
<th>RF Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>30</td>
<td>60</td>
<td>RFM1</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>10</td>
<td>5</td>
<td>RFM1, RFA1</td>
</tr>
<tr>
<td>3</td>
<td>99</td>
<td>66</td>
<td>99</td>
<td>RFM3, RFA1</td>
</tr>
<tr>
<td>4</td>
<td>83</td>
<td>61</td>
<td>152</td>
<td>RFM4, RFA2</td>
</tr>
<tr>
<td>5</td>
<td>187</td>
<td>127</td>
<td>156</td>
<td>RFM5, RFA3</td>
</tr>
</tbody>
</table>

*The serum pools were prepared by mixing roughly equal volumes of sera from RA patients with high titers of ACPA.

**The RF1 fraction was obtained after combining the IgG- and IgM-depleted serum pools of serum pools 2 and 3.

For all chromatographies performed with this serum pool, washing included 3 column volumes of phosphate buffer containing 0.15 M NaCl and 0.1% Triton X-114 to minimize contamination by endotoxins (38).
Table II. RF activity and endotoxin content of the IgM and IgA RF and RF-depleted fractions purified from RA serum pools

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Relative RF Activity</th>
<th>RF Amount (mg)</th>
<th>Endotoxin Concentration (Endotoxin Unit/μg g)</th>
<th>Maximal Endotoxin Concentration in Macrophage Stimulation Assay (Endotoxin Unit/ml)?</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFM1</td>
<td>0.38</td>
<td>0.026</td>
<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>RFM2</td>
<td>0.17</td>
<td>0.04</td>
<td>0.5</td>
<td>12</td>
</tr>
<tr>
<td>RFM3</td>
<td>0.07</td>
<td>0.08</td>
<td>2.5</td>
<td>63</td>
</tr>
<tr>
<td>RFM4</td>
<td>0.29</td>
<td>0.39</td>
<td>3</td>
<td>74</td>
</tr>
<tr>
<td>RFM5</td>
<td>0.45</td>
<td>0.18</td>
<td>0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>RFA1</td>
<td>1.00</td>
<td>1.6</td>
<td>0.007</td>
<td>0.2</td>
</tr>
<tr>
<td>RFA2</td>
<td>3.96</td>
<td>1.8</td>
<td>0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>RFA3</td>
<td>8.90</td>
<td>11.7</td>
<td>0.0001</td>
<td>0.003</td>
</tr>
<tr>
<td>RF IgM1</td>
<td>0.0003</td>
<td>—</td>
<td>0.018</td>
<td>1.2</td>
</tr>
<tr>
<td>RF IgM2</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>RF IgM3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>RF IgM4</td>
<td>0.0082</td>
<td>—</td>
<td>3.75</td>
<td>203</td>
</tr>
<tr>
<td>RF IgM5</td>
<td>0.0004</td>
<td>—</td>
<td>0.008</td>
<td>1</td>
</tr>
<tr>
<td>RF IgA1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>RF IgA2</td>
<td>0.0034</td>
<td>—</td>
<td>0.016</td>
<td>0.3</td>
</tr>
<tr>
<td>RF IgA3</td>
<td>0.0030</td>
<td>—</td>
<td>0.006</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*IgM or IgA RF activities were evaluated by ELISA (see Materials and Methods and supplemental material) using HUL and the first purified IgA RF fraction (RFA1) as calibrators, respectively, and the relative RF activity was calculated by dividing the IgM or IgA RF activity by the relevant Ig (IgM or IgA) mass concentration evaluated by dot blot assay.

**The endotoxin concentration (endotoxin unit) was evaluated by a kinetic Limulus amebocyte lysate chromogenic assay.

The value corresponds to the most unfavorable hypothesis in which the entire amount of endotoxins present in the fractions used at maximal concentration remained in the culture well despite the washes that followed IC formation.

*ND in the fractions were discarded because of bacterial or RF contamination (IgM or IgA RF reactivity representing >1% of the IgM or IgA, respectively).

with PBS containing 0.05% Tween 20 (PBST), samples (serial dilutions of the Ig fractions in PBS-BSA containing 2 M NaCl) were incubated for 2 h. After washing in PBST, the HRP-conjugated goat F(ab\(^{-}\))\(_2\) fragments to human IgM (1/16,000 in PBS-BSA) or IgA (1/4,000 in PBS-BSA) were added for the detection of RF of the IgM or IgA class, respectively. HRP activity was revealed using ortho-phenylenediamine dihydrochloride and the OD at 492 nm measured. For interpretation of results, the OD values considered were the mean OD of at least three independent determinations. These values were relative to the particular specific RF activity of the calibrator used and therefore cannot be considered as absolute estimates of the IgM or IgA RF mass concentrations in the samples.

**Macrophage stimulation**

Monocytes were purified and differentiated into macrophages as formerly detailed (21). Stimulation by ACPA-IC formed in the absence or presence of RF was performed essentially as previously described (33). Briefly, 96-well plates were coated with C-FBG (0.5 μg/well) and IC were generated by incubation with ACPA\(^{-}\) IgG (2.5 mg/ml) supplemented or not with an IgM or IgA RF fraction at the indicated RF mass concentration. All incubations were separated by washes in Dulbecco’s PBS without calcium and magnesium (D-PBS; Life Technologies) containing 0.1% Triton X-114. After final washes in D-PBS, each well received 50,000 cells in a serum-free medium (macrophage-SFM; Life Technologies). ACPA\(^{-}\) IgG and RF-negative IgM or IgA were used as controls. The later corresponded to the purchased IgM or IgA or to the RF-depleted IgM or IgA fractions. Stimulation by ACPA-IC formed in the presence of HUL were also performed. Other assays consisted in placing macrophages on wells coated with increasing concentrations of HAGG or the purchased IgA as in the presence of increasing concentrations of LPS (from Escherichia coli strain 055:B5; Sigma-Aldrich). Alternatively, macrophages were placed on a combination of passively absorbed HAGG and the purchased IgM or IgM-Fc\(\gamma\)R\(_2\) fragments, obtained by sequential coating. To inhibit FcγRI occupancy, peptide M was incubated for 1 h with either the passively adsorbed IgA or the preformed IgA RF-containing ACPA-IC prior to washing and macrophage addition. For all stimulations, after 6 h culture, supernatants were harvested and stored at −30°C until measurement of cytokine concentrations in simplex or multiplex assays, as previously described (33).

