IgA complexes in plasma and synovial fluid of patients with rheumatoid arthritis induce neutrophil extracellular traps via FcαRI

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Abstract
Autoantibodies, including rheumatoid factor (RF), are an important characteristic of rheumatoid arthritis (RA). Interestingly, several studies reported a correlation between the presence of immunoglobulin A autoantibodies and worse disease course. We previously demonstrated that triggering the IgA Fc receptor (FcαRI) on neutrophils results in neutrophil recruitment and release of neutrophil extracellular traps (NETs). As this can lead to tissue damage, we investigated whether IgA immune complexes in plasma and synovial fluid of RA patients activate neutrophils. RF isotypes were measured with ELISA and immune complexes were precipitated using polyethylene glycol (PEG) 6000. Isolated neutrophils were incubated with immune complexes and activation and release of NETs was determined in the presence or absence of FcαRI blocking antibodies. Plasma and SF of RA patients contained IgM RF, IgG RF and IgA RFs. Patient plasma IgA RF and IgM RF showed a strong correlation. No uptake of IgM and minimal endocytosis of IgG immune complexes by neutrophils was observed, in contrast to avid uptake of IgA complexes. Incubation of neutrophils with immune complexes resulted in production of reactive oxygen species, release of NETs, lactoferrin and chemotactic stimuli. Importantly, activation of neutrophils was reduced when FcαRI was blocked. Neutrophils were activated by IgA immune complexes, which suggests that neutrophils play a role in inducing joint damage in RA patients who have IgA autoantibody complexes, hereby increasing severity of disease. Blocking FcαRI inhibited neutrophil activation and as such may represent an attractive novel additional therapeutic strategy for the treatment of RA.
Introduction
Rheumatoid arthritis (RA) is a systemic chronic autoimmune disease, which is characterized by inflammation of the joints \(^1\). This disease leads to long-term joint damage, resulting in chronic pain, loss of function and disability in patients. RA has a prevalence of 1\% and is two to three times more common in women than men. The cause of RA is unknown and the molecular pathology is still poorly understood, but it is likely a multifactorial disease in which both genetics and environmental factors play crucial roles \(^2\). An important characteristic of RA is the presence of autoantibodies. These include rheumatoid factors (RF), which are autoantibodies specifically directed against the Fc domain of immunoglobulin G (IgG) \(^3,4\). A second type of RA-related autoantibodies are anti-citrullinated protein antibodies (ACPA) \(^5,6\). The combination of RF and ACPA is generally accepted as a diagnostic tool by most rheumatologists.

RF and ACPA can be of the IgM, IgG or IgA antibody subclasses. Although some conflicting results were reported \(^7\), most studies suggested a correlation between the presence of IgA RF in blood or synovial fluid and worse disease prognosis as well as severe manifestations in RA \(^8,9,10,11,12\). A recent study showed that the amount of IgA RF was significantly associated with severity of disease in RA patients that were IgM RF negative. No correlation was found between IgG RF levels and the severity of RA disease \(^10\). Furthermore, IgA RF were also implicated in extra-articular disease manifestations \(^11\). In a large patient cohort study, a positive correlation was found between increased serum IgA and the grade of cartilage damage in active RA \(^12\). Anti-tumor necrosis factor (TNF-\(\alpha\)) treatment of RA patients was shown to reduce RF levels and alleviated disease \(^16,17\). However, patients with high pre-treatment IgA RF levels had a poor clinical response to TNF-\(\alpha\) inhibitors \(^16,17\).

IgA RF or IgA anti-CCP antibodies have been reported to possess predictive value for the progression of disease \(^13\).