**Flow cytometry**

Monocytes and monocyte-derived macrophages were routinely checked for the expression of FcγRI, FcγRII, and FcγRIII as previously described (21). Some macrophages and their monocyte precursors (monocyte and macrophage pairs) were also stained with mouse mAbs reactive with FcγRI (FITC-labeled clone MIP8a; AbD Serotec), Fcγ/μR (PE-labeled clone TX61; BioLegend), or FcγR (unlabeled clones IE4 or RR16 from Novus and Santa Cruz Biotechnology, respectively). Abs were diluted in D-PBS supplemented with 2% human serum (Sigma-Aldrich). Nonspecific staining was systematically controlled using mAbs of matching isotype and labeling configuration. Unlabeled clones were detected with an Alexa Fluor 488-labeled goat anti-mouse IgG (H+L) (Molecular Probes). Samples were acquired on a FACScan flow cytometer (Becton Dickinson) and data analyzed using FlowJo software (Tree Star) after gating on live cells using forward and side scatter parameters.

**ELISA analysis of the IgG content of ACPA-IC formed in the absence or presence of RF**

ACPA-IC were formed in 96-well culture plates in the absence or presence of RF as described for the macrophage stimulation assay except that the concentrations of Ag and IgG were proportionately reduced. Briefly, the plates were coated with 0.03 μg/well C-FBG and ACPA\(^{-}\) IgG were used at 0.15 mg/ml supplemented or not with the specified IgM or IgA RF fraction at the indicated concentration. ACPA\(^{-}\) IgG and RF-negative IgM or IgA were used as controls. The presence of IgG and of IgM or IgA in the formed IC was then assayed in parallel by 1-h incubation with HRP-conjugated secondary Abs, that is, a mouse monoclonal directed to the human IgG Fd portion (Cedarlane, 1/5000), the goat F(ab\(^{-}\))\(_2\) fragments directed to human IgM (1/8000) or the goat F(ab\(^{-}\))\(_2\) fragments directed to human IgA (1/40000), as appropriate. Blocking, washing, and detection of HRP activity were performed as indicated above for the RF ELISA. The OD values considered were the mean OD of triplicate measurements minus the mean OD of triplicate blank wells where, during IC formation, buffer replaced all Abs.

**Complement activation assay**

The assay was adapted from a method described by Lood et al. (40). Briefly, microtiter plates (MaxiSorp; Nunc) were coated with C-FBG (10 μg/ml in PBS; 100 μl/well) and blocked with D-PBS containing 1% BSA. ACPA-IC were then generated by 2-h incubation with ACPA\(^{-}\) IgG at 2.5 mg/ml in D-PBS/1% BSA/2 M NaCl supplemented or not with the RFM3 or the RFA3 fraction. In control conditions, ACPA\(^{-}\) IgG replaced the ACPA\(^{-}\) IgG...
and/or an RF-depleted IgM or IgA fraction (as appropriate) replaced the tested RF fraction. Then, 100 μl normal human serum (Quidel), diluted 1:4 in 0.9 M veronal buffered saline (pH 7.2) containing 0.25 mM CaCl₂ and 1.8 mM MgCl₂ (VBSCaMg), was incubated for 30 min at 37°C. Formation of the terminal C5b-9 complex was detected using a biotin-conjugated anti–SC5b-9 mAb (Quidel), diluted 1:1000 in VBSCaMg containing 1% BSA (VBSCaMg-BSA) and incubated for 2 h at room temperature followed by HRP-conjugated avidin (eBioscience; 1:500 in VBSCaMg-BSA) for 30 min at room temperature. Between each step, the plates were washed in D-PBS containing 0.05% Tween 20. HRP activity was revealed with ortho-phenylenediamine dihydrochloride and the OD at 492 nm was measured. The OD values considered were the OD of sample minus that of a blank well where, during IC formation, buffer replaced all Abs.

Data analysis

For each experiment details on the numbers of parallel and separate replicates made to ascertain the validity of findings are indicated in the corresponding Materials and Methods sections and in the figure legend. In Fig. 1, differences between cytokine secretions were assessed for significance using the paired Wilcoxon’s test, and in Fig. 2, for the calculation of cytokine ratios, values < 1 pg/ml were replaced by 1 pg/ml.