Taken together, these data prompted us to hypothesize that IgA autoantibodies might be involved in causing joint cartilage erosion in active RA disease, as we previously demonstrated that IgA antibodies potently activate polymorphonuclear cells (PMNs; neutrophils) \(^18,19\). These are abundant circulating effector cells of the innate immune system that have long been viewed as short-lived cells with a primary function in bacterial elimination \(^20\). However, excessive neutrophil activation can result in severe tissue damage and chronic inflammation as they contain a multitude of granules containing cytotoxic molecules \(^21\). We demonstrated that crosslinking of Fc\(\alpha\)RI by IgA complexes induced release of the chemoattractant leukotriene B4 (LTB4), leading to amplification of neutrophil migration \(^18\). Furthermore, the hypothesis that IgA–Fc\(\alpha\)RI interactions play a harmful role in autoimmune diseases is supported by our previous results investigating the rare skin blistering disease Linear IgA Bullous Disease (LABD) \(^22\). LABD is characterized by IgA autoantibodies directed against collagen XVII and neutrophil influx in the skin of patients, as well as extensive skin damage and blister formation. We previously demonstrated that neutrophil activation and concomitant tissue damage is a direct consequence of Fc\(\alpha\)RI triggering through IgA autoantibodies \(^19\). Moreover, nowadays it is more appreciated that neutrophils can secrete a multitude of anti- and proinflammatory mediators and play a role in inducing (in)appropriate innate and adaptive immune responses \(^23\). Neutrophils express
FcαRI, which is a member of the Fc receptor Ig superfamily. Crosslinking of FcαRI by IgA complexes induces multiple proinflammatory functions, including phagocytosis, respiratory burst, degranulation, antibody-dependent-cellular cytotoxicity, release of cytokines and inflammatory mediators. Additionally, neutrophils have the ability to release neutrophil extracellular traps (NETs), which is increased after crosslinking of FcαRI by IgA antibodies. NETs are web-like structures that can trap and possibly kill bacteria and fungi. NETs contain DNA, histones and several granular and cytoplasmic antimicrobial proteins, like elastase and myeloperoxidase (MPO). NETs release has been implicated in the pathology of different autoimmune disorders, such as systemic lupus erythematosus (SLE) and RA. As neutrophils are found in high numbers in the SF of RA patients, we investigated whether IgA autoantibody complexes in plasma and SF of RA patients activate neutrophils and induce NETs release.

**Materials and Methods**

**Patients**

Plasma (21 patients) and synovial fluids (5 patients) of diagnosed RA patients were collected during their visit to the rheumatology department at VU University Medical Center (see Table I for patient characteristics). Patients gave their written informed consent to their involvement in the study in accordance with the guidelines of the Medical Ethical Committee of the VU University Medical Center (The Netherlands).

**Isolation of human PMNs from healthy controls**

PMNs were isolated from peripheral blood that was obtained from healthy donors using Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation, after which erythrocytes were lysed in ammonium chloride buffer (155 mM NH₄Cl, 10 mM KHCO₃ and 0.11 mM EDTA, 10 minutes, RT). After lysis, PMNs were washed with phosphate-buffered saline (PBS; B.Braun, Melsungen, Germany). Cells were resuspended in RPMI 1640 (Gibco BRL, Paisley, UK) supplemented with glutamine and antibiotics. Additionally, a low concentration of 1% heat-inactivated fetal calf serum was added (60 minutes, 70 °C), because fetal calf serum contains heat stable nucleases that can interfere or break down NETs (medium hereafter referred to as RPMI 1%). Before experiments, isolated PMNs were allowed to settle for 1 hour at 37 °C. Blood samples were collected after donors gave informed consent, in accordance with the guidelines of the Medical Ethical Committee of the VU University Medical Center (The Netherlands). For plasma collection (8 healthy volunteers), blood was diluted 1:1 with PBS, and Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation was performed. The plasma fraction (1:1 diluted in PBS) was collected and stored at -80 °C in aliquots until use.

**Preparation of plasma and SF**

After SF collection, samples were centrifuged at 3420 x g for 5 minutes. The supernatant was removed and stored at -80 °C in aliquots until use. Blood was diluted 1:1 with PBS, after which Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation was performed. The plasma fraction was collected and stored at -80 °C in aliquots until use.
Measurement of RF antibody isotypes with ELISA
IgA RF, IgM RF and IgG RF were measured in duplo’s in plasma and SF with ELISA, adapted from 11. Briefly, flat well microtitre ELISA plates (Nunc-Immuno MaxiSorp, Roskilde, Denmark) were coated with 100 µl rabbit IgG (40 µg/ml, Sigma-Aldrich, St. Louis, MO). Non-specific binding sites were blocked by incubation with PBS containing 0.05% Tween-20 (PBST, 200 µl) and 1% bovine serum albumin for 2 hours at room temperature (RT). After washing with PBST, plates were incubated with 1:40 diluted (in PBST, 100 µl) plasma or SF for 2 hours at RT. Plates were washed, after which they were incubated with horseradish peroxidase (HRP) conjugated F(ab')2 fragments of goat anti-human IgA (1:500; Thermo Fisher Scientific Incl., Waltham, MA, USA), F(ab')2 fragments of goat anti-human IgM (1:500; Thermo Fisher Scientific) or F(ab')2 fragments of goat anti-human IgG (1:500; Thermo Fisher Scientific) for 1 hour at 37 °C. After addition of the substrate ((3,3', 5,5')-tetramethylbenzidine), plates were read with a microplate reader (Bio-Rad, Berkely, CA) at 450 nm. The results of the individual test samples were read and expressed in optical density (OD). Experiments were repeated 3 times.