Results

IgM RF and IgA RF from RA patients drive macrophage cytokine secretion induced by ACPA-IC toward a more pronounced proinflammatory profile

The typical evolution of the TNF-α response of macrophages to ACPA-IC when they were formed in the presence of increasing doses of an IgM or IgA RF fraction from RA serum pools is illustrated with assays involving the RFM5 and the RFA3 fractions in Fig. 1A and 1B, respectively. Under control conditions where no IC was formed, that is, when C-FBG was incubated with control IgG fractions devoid of ACPA reactivity (ACPAb− IgG) in the absence or presence of RF, no or very low TNF-α secretion was detected. Conversely, TNF-α secretion increased when, following incubation of C-FBG with ACPAb+ IgG, ACPA-IC were formed (957 ± 186 and 173 ± 61 pg/ml in Fig. 1A and 1B, respectively). Moreover, when ACPA-IC formed in the presence of RFM5 at 2.5 or 25 μg/ml, TNF-α secretion increased ~8- and 27-fold, respectively. In contrast, it was not amplified when ACPA-IC formed in the presence of the RF-depleted −RF IgM5 fraction, showing that the effect of RFM5 depended on its RF activity. RFA3 also induced an amplification of the TNF-α secretion that was not observed with the control RF-depleted IgA (−RF IgA3), although it occurred only when used at 25 μg/ml. Fig. 1C and 1D show the dose-dependent variations of the amplification of TNF-α secretion observed in assays using each of the IgM RF or IgA RF fractions, respectively. Both at 2.5 and 25 μg/ml, the IgM RF fractions systematically amplified TNF-α secretion (p = 0.016 for both doses, in statistical comparisons compiling all assays), as did a monoclonal IgM RF termed HUL derived from a patient with mixed cryoglobulinemia (p = 0.0001 for both doses), with systematically higher amplifications at the highest concentrations (p = 0.016 and p = 0.0001 for the RF IgM fractions and HUL cryoglobulin, respectively). In contrast, there was no amplification with a purchased RF-negative IgM (p = 0.94, not plotted). Similarly, the IgA RF fractions also amplified TNF-α secretion, although only when used at the highest 25 μg/ml dose (Fig. 1D, p = 0.016), whereas no amplification occurred with the purchased RF-negative IgA (p = 0.67, not plotted).

TNF-α, IL-1β, IL-6, IL-8, IL-1Ra, and IL-10 were simultaneously measured in assays of amplification by an IgM RF or an IgA RF fraction from RA sera at the indicated concentration (μg/ml). In control conditions, an ACPAb− IgG fraction (ACPAb− IgG) and the concomitantly obtained RF-depleted IgM (−RF IgM5) (A) or IgA (−RF IgA3) (B), at a concentration matching the protein concentration of the corresponding RF fraction tested, replaced the ACPAb+ IgG and RFM5 or RFA3 fractions, respectively. Bars represent mean concentrations (error bars are SD) of triplicate culture assays performed with monocyte-derived macrophages from one blood donor. (C and D) Amplification of adjusted TNF-α secretion induced by various RF fractions of IgM (C) or IgA (D) class at two concentrations (2.5 and 25 μg/ml) in assays performed with the macrophages of different blood donors. The number in brackets refers to the sequence number of the assay for the related RF fraction. Adjusted TNF-α secretion was calculated by subtracting any generally very low background TNF-α secretion measured when ACPAb− IgG replaced ACPAb+ IgG. Amplification is the ratio of adjusted TNF-α secretion in the presence of RF to the adjusted secretion in its absence. In (C), the median amplification of 14 assays performed with the RF IgM monoclonal HUL is also plotted.

FIGURE 1. Influence of RF of the IgM and IgA classes from RA patients on the TNF-α secretion of macrophages in response to ACPA-IC. (A and B) TNF-α secretion of macrophages stimulated by IC formed by incubating passively adsorbed C-FBG with an ACPAb+ IgG fraction not supplemented (−) or supplemented with a RF fraction of the IgM (RFM5) (A) or IgA (RFA3) (B) class purified from RA sera at the indicated concentration (μg/ml). In control conditions an ACPAb− IgG fraction (ACPAb− IgG) and the concomitantly obtained RF-depleted IgM (−RF IgM5) (A) or IgA (−RF IgA3) (B), at a concentration matching the protein concentration of the corresponding RF fraction tested, replaced the ACPAb+ IgG and RFM5 or RFA3 fractions, respectively. Bars represent mean concentrations (error bars are SD) of triplicate culture assays performed with monocyte-derived macrophages from one blood donor. (C and D) Amplification of adjusted TNF-α secretion induced by various RF fractions of IgM (C) or IgA (D) class at two concentrations (2.5 and 25 μg/ml) in assays performed with the macrophages of different blood donors. The number in brackets refers to the sequence number of the assay for the related RF fraction. Adjusted TNF-α secretion was calculated by subtracting any generally very low background TNF-α secretion measured when ACPAb− IgG replaced ACPAb+ IgG. Amplification is the ratio of adjusted TNF-α secretion in the presence of RF to the adjusted secretion in its absence. In (C), the median amplification of 14 assays performed with the RF IgM monoclonal HUL is also plotted.
IgA RF fraction at 25 μg/ml (two and three assays, respectively) (Fig. 2). Both RF classes increased the secretion of the proinflammatory cytokines IL-1β, IL-6, and IL-8 but also that of the regulatory cytokines IL-10 and IL-1Ra. However, regularly, the TNF-α/IL-10 ratio increased and the IL-1Ra/IL-10 ratio decreased, showing a shift toward the proinflammatory mediators with both classes of RF.

Whereas IgM Fc receptors do not participate in the amplification of the cytokine response of macrophages by IgM RF, FcαRI contributes to amplification by IgA RF.

To investigate the mechanisms underlying amplification of IC-induced responses by RF of the IgM and IgA classes, the expression of established leukocyte-expressed receptors for the Fc portion of IgM and/or IgA by the macrophages used in the ACPA-IC stimulation model was investigated. Paired monocytes and macrophages were analyzed by flow cytometry after staining by mouse mAbs reactive with extracellular epitopes of FcαRI (Fig. 3A). FcαRI was clearly expressed at the surface of both monocytes and macrophages, whereas FcγRI was not expressed by macrophages and was faintly expressed by monocytes. FcγRI was totally absent from both cell types. This was confirmed using a second mAb targeting another extracellular portion of FcγRI (not shown). Additionally, definitively excluding expression of any known or yet undescribed signaling receptor for IgM, macrophages did not secrete any TNF-α in response to saturating amounts of passively adsorbed plate-bound human HAGG was not modulated by the simultaneous presence of 10-fold greater amounts of adsorbed IgM or FcγRI (Fig. 3B). In contrast, fitting with their expression of FcαRI, macrophages secreted TNF-α in response to passively adsorbed plate-bound IgA and this was inhibited dose-dependently when they were preincubated with up to 1 nM of an IgA Fc-binding peptide able to prevent the interaction with FcαRI (Fig. 3C). Moreover, this peptide also dose-dependently inhibited amplification of the TNF-α response of macrophages to ACPA-IC by an IgA RF fraction (Fig. 3D).

**ACPA-IC formed in the presence of IgM RF contain more IgG**

In our macrophage stimulation assay, to reflect what could actually occur in RA synovial membranes, the RF-containing ACPA-IC are formed in the course of simultaneous incubation of C-FBG with the ACPA+ IgG and the IgM or IgA RF. The IC therefore not only include C-FBG, the ACPA captured by this autoantigen, and the RF captured by ACPA, but they could also incorporate additional IgG immunocaptured by the RF that could participate in the stimulation of macrophages. Whether the presence of IgM or IgA RF entailed recruitment of more IgG into the formed ACPA-IC was investigated by ELISA. ACPA-IC were formed in culture plates exactly as for the macrophage stimulation assay except that the amounts of C-FBG and IgG were proportionately reduced by ~17-fold. Formation of ACPA-IC in the presence of RFM5 at increasing concentrations resulted in a dose-dependent increase of not only their IgM but also their IgG content as indicated by the increased detection of IgM and IgG bound at the bottom of the C-FBG–coated culture plate (Fig. 4A). An identical observation was made when HUL replaced the RFM5 fraction (data not shown).

Conversely, formation of ACPA-IC in the presence of RF3, even at a very high concentration that, in the macrophage stimulation assay, would be tantamount to using it at 625 μg/ml, was not associated with any noticeable incorporation of more IgG (Fig. 4B). An identical observation was made with the RFA1 and RFA2 fractions, used at up to 15 μg/ml, representing a 250 μg/ml concentration for the macrophage stimulation assay (data not shown).

**IgM and IgA RF amplify the proinflammatory response of macrophages independently of a possible contamination by endotoxins**

In view of its great capacity to prompt proinflammatory cytokine responses in human macrophages, it was important to rule out that amplification of the cytokine responses of macrophages by the RA-derived RF fractions was not an artifact attributable to their contamination by endotoxins (Table II) that would persist despite the numerous washes with the endotoxin-dispersing surfactant Triton X-114 (38) performed before macrophage addition. For this reason, we also used the concomitantly purified RF-depleted IgM or IgA fractions as controls, as we had observed that their endotoxin content was similar to that found in the matched RF fraction (Table II). The fact that they did not enhance macrophage stimulation by ACPA-IC argues against an effect exclusively mediated by endotoxins contaminating the RF fractions (Figs. 1A, 1B, 5). However, the enhancement of cytokine production could be the result of a cooperation between Ig-mediated and endotoxin-mediated signals, as suggested by the capacity of LPS added in the culture medium to further enhance amplification of TNF-α secretion induced by IgM RF or IgA RF (Fig. 6A, 6B). We therefore determined the maximal endotoxin content that could be tolerated in the RF fractions without risking interference by such cooperation. Fig. 6C and 6D show that LPS added in the culture
medium dose-dependently amplified the TNF-α secretion induced by increasing amounts of plate-bound HAGG or IgA, respectively. However, for all doses of both Igs, this did not occur for LPS concentrations of ≥1 endotoxin unit/ml. Therefore, in view of the endotoxin concentrations measured in the different IgM and IgA RF fractions (Table II), its contribution to amplification of the macrophage cytokine response by all IgA RF fractions and by the RFM1 and RFM5 fractions could be excluded. Of note, with these fractions, the amplifications of cytokine secretions were similar to those prompted by the endotoxin-free RF IgM HUL (Figs. 1C, 2). For the RFM2, RFM3, and RFM4 fractions, it could not be ruled out that some “wash-resistant” endotoxin may have partially contributed to their comparatively higher amplifications of TNF-α secretion.

IgM RF and IgA RF amplify complement activation mediated by ACPA-IC

The capacity for complement activation by ACPA-IC formed in the absence or presence of IgM or IgA RF was measured in a complement activation ELISA (Fig. 7). Confirming previous results (33), complement activation was specifically prompted by IC formed by incubating ACPA+ IgG and plate-bound C-FBG, their main synovial target, with clearly increased levels of deposition of the terminal C5b–9 complex when C-FBG was incubated with ACPA+ IgG in comparison with deposition prompted by C-FBG alone or incubated with ACPA+ IgG. Moreover, complement activation increased when ACPA-IC formed in the presence of the RFM3 fraction at 2.5 or 25 μg/ml of or the RF3A fraction at 25 μg/ml, whereas no increase occurred when they formed in the presence of control RF-depleted IgM or IgA fractions at 25 μg/ml. Finally, C-FBG incubated with ACPA+ IgG and the RF fractions did not activate complement, showing that the amplification of complement cascade activation by ACPA-IC formed with the RF fractions did not result from their direct, nonspecific binding to C-FBG.