Precipitation of RF immune complexes from plasma and SF
Immune complexes were precipitated from plasma and SF with polyethylene glycol (PEG) 6000. Plasma and SF were centrifuged at 3420 x g for 5 minutes before use. Supernatants were mixed with an equal volume of 10% PEG6000 (final concentration of 5% PEG 6000). After overnight incubation at 4°C, precipitates were collected by centrifugation for 10 minutes at 9560 x g. Precipitates were solved in PBS to the original volume of the plasma or SF and placed on ice for use at the same day in experiments.

Preparation of soluble heat-aggregated IgA
Aggregation of IgA was performed by diluting serum IgA (Cappel, MP Biomedicals, Santa Ana, CA) in PBS and by heating for 30 minutes at 63 °C. After cooling down to RT, the protein was centrifuged at 10,000 x g for 2 minutes to remove the insoluble aggregates. The supernatant was then used as the heat-aggregated IgA.

Live cell imaging
For monitoring of NET release in time, nuclei of isolated live PMNs were labeled with Hoechst nucleic acid stain (1:1000; Invitrogen Life Technologies, Carlsbad, CA) in RPMI 1% (30 minutes, 37°C). Cells were washed and allowed to settle (1 hour, 37°C), after which they were incubated in Ibidi µ-slides (Ibidi, Martinsried, Germany) (2x105/well). Additionally, the nucleic acid label SYTOX green (2.5 µg/ml; Invitrogen Life Technologies) was added to the medium to detect the presence of extracellular DNA during recording. PMNs were stimulated with 1-5% PEG 6000 precipitates of RA patients’ plasma or SF and monitored in time at 37°C with live cell microscopy (Olympus IX81, Tokyo, Japan).

Immunofluorescence staining of NETs
PMNs were added to Lab-Tek Chamberslides (1.5x105/well; Nalge NUNC, Rochester, NY) and stimulated with non-precipitated plasma or SF as well as 1-5% PEG 6000 precipitates
for 3 hours at 37°C. After incubation, cells were fixed for 15 minutes at RT with 4% paraformaldehyde, after which the fixative was removed. Slides were stained with DAPI (1:10000; Invitrogen Life Technologies) to visualize DNA and with anti-MPO or Anti-Neutrophil Elastase rabbit polyclonal antibodies (1:50; Calbiochem, Millipore, Billerica, MA) followed by incubation with goat anti-rabbit IgG secondary antibody labeled with fluorescent Alexa Fluor 488 dye (1:400; Invitrogen Life Technologies). Cells were visualized using a Leica DM6000 microscope (Leica, Solms, Germany).

Quantitative fluorometric analysis of NETs release
PMNs (1x10^5) were incubated in RPMI 1% in black 96 wells plates (FLUOTRAC 200, Greiner Bio-One) with non precipitated RA patients plasma or SF or 1-5% PEG 6000 precipitates or heat-aggregated IgA for 3 hours at 37°C. Where indicated, either DNase I (0.1 mg/ml; Roche, Penzberg, Germany) was added or PMNs were pre-incubated with anti-FcαRI mAb MIP8a (20-60 µg/ml; AbD Serotec, Raleigh, NC) for 20 minutes on ice to block FcαRI or with an isotype control (Ultra-LEAF™ Purified Mouse IgG1, κ Isotype Ctrl Antibody, Biolegend). Release of extracellular DNA was detected by adding nucleic acid label SYTOX green (2.5 µg/ml; Invitrogen Life Technologies;). Optical density was measured using a fluorimeter (FLUOstar/POLARstar BMG Labtech GmbH, Offenburg, Germany) at 480nm excitation, 520nm emission.

Binding and uptake of complexes by neutrophils
PMNs were incubated with PEG 6000 precipitated samples or heat-aggregated IgA at 4°C for 1 hour, followed by incubation at 37°C for 30 minutes. After incubation, cells were washed and cytopsins were made. Slides were fixed for 10 minutes at RT with acetone. Immunofluorescence was performed and cytopsins were stained for IgA (Polyclonal F(ab’)2 Rabbit Anti-Human IgA-FITC, DAKO, Glostrup, Denmark), IgG (Polyclonal IgG F(ab’)2 Goat Anti-Human-FITC, AbD Serotec, Raleigh, NC) or IgM (Polyclonal Rabbit Anti-Human IgM/HRP, DAKO, Glostrup, Denmark and Goat Anti-Rabbit-alexa fluor 488), FcαRI (Monoclonal Mouse Anti-Human CD89-PE, BD Pharmingen) and DNA (DAPI, Invitrogen Life Technologies).