Discussion

Previously, to evaluate the effect of incorporation of IgM RF on the proinflammatory potential of ACPA-IC, we had stimulated macrophages by IC formed following coincubation of C-FBG, ACPA+ IgG, and one of several monoclonal IgM RF from patients with mixed cryoglobulinemia. This amplified the macrophage cytokine secretion and skewed it in favor of proinflammatory cytokines, leading to an increased capacity to prompt IL-6 secretion by RA synoviocytes (33). Although interaction sites on human IgGs for FcγRs are primarily situated in their lower hinge region, residues in the Cy2 domain are also involved in the interaction (43). RF is known to interact with epitopes located at the Cy2/Cy3 interface, but variations in the antigenic specificity have been reported, not only for monoclonal cryoglobulinemia-associated IgM RF but also for polyclonal RF or IgM RF clones from RA patients (44–47). The proximity or potential overlap between binding sites for RF and for FcγRs suggests that some RF clones could hinder FcγR engagement by the IgG they bind and thereby inhibit
cytokine induction via this pathway (48). As the epitope specificities of the polyclonal IgM RF present in the serum of RA patients most likely differed from that of the previously tested monoclonal IgM RF, it was therefore important to examine the influence of IgM RF from RA patients. Five different IgM RF fractions isolated from numerous RA sera, most probably targeting a high diversity of epitopes on IgG Fc regions, induced an augmentation of the TNF-α responses, with a similar shift of the cytokine balance toward inflammation by the endotoxin-free IgM RF fractions and the HUL cryoglobulin. This definitively establishes the enhanced proinflammatory potential of ACPA-IC incorporating RA-associated IgM RF. Although beyond the scope of the current study, assessing the global proinflammatory potential of the IgM RF fine specificities coexisting in single patients may unravel interindividual differences for which it would be interesting to investigate the existence of an association with RA severity.

With the monoclonal IgM RF HUL, we had previously demonstrated that TNF-α secretion by macrophages did not occur when interaction with IgG did not take place (33). However, a direct interplay of the IgM RF with a receptor that could strengthen the interaction of cells with IgM RF-containing IC had not been excluded. To rule this out, and because it had not previously been reported, we investigated whether our in vitro M-CSF–generated macrophages expressed the recently identified hematopoietic receptors for IgM Fc, that is, the receptor FccαRIIa, or FcαRIIb, which are all known to be expressed by the M-CSF–generated macrophages (21), could help delineate their respective participations. Given its contribution in the macrophage response to ACPA-IC formed in the absence of RF using an ELISA. The results showed that the presence of IgM RF specifically induced the recruitment of more IgG into the IC, as reflected by an increased detection of IgG bound on the C-FBG–coated culture plate. The pentameric structure of the IgM RF most probably contributes to cross-linking these supplementary IgGs and this contributes to crosslinking FcγR, probably playing a large role in the amplification of the macrophage cytokine response. Disrupting the pentameric structure, for example by the generation of F(ab')2 fragments, should permit further investigations. Nonetheless, taking all data into account, because performing stimulation in the absence of complement components excluded the participation of complement receptors, it is clear that the increased potency for macrophage activation of ACPA-IC formed in the presence of IgM RF involved increased signaling downstream of FcγR. Further experiments, using endotoxin-free Fab fragments of Abs or peptides specifically blocking FcγRI, FcγRIIa, or FcγRIII, which are all known to be expressed by the M-CSF–generated macrophages (21), could help delineate their respective participations. Given its contribution in the macrophage response to ACPA-IC formed in the absence of RF (21, 22), a role for FcγRIIa is to be expected. Recently, a very elegant study used a comprehensive array of

FIGURE 4. IgG content of ACPA-IC formed in the presence of IgM or IgA RF. The amount of IgG incorporated into ACPA-IC formed by incubating C-FBG and ACPA+ IgG in the absence or presence of RFM5 (A) or RFA3 (B) was assayed by ELISA using an HRP-conjugated anti-IgG Fd mAb (filled bars, left-hand y-axes). The incorporation of the tested RF was detected in parallel in identically processed wells, using HRP-conjugated F(ab')2 fragments directed to IgM or IgA, as appropriate (open bars, right-hand y-axes). Bars represent one of at least two representative experiments for each RF dose.

FIGURE 5. Influence of the RFM4 IgM RF fraction in comparison with the matched concomitantly obtained RF-depleted fraction. TNF-α secretion of macrophages from a blood donor stimulated by IC formed by incubating passively adsorbed C-FBG with ACPA+ IgG not supplemented (−) or supplemented with the RFM4 fraction at the indicated concentrations (μg/ml) is shown. In control conditions ACPA+ IgG and the concomitantly obtained RF-depleted IgM (−RF IgM4) replaced the ACPA+ IgG and the RFM4 fractions, respectively. Bars represent mean concentrations (error bars are SD) of triplicate culture assays performed with monocyte-derived macrophages from one blood donor.
isotype variants of a human anti-TNP monoclonal IgG and cells stably transfected with the different human FcγRs and their common allelic variants to examine the impact of IC size on their binding to FcγR (53). Of particular interest for our study that involves autoantibodies that are mainly of the IgG1 and IgG4 subclasses (54) is the observation that an increase in the size of IgG1-IC and IgG4-IC enhanced their binding to all low-affinity receptors, including both the 131H and the 131R variants of FcγRIIA (53). Moreover, very interestingly, as in our study, a positive correlation was observed between the IC size and the capacity to trigger cytokine release, as only the large IgG1-IC could trigger IL-6 production by human PBMCs.

With three IgA RF fractions from RA patients we observed that IgA RF incorporation into ACPA-IC also enhanced the proinflammatory response to these complexes, although to a lower extent than did the IgM RF fractions, with enhancement not only of the TNF-α secretion but also of those of IL-6 and IL-8, as well as shifts of the TNF-α/IL-10 and IL-1Ra/IL-1β ratios in favor of the proinflammatory mediators. This demonstrates the proinflammatory potential of the RA-associated IgA RF. Because we showed that it is not present at the surface of M-CSF–derived macrophages, the contribution of Fcα/μR in this proinflammatory potential could be excluded. However, in accordance with observations by Hamre et al. (55), we confirmed that FcαRI, the only other myeloid-expressed IgA receptor, was highly expressed by monocytes and that it persisted at the surface of M-CSF–generated macrophages. In monocytes, FcαRI cross-linking by specific mAb or by IgA IC results in the secretion of proinflammatory mediators (56). Fitting with their expression of FcαRI, we found that immobilized IgA prompted a significant TNF-α response of the M-CSF–generated macrophages that was inhibited up to 80% when the IgA were incubated with up to an ~40-fold molar excess of a peptide preventing IgA–FcαRI interaction (37). This peptide was not contaminated by endotoxins, but for some unknown reason, instead of leading to a complete inhibition, higher doses of this peptide increased the cytokine release. This prevented us from determining a dose at which cytokine induction by plate-bound IgA is totally inhibited and from using that dose in the experiment with ACPA-IC formed in the presence of IgA RF in an attempt to dissect the respective contributions of the FcαRI and Fcγ receptors to the amplification of cytokine secretion. Nonetheless, at the lowest doses this peptide also dose-dependently inhibited the TNF-α response of macrophages to ACPA-IC formed in the presence of IgA RF. Moreover, we also observed that the coin-

**FIGURE 6.** Influence of costimulation by LPS on the macrophage TNF-α secretion induced by IC. (A and B) TNF-α secretion after stimulation by ACPA-IC formed in the presence of HUL (A) or of the RFA3 fraction (B) both at 25 μg/ml and with or without LPS addition in the culture medium at the indicated concentration (endotoxin unit [EU]/ml). Control conditions notably included ACPA-IC formation in the absence of RF. Bars represent mean concentrations (error bars are SD) of triplicate culture assays performed with the macrophages of one blood donor. (C and D) Dose-dependent variations of the amplification by LPS of TNF-α secretion induced by culture on wells where HAGG (C) or human IgA (D) had previously been adsorbed at the indicated concentrations. The amplification is the ratio of TNF-α secretion in the presence of LPS in the culture medium to TNF-α secretion in its absence. The results correspond to median values of three independent experiments performed with the macrophages of different blood donors.

**FIGURE 7.** Influence of RF of the IgM and IgA classes from RA patients on the complement activation prompted by ACPA-IC. ELISA of the formation of the terminal C5b–9 complement component, induced upon incubation of complement-active normal human serum with ACPA-IC formed by incubating plate-bound C-FBG with ACPA+ IgG in the absence (−) or presence of the RFM3 or RFA3 fraction at the indicated concentration (μg/ml). ACPA+ IgG and RF-depleted IgM (−RF IgM5) or IgA (−RF IgA3) fractions were used as controls at a protein concentration identical to that of the corresponding RF fraction, tested at the highest concentration. Data shown correspond to the mean ± SD of OD values obtained in two independent experiments.
cubation of ACPA+ IgG and IgA RF did not lead to increased recruitment of IgG into the IC. Owing to the limited amounts of available IgA RF we could not generate F(ab\(^{\prime}\))\(_2\) fragments and thereby directly confirm the importance of the IgA Fc signaling in the amplification of the inflammatory response of macrophages to ACPA-IC. However, taken together, our data clearly point toward a very prominent role played by signaling downstream of FcγR in this IgA RF-mediated amplification, in conjunction with ACPA IgG-mediated signaling through activating FcγR.

In view of the suspected participation in RA pathophysiology of TLR4 engagement by endogenous, mostly inflammation-associated, ligands (7), it was interesting to note that LPS substantially enhanced induction of macrophage TNF-α secretion by ACPA-IC containing IgM or IgA RF. FcR engagement by these RA-associated IC and TLR4 ligation by endogenous ligands could therefore cooperate in promoting synovial tissue inflammation. Moreover, fibrinogen has been proposed as an endogenous TLR4 ligand inducing proinflammatory chemokine and cytokine secretion by monocytes or macrophages (22, 57, 58). Additionally, in murine macrophages, citrullination was proposed to enhance the TNF-α inducing potential of fibrinogen and it was reported that IC containing C-FBG stimulate TNF-α production by human M-CSF–generated macrophages both via TLR4 ligation and FcγR engagement (22). However, in our hands, macrophages cultured onto passively adsorbed C-FBG do not secrete more TNF-α than do cells cultured on uncoated wells (mean ± SD, 5 ± 8 versus 8 ± 13 pg/ml with macrophages from 43 blood donors; see also our previous work in Refs. 21 and 33). Moreover, as for many other recently proposed endogenous TLR4 ligands, the direct biochemical interaction of (citrullinated) fibrinogen with this receptor has not been established and it was suggested that, rather, fibrinogen could act as a molecule that directly or indirectly binds its ligands (59). This leaves the interesting possibility that in IC where RF is associated with ACPA that have engaged citrullinated fibrin, this particular ACPA target significantly contributes to further boost macrophage activation by increasing their stimulation via TLR4 ligation, at least indirectly, when exogenous or endogenous agonists of this receptor are present. As yet, this has not been proposed for other established articular ACPA targets.

The aim of our study was to evaluate the proinflammatory properties of the IC formed in rheumatoid synovial membranes by RF, ACPA, and citrullinated fibrin deposits, a major ACPA target in this tissue. That is the reason why we set up an activation test in which such IC are artificially reconstituted (using citrullinated fibrinogen that selectively immunocaptures ACPA) and are presented to macrophages (not monocytes) under an immobilized form. However, as soluble ACPA- and/or RF-containing IC may also stimulate immune cells, notably in the cavity of RA joints, it would be interesting to explore cytokine responses to soluble IC formed by coincubating citrullinated ACPA targets, RF, and pure ACPA IgG. Moreover, we only used M-CSF–generated macrophages, as they may correspond to a good model for at least some of the RA synovial tissue macrophages, which are known to be exposed to this cytokine (60, 61). Because the presence of different macrophage subtypes with different FcR expression profiles in the RA synovial joint is highly probable, it will be important to investigate the cytokine responses to (RF-containing) ACPA-IC not only in differentially polarized monocyte-derived macrophages but also in synovial tissue macrophages.