Reactive oxygen species (ROS) production
PMNs were preloaded for 20 minutes at 37°C with the fluorescent probe CM-H_2DCFDA (10 μM; Invitrogen Life Technologies) according to the manufacturer’s guidelines. After incubation, cells were washed twice and carefully resuspended in RPMI 1% in black 96 wells plates (FLUOTRAC 200, Greiner Bio-One). PMNs (1x10^5) were stimulated with 1-5% PEG 6000 precipitates from RA patients plasma or SF or heat-aggregated IgA. Optical density was measured every 5 minutes for 3 hours in a preheated fluorimeter at 37°C (FLUOstar/POLARstar) at 480 nm excitation, 520 nm emission.

Measurement of lactoferrin in supernatant of stimulated cells
Lactoferrin was measured in the supernatants of PMNs (in duplicate) that had been stimulated for 3 hours with immune complexes of RA patients’ plasma or SF or heat-
aggregated IgA at 37 °C. Flat well microtitre ELISA plates (Nunc-Immuno MaxiSorp, Roskilde, Denmark) were coated with 100 µl of rabbit anti-human lactoferrin antibodies (50 µg/ml; Sigma-Aldrich. St. Louis, MO) followed by a blocking step with 200 µl PBST containing 0.5% bovine serum albumin for 1 hour at 37 °C to block non-specific binding sites. Plates were then incubated with 2x diluted supernatant for 1 hour at 37 °C, followed by incubation with alkaline phosphatase–labeled rabbit anti-human lactoferrin antibodies (1:2500; MP Biomedicals, LLC, Solon, OH) for 1 hour at 37 °C. Plates were washed between incubation steps with PBST. After addition of the chromogenic substrate P-nitrophenyl phosphate (Sigma-Aldrich. St. Louis, MO), plates were read with a microplate reader (Bio-Rad) at 405 nm. Purified human lactoferrin (Sigma-Aldrich. St. Louis, MO) was used as a standard to calculate the amount of lactoferrin in the measured samples. Experiments were repeated 3 times.

**Chemotaxis assay**
PMNs were fluorescently labeled for 30 minutes at 37°C with the fluorescent probe calcein-AM (1 µM; Molecular Probes, Eugene, OR). After incubation, cells were washed twice and carefully resuspended in RPMI 1%. To measure chemotaxis (directed migration), bottom wells of blind well chambers were filled with 26 µl supernatants of stimulation experiments (see above) or purified LTB4 (Sigma-Aldrich. St. Louis, MO, 10^{-7} M) as positive control. Calcein AM-labeled neutrophils (50 µl, 5x10^5) were added to top wells. After incubation for 40 minutes at 37°C, cells that had migrated towards the lower compartments were quantified in black 96 wells plates (FLUOTRAC 200, Greiner Bio-One) with a fluorimeter (FLUOstar/POLARstar) at 480 nm excitation, 520 nm emission.

**Statistical analysis**
Statistical analysis was performed using GraphPad Prism version 4 software (GraphPad Software, San Diego, CA). Statistical differences were determined using unpaired Student two-tailed t test (2 groups), or with ANOVA (>2 groups), or with non-parametric wilcoxon matched pairs one-tailed (matched samples). Correlations were determined using non-parametric Spearman correlation coefficient (r). A p value <0.05 was considered statistically significant.
Results

Detection of RF in plasma and synovial fluid of RA patients

The presence of IgM RF, IgG RF and IgA RF was determined in non paired plasma and SF samples of RA patients (see Table I for patient characteristics). Most patients’ plasma and SF contained increased IgA RF and IgM RF compared to healthy donors (Figure 1A). In contrast, overlap in IgG RF levels was observed between patient and healthy donor plasma. A strong correlation between presence of IgA RF and IgM RF in patients plasma was observed, but only a weak correlation of IgG RF with either IgA RF or IgM RF was found (Figure 1B).

<table>
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<td>Disease duration, years</td>
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<td>Anti-CCP positivity at time of diagnosis (pos/neg/not known)</td>
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<td>Biologics (yes/no)</td>
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Table I: Rheumatoid arthritis patient characteristics. Blood was sampled during a routine visit to the polyclinic. Data are presented as mean ± SD or number. F, female; M, male; pos, positive; neg, negative; anti-CCP, anti-cyclic citrullinated peptide antibodies; RF, rheumatoid factor; DMARDs, disease-modifying anti-rheumatic drugs. Of note, synovial fluid was taken from patients in active disease to alleviate their symptoms. Samples were received anonymously. As such, patient characteristics are not included in the table.