Within RA synovial membranes, it is also highly probable that RF of the IgG class also incorporates into IC including ACPA and citrullinated fibrin. Even though it was not possible in the present study given difficulties associated with the purification and dosage of this particular RF class, it would be interesting to test its capacity to enhance the proinflammatory response of macrophages to ACPA-IC.

There is solid evidence that both the classical and the alternative pathways of complement are activated during RA (62, 63). Therefore, besides FcR-mediated responses, it was also important to evaluate the influence of RF on complement activation by ACPA-IC. It was shown for both ACPA and RF that they could independently induce complement activation (20, 64, 65). Besides, we showed that in vitro complement activation by immobilized ACPA-IC increased upon incorporation of the HUL RF (33). Consistently, in the present study we found that incorporation of RA-associated IgM RF enhanced complement activation by ACPA-IC. We also report, to our knowledge for the first time, that this is also true for IgA RF, although this occurs at a higher concentration. Therefore, ACPA-IC incorporating IgM or IgA RF probably participate significantly in the triggering of the inflammation-promoting activation of complement cascades occurring in the RA joint.

We have previously shown that, when conjugated to non-ACPAs, IgG, IgM RF paraproteins also could trigger TNF-α secretion by macrophages (33). Additionally, RA-associated IgM or IgA RF fractions amplify the TNF-α response to non-ACPAs, serum IgG from healthy individuals or non-RA rheumatology patients (Supplemental Fig. 4). However, as mentioned in the Introduction, it remains that more subtle differences (possibly arising from divergent glycosylation profiles) may exist that could contribute to variable avidities of the RA-associated RF for IC containing ACPA IgG or non-ACPAs IgG. This fascinating question was beyond the scope and the immunological tools available for the present study. However, clearly, RA is not the only disease associated to RF production, as its serum concentration notably increases in the course of other autoimmune or infectious diseases. Most likely, and this obviously via binding to non-ACPAs IgG, RF has an impact into the inflammatory effects of the cryoprecipitable IC that mediate vasculitis in the mixed cryoglobulinemia syndrome associated to hepatitis C virus infection.

Finally, regarding consistency between our in vitro observations and RA pathophysiology, it would be informative to examine whether RF production, which presumably occurs as a response to the appearance of the disease-specific ACPA-IC (66–68), aggravates the disease in comparison with RA where only ACPA develops. Of note, the concordant presence of IgM RF and ACPA, which occurs in a very large majority of patients, was very recently reported to associate with increased disease activity (69). Even more recently, it was reported that an increase in RF titer enhanced the size and number of erosions in ACPA+ but not ACPA− patients (70). In any event, consistent with our demonstration of the proinflammatory potential of IC including both ACPA and RF is the already firmly established individual association of both autoantibody families with the most severe forms of RA that our studies elucidate, shedding new light on the largely accepted but poorly mechanistically documented role of RF in RA pathophysiology, and pointing its fixation to ACPA bound to citrullinated fibrin as potentially decisive.

Acknowledgments
We thank J.L. Pasquali (Unité Propre de Recherche Centre National de la Recherche Scientifique 9021, Strasbourg, France) for the gift of the HUL paraprotein and A. Shibuya (University of Tsukuba, Ibaraki, Japan) for the gift of the unlabeled anti-FcμR antibody (TX61clone) used in preliminary experiments. The technical assistance of L. Ceccato, E. Parra, M.-F. Isaia, and M.-P. Henry is gratefully acknowledged. We also thank A. Desquesnes and L. Monbrun (Phenotype Analysis Service of the Anexpol platform in Life Sciences of Toulouse Genopole) for performing multiplex
cytokine assays, and F-E. L’Faqihi-Olive, V. Duplan-Eche, and D. Destrade (Cytometry and Cell Sorting Service of the Toulouse RIO imaging platform) for assistance in flow cytometry analyses.

Disclosures
M.S. and G.S. are coinventors of patents that are licensed to companies selling ACPA assays. However, the financial relationship with the companies selling the assays is with Toulouse University and not directly with the authors. The work was not commissioned by any of these companies. None had a role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The remaining authors have no financial conflicts of interest.

References


49. Vire, B., A. David, and A. Wiestner. 2011. TOSO, the Fcγ receptor, is highly expressed on chronic lymphocytic leukemia B cells, internalizes upon IgM binding, shuttles to the lysosome, and is downregulated in response to TLR activation. J. Immunol. 187: 4040–4050.


SUPPLEMENTARY DATA
For “IgM and IgA rheumatoid factors purified from rheumatoid arthritis sera boost the Fc receptor- and complement-dependent effector functions of the disease-specific anti-citrullinated protein autoantibodies”

SUPPLEMENTARY FIGURE 1: Affinity chromatography workflows. Diagrams depicting the sequential affinity chromatographies performed for (A) the isolation of ACPA-containing IgG fractions (ACPA⁺ IgG), of purified RF fractions of the IgM and IgA class (IgM RF and IgA RF, respectively) and of IgM and IgA fractions devoid of RF reactivity (RF-depleted IgM and RF-depleted IgA, respectively) starting from pools of ACPA-positive RA sera, (B) the isolation of IgG solutions devoid of ACPA reactivity (ACPA⁻ IgG) starting from pools of ACPA-negative sera. The different affinity matrixes used are symbolized by grey boxes with a white-printed ligand name. The starting material and content of the fractions that flowed through, were pre-eluted or were eluted from the columns are indicated in the white boxes. The fractions used in macrophage stimulation assays are bold-printed.
SUPPLEMENTARY FIGURE 2: Effect of a pre-elution step on the IgM RF and IgA RF content of the ACPA+ IgG solutions eluted from the protein G column. The IgG fraction of a pool of 33 ACPA-positive RA sera was purified by affinity chromatography on a protein G column in which bound Ig were eluted using 0.2 M glycine-HCl at pH 2.7 either directly (no pre-elution) or after a pre-elution using 0.2 M glycine-HCl at pH 4.5, 4.0 or 3.5. The pre-elution and elution fractions were collected and their content in IgG, IgM and IgA and in IgM RF and IgA RF were determined by quantitative dot blot assay or ELISA, respectively. The IgG ACPA reactivity was assayed in serial dilutions by ELISA on in vitro citrullinated human fibrinogen (Chapuy-Regaud et al. Clin Exp Immunol, 139:542, 2005). The IgG ACPA reactivity in the fraction was then calculated by multiplying the fraction volume by its titre-like value, obtained by multiplying the highest OD that fell within the linear portion of a standard curve plotted using a pool of ACPA-positive RA sera by the corresponding dilution factor. For each chromatography condition, the data of the pre-eluate and of the eluate were summed and their relative proportions (in %) were plotted. Part of the IgM and IgA present in the serum pool loaded on the column did not directly flow through it and co-eluted with IgG when directly using glycine-HCl at pH 2.7. Most of these IgM and IgA probably correspond to RF bound to the protein G-captured IgG, since RF of the IgM and IgA class was detected in this fraction. When a pre-elution step at pH 4.5 preceded elution, not only most IgG but also most IgM and IgA remained in the elution fraction. Conversely, with a pre-elution step at pH 4.0 or at pH 3.5, while most IgG remained in the elution fraction, most ‘contaminating’ IgM and IgA and RF of the IgM and IgA class exited the column in the pre-elution fraction, the proportion in the pre-elution fraction being higher at pH 3.5 than at pH 4.0. However, pre-elution at these pH also led to a loss of ACPA IgG that was more important at pH 3.5 than at pH 4.0. Out of the tested protocols, pre-elution at pH 4.0 was therefore the best compromise for getting rid of most IgM and IgA RF reactivities with minimal loss of ACPA.
SUPPLEMENTARY FIGURE 3: Purity and RF activity in the RF and RF-depleted fractions. (A) The purity of the IgM or IgA RF fractions (RFM2-5 and RFA1-3, respectively) and of the RF-depleted IgM or IgA fractions (-RF IgM4 or -RF IgA2 and -RF IgA3, respectively) was checked by subjecting 1 µg of each protein solution to SDS-PAGE using miniature 4-15% gradient gels ran on a PhastSystem (GE-Healthcare), along with the purchased monoclonal IgM and polyclonal IgA (mIgM and pIgA, respectively, 1 µg each), followed by Coomassie blue staining that showed the absence of undesired protein contaminants. (B) The relative proportions of IgM, IgA and IgG in the different obtained IgM or IgA RF fractions (RFM1-5 and RFA1-3, respectively) were evaluated using quantitative dot blot. Labels indicate the proportion of the most abundant Ig class. The RF activities of the IgM (C) and IgA (D) class present in the different purified Ig fractions were evaluated in ELISAs that used human IgG Fc fragments as the immunodetected Ag. OD values minus blank are shown. Reactive or unreactive reagents (RA serum pool and serum from an healthy individual for both ELISAs, the cryoglobulinemia-derived monoclonal IgM RF HUL and the purchased myeloma-derived RF-negative IgM (mIgM) in the IgM RF ELISA, and the purchased polyclonal RF-negative IgA in the RF IgA ELISA (pIgA)), allowed verifying that both assays had adequate sensitivity and specificity. As examples, the OD values minus blank given by serial dilutions of the RFM5 and RF-depleted IgM5 fractions in the IgM RF ELISA (C) and by serial dilutions of the RFA2 and RF-depleted IgA2 fractions in the RF IgA ELISA (D) are shown. Note that for these fractions, the indicated concentrations correspond to the protein concentrations estimated by dot-blot assay.
SUPPLEMENTARY FIGURE 4: Influence of IgM and IgA RF from RA patients on the TNF-α secretion of macrophages in response to non-ACPA IgG. Ninety six-well culture plates were coated with non-ACPA IgG (serum IgG from healthy donors from Sigma-Aldrich, 0.1 µg/well) and blocked with D-PBS containing 2 % BSA. IC containing RF and the non-ACPA IgG were then generated by incubation with the indicated IgM or IgA RF fraction at the indicated concentration (µg/ml) in the presence of the non-ACPA IgG at 0.5 mg/ml (IgG). In control conditions, the IgG and/or RF fraction was omitted (-) or the concomitantly obtained RF-depleted IgM (-RF IgM5) or IgA (-RF IgA3) fraction was used, at a protein concentration matching that of the related RF tested at maximal concentration. All incubations were separated by washes in D-PBS containing 0.1 % Triton X-114. After final washes in D-PBS, each well received 50,000 cells in Macrophage-SFM medium and, after 6-h culture, supernatants were harvested for TNF-α quantitation. Bars represent mean concentrations (error bars are SD) of triplicate culture assays and the results shown are representative of data from two experiments independently conducted with the macrophages from different blood donors, testing each time not only the above-mentioned serum IgG from healthy donors but also the ACPA- IgG, prepared as described in the Materials and Methods section of the manuscript, from pools of ACPA-negative sera of non-RA rheumatology patients.