Plasma and synovial fluid of RA patients induce NETs release by neutrophils

To investigate the ability of patients’ plasma and SF to activate neutrophils, isolated peripheral blood neutrophils were incubated with plasma or SF. Minimal release of extracellular DNA, indicative of NETs formation, was observed when neutrophils were incubated with plasma from healthy controls, which was comparable with resting neutrophils that had been kept in culture medium (Figure 2A). By contrast, incubation with plasma or SF of RA patients resulted in the formation of NETs, which were destroyed when cell stimulation occurred in the presence of DNase and therefore could not be detected (Figure 2A). MPO and elastase are granular proteins that are components of NETs. The presence of extracellular DNA that stains positive for these granular markers is consistent with the process of NET formation 30. To confirm the release of NETs, we therefore performed immunostaining, which demonstrated that extracellular DNA stained positive for both elastase (Figure 2B) and MPO (Figure 2C) when neutrophils were incubated with plasma or SF of RA patients. Resting neutrophils or cells that had been stimulated with plasma from healthy controls did not release NETs.
IgA immune complexes induce NETs

Figure 1: RF presence in non-paired samples of plasma and synovial fluid from RA patients. (A) The presence of IgA RF (left panel), IgG RF (middle panel) and IgM RF (right panel) in RA patients’ plasma and SF was determined with ELISA. n.s.: non significant; ***p < 0.05; ****p < 0.0001. (B) Correlation between IgA RF, IgG RF and IgM RF in RA patients plasma and SF. Each point represents the mean of triplicates of one patient sample. Experiments were repeated three times. One representative experiment is shown.

Figure 2: Plasma and synovial fluid of RA patients induce NETs formation. (A) Quantification of extracellular DNA with sytox green (which reflects NETs) after 3h when PMNs were non stimulated (●, dotted line represents average of NETs release by non-stimulated PMNs) or stimulated with healthy controls plasma (△), RA patients plasma (●) or patients SF (▲). Extracellular DNA was also measured after stimulation of cells with RA patients’ plasma or SF in the presence of DNase (x). ***p < 0.001. (B+C) After incubation for 3 hours in different conditions, PMNs were stained for (B) elastase and (C) MPO in red. DNA was stained with DAPI (blue). Scale bar, 10 μm. Experiments were repeated three times, yielding similar results.
**IgA autoantibodies contribute to NETs release that is dependent on FcαRI**

We have previously shown that activation of neutrophils via IgA antibodies is mediated through FcαRI. Interestingly, crosslinking of FcαRI by IgA-coated particles induced NETs release, which could be inhibited by a FcαRI blocking mAb. To test the hypothesis that the presence of IgA autoantibodies can lead to NETs release, we incubated neutrophils with plasma or SF in the presence or absence of the FcαRI blocking mAb MIP8a. Incubation of cells with MIP8a, followed by stimulation with plasma from healthy controls did not affect NETs release (data not shown). However, blocking FcαRI resulted in decreased NET formation by neutrophils after incubation with 14 out of 21 plasma samples of RA patients (Figure 3A) and in 4 out of 5 SF samples (Figure 3C). In general, the presence of a higher score of IgA RF in plasma (Figure 3B) and SF (Figure 3D) of patients resulted in more NETs release, which were also responders to MIP8a. By contrast, when a lower amount of IgA RF was present in the plasma, generally less NETs release was observed, which could also not be blocked by incubation of neutrophils with MIP8a.

![Figure 3: NETs formation is partly inhibited by blocking FcαRI. (A+C) Quantification of extracellular DNA with sytox green (which reflects NETs) after 3h when PMNs were not stimulated (*, dotted line represents average of NETs release by non-stimulated PMNs) or stimulated with (A) RA patients’ plasma (●) or (C) SF (■). PMNs were also stimulated with plasma (○) or SF (□) of RA patients in the presence of MIP8a. *p < 0.05. (B+D) Correlation between the presence of IgA RF and NET release of responders (♦) and non responders to MIP8a (◊) in (B) plasma or (D) SF.](image)

**Isolated IgA immune complexes induce release of ROS, lactoferrin and chemotactic factors**

To establish the contribution of autoantibody complexes present in plasma or SF in activation of neutrophils, immune complexes were precipitated from RA patients’ samples.
Moreover, IgA antibodies bind to FcαRI with moderate affinity, while larger IgA-immune complexes bind avidly. For this reason, large immune complexes present in the plasma or SF of RA patients were isolated by PEG 6000 precipitation. The majority of these precipitates contained IgA RF, IgM RF and IgG RF (data not shown). When neutrophils were incubated with these immune complexes, no binding or uptake of IgM was observed. Additionally, minimal uptake of IgG immune complexes occurred, in contrast to IgA immune complexes, which were taken up avidly by neutrophils (Figure 4A). First, heat-aggregated IgA was used as surrogate IgA immune complexes to show that they are able to induce activation and NETosis (Supplementary Figure 1). Next, we studied activation of neutrophils by isolated immune complexes from RA patients’ plasma or SF. The production of ROS is an important step in the formation of NETs. As such, we investigated ROS production by neutrophils after addition of isolated immune complexes. Minimal ROS production was observed over time in resting neutrophils or in cells that were incubated with precipitates of healthy controls (Figure 4B). In contrast, when neutrophils were incubated with precipitates of RA patients’ plasma or SF, a clear increase in ROS production was observed over time (Figure 4B). Furthermore, stimulation of neutrophils with isolated immune complexes of RA patients’ plasma or SF resulted in degranulation of cells, which is reflected by lactoferrin release (present in secondary granules) (Figure 4C). Supernatants were collected to investigate whether chemotactic stimuli had been released by neutrophils after addition of isolated immune complexes of RA patients’ plasma or SF. Neutrophils did not migrate towards supernatants of experiments in which neutrophils were not stimulated or stimulated with plasma of healthy controls. By contrast, supernatants of experiments in which neutrophils were stimulated with isolated immune complexes of RA patients’ plasma or SF showed chemotactic activity (Figure 4D).

**Isolated immune complexes of patients’ plasma or SF induce NETs release partly via FcαRI**

NETs release was visualized with fluorescent microscopy using real-time video recording after stimulation of neutrophils with isolated immune complexes of RA patients’ plasma or SF. Minimal NET formation was demonstrated by unstimulated neutrophils over time (Figure 5A and Video 1), in contrast to neutrophils that had been exposed to RA patients’ plasma or SF (data not shown) or isolated immune complexes of RA patients’ plasma or SF (Figure 5A and Video 2&3). To confirm release of NETs, we performed immunostaining, which demonstrated that extracellular DNA stained positive for both elastase (Figure 5B) and MPO (Figure 5C). Blocking FcαRI resulted in decreased NET formation after stimulation of neutrophils with isolated immune complexes of RA patients’ plasma (Figure 5D) or SF (Figure 5E), which was not seen when an isotype control antibody was used. Furthermore, NET release was abrogated in the presence of DNase. Thus, NET release was partly inhibited by blocking FcαRI, supporting that neutrophils are activated via FcαRI by IgA complexes present in plasma and SF of most RA patients.
Figure 4: Isolated immune complexes from RA patients’ plasma and synovial fluid activate neutrophils and induce migration. (A) IgA, IgG and IgM complexes uptake by neutrophils. PMNs were incubated with PEG 6000 precipitated samples at 4°C for 1 hour, followed by incubation at 37°C for 30 minutes. Cytospins were stained for IgA, IgG or IgM (green), FcγRI (red) and DNA (blue). (B) ROS production was measured over time (3h) in unstimulated PMNs (○, dotted line) or after stimulation with PEG 6000 precipitated immune complexes of healthy controls’ plasma (▲), or plasma (blue ●, left panel) or SF ( blue ■, right panel) of RA patients. (C) Lactoferrin release (reflecting degranulation of PMNs) was measured by ELISA in duplicates of supernatants of non-stimulated neutrophils (*, dotted line represents average of NETs release by non-stimulated PMNs) or after stimulation of neutrophils with PEG 6000 precipitated immune complexes of RA patients’ plasma (●) or SF (■). Experiments were repeated three times. One representative experiment is shown. **p < 0.05 (D) Chemotaxis towards supernatants of neutrophils that had been stimulated with PEG 6000 precipitated immune complexes for 3 hours. LTB4 served as a positive (pos) and medium as negative (neg) controls. *p < 0.05.
IgA immune complexes induce NETs

Figure 5: Isolated immune complexes induce NETs formation partly via FcεRI. (A) Images of live PMNs stained with a nuclear dye (Hoechst, blue), which were incubated in culture medium containing sytox green (stains extracellular DNA). Cells were followed over time with live-cell microscopy after stimulation with isolated immune complexes from RA patients’ plasma (middle panels) or SF (lower panels). Non stimulated PMNs served as control (upper panels). PMNs were stained for (B) elastase and (C) MPO in red and DNA was stained with DAPI (blue), after stimulation of PMNs with PEG 6000 precipitated immune complexes from RA patients’ plasma or SF compared to non stimulated cells. Scale bar, 10 μm. (D-E) Quantification of extracellular DNA with sytox green (which reflects NETs) after 3h when PMNs were non stimulated (*, dotted line represents average of NETs release by non-stimulated PMNs) or stimulated with PEG 6000 precipitated (D) RA patients’ plasma (●) (E) or SF (■). PMNs were also stimulated with immune complexes of (D) RA patients’ plasma (○) or (E) SF (△) in the presence of MIP8a or an isotype control (▽). Extracellular DNA was also measured after stimulation of PMNs with immune complexes of RA patients’ plasma or SF in the presence of DNase (x). n.s.: non significant; *p < 0.05; **p > 0.05. Experiments were repeated three times, yielding similar results.
Discussion

Recently it was shown that stimulation of macrophages with ACPA immune complexes in the presence of IgM RF or IgA RF, resulted in increased concentrations of the pro-inflammatory cytokine TNF-α. The presence of IgM RF or IgA RF also enhanced the capacity of ACPA immune complexes to activate the complement cascade. Blocking the interaction of IgA RF with macrophage FcαRI decreased TNF-α secretion, suggesting that macrophages may play a detrimental role in maintaining inflammation.

The contribution of neutrophils to the pathology of RA has not yet completely been elucidated. Neutrophils are recruited quickly towards sites of infection or after tissue damage and the number of infiltrating cells accumulates over time in inflamed tissue to destroy infectious threats like bacteria. They release different anti-bacterial products, which are able to efficiently kill pathogens. They can produce ROS, release powerful proteases and form NETs. As such, neutrophils are filled with toxic components and their activation needs to be controlled tightly to prevent collateral damage to normal host tissue. Neutrophils normally have a short half-life in the circulation (6-18 hours), but it has been demonstrated that within SF in the presence of a physiological oxygen concentration or other survival signals in the inflammatory milieu, the lifetime of neutrophils is extended, which will increase their potential to cause damage and promote inflammation. We now demonstrate that IgA immune complexes that are present in plasma and SF of patients with RA potently activate neutrophils, which results in degranulation (reflected by lactoferrin release) and the release of NETs.

In recent years, the formation of NETs has increasingly been associated with pathogenesis in different autoimmune diseases, such as SLE, RA, psoriasis and vasculitis. NETs can cause tissue damage. For instance, it was demonstrated that histones, which are major components of NETs, injure the endothelium. Furthermore, the formation of NETs results in exposure of intracellular self molecules which can serve as auto-antigens. This leads to production of autoantibodies that contribute to autoimmunity, which is most evident in SLE. Immunogenic complexes composed of neutrophil-derived anti-microbial peptides and self-DNA were identified in the sera of SLE patients. Activated neutrophils release these autoantigens, which efficiently trigger innate plasmacytoid dendritic cell (pDC) activation. SLE patients develop autoantibodies to self-DNA and anti-microbial peptides present in NETs, which suggests that these autoantigens are able to trigger B cell activation. It was shown that neutrophils of SLE patients release more NETs than those from healthy controls. Moreover, NETs release is also stimulated by the presence of anti-microbial autoantibodies. Thus neutrophils are able to activate pDCs through the release of NETs and a dysregulation of this pathway may drive chronic pDC activation and auto-immunity in SLE. Additionally, neutrophils in blood and SF of RA patients show enhanced NETs formation compared to healthy controls. Sera from RA patients with high levels of ACPA and/or RF enhanced NETs formation. Accelerated NETs formation has been implicated in RA pathogenesis through externalization of citrullinated autoantigens that may promote aberrant immune responses in the joint and in the periphery. NETs formation thus likely plays a pathogenic role in RA, although it is unknown whether NETs are able to induce tissue damage as suggested in SLE. Nonetheless, the formation
IgA immune complexes induce NETs

of NETs in response to plasma or SF of RA patients indicates activation of neutrophils by circulating factors. In plasma and particularly in SF of RA patients different cytokines are present that might also promote neutrophil activation. To establish the contribution of autoantibody complexes, we used PEG 6000 to precipitate immune complexes from plasma and SF of RA patients. These precipitates contained high molecular weight proteins, like IgM RF, IgG RF and IgA RFs. Stimulation of neutrophils with isolated immune complexes resulted in activation as demonstrated by NETs formation and ROS production. Especially (large) IgA immune complexes bind to neutrophils via the low to moderate affinity receptor FcαRI. Monomeric IgA antibodies bind poorly, whereas large IgA immune complexes bind avidly to FcαRI. RA patients’ plasma and SF likely contains a range in size of immune complexes. Importantly, NETs release was blocked in the presence of the anti-FcαRI mAb MIP8a after incubation with the majority of RA patients’ plasma and SF immune complexes, supporting the role of IgA in neutrophil activation. Only minimal uptake of IgG complexes was observed. In alternative experiments with antibody-coated bead or antibody-coated bacteria, IgG opsonization induced similar phagocytosis compared to IgA opsonization (data not shown). As such, the minimal uptake of IgG complexes by neutrophils suggests that low amounts of IgG immune complexes were present in PEG 6000 precipitates.

The presence of IgA RF was observed in the majority of RA patients’ plasma and SF. Both IgA RF and IgM RF were more discriminative compared to IgG RF, as less overlap was seen between healthy controls and RA patients’ plasma. Measurement of IgA RF may therefore be a helpful addition to the measurement of IgM RF as a diagnostic tool, but this needs to be confirmed in large series of samples of RA patients, patients with other diseases and of healthy controls. However, activation of neutrophils by IgA autoantibodies was not observed in all patients. Especially samples with high amount of IgA RF correlated with high neutrophil activation, which could be blocked in the presence of anti-FcαRI mAb MIP8a. Thus, it seems that a threshold of high IgA autoantibodies is required for neutrophil activation, which could explain the findings in previous studies in which presence of IgA RF and IgA ACPA correlated with worse disease course in RA patients. It was previously reported that monovalent targeting of FcαRI can induce inhibitory ITAM signaling, which is referred to as ITAMI signaling. As such, it could be possible that the anti-FcαRI mAb MIP8a did not block binding and activation through FcαRI, but induced ITAMI signaling, which would decrease activation via binding of IgG RF to FcγR. To exclude the latter possibility we performed a phagocytosis experiment with either IgA- or IgG-coated beads in the presence or absence of MIP8a. Phagocytosis of IgA-coated beads was decreased in the presence of MIP8a, in contrast to phagocytosis of IgG-coated beads (data not shown), which supports that MIP8a specifically blocks IgA-mediated activation and NETs release, but did not induce ITAMI signaling.

In addition to NETs release, also secretion of chemotactic factors was observed after incubation of neutrophils with isolated immune complexes from plasma or SF of RA patients. Previously, we demonstrated that crosslinking of neutrophil FcαRI by IgA immune complexes resulted in release of the potent neutrophil chemoattractant LTβ4 and concomitant migration, which led to increased recruitment of neutrophils. Interestingly,
LTB4 concentration is increased in both serum and SF of RA patients, supporting the involvement of neutrophils in joint inflammation. We now show that IgA RF autoantibodies present in RA patients’ plasma and SF potently activate neutrophils, which in response secrete chemoattractants to attract more neutrophils. We previously demonstrated that neutrophil activation and concomitant tissue damage is a direct consequence of FcαRI triggering through IgA autoantibodies in the rare skin blistering disease Linear IgA Bullous Disease (LABD). Patients with this disease have IgA autoantibodies directed against collagen XVII as well as neutrophil accumulations in the skin, which ultimately leads to extensive skin damage and blister formation. When skin cryosections were incubated with neutrophils and serum of LABD patients (which contained anti-collagen XVII IgA autoantibodies), separation of the dermis and epidermis was observed, which reflects blister formation. Importantly, IgA-induced tissue damage was prevented by blocking FcαRI using an anti-FcαRI mAb. It is likely that presence of IgA immune complexes in SF can directly contribute to activation and recruitment of neutrophils into the joints of RA patients through interaction with FcαRI. The newly recruited neutrophils will then also encounter these complexes and get activated, which will lead to secretion of more chemoattractants, resulting in amplification of neutrophil migration to the joints of RA patients. Moreover, degranulation and release of NETs may result in increased destruction of joint tissue, which may explain why RA patients with high levels of IgA RF immune complexes have worse prognosis. As such, IgA autoantibodies are not only a predictor for the development of erosive disease of the joints, but may in fact be the cause of tissue destruction.

In conclusion, our results show that neutrophils are activated by IgA RF complexes via FcαRI. Since neutrophils are found in high numbers in the SF of RA patients and IgA complexes are able to recruit and activate neutrophils, this supports their role in inducing joint damage and in this way worsen disease in RA patients. IgA-induced tissue damage by neutrophils might be prevented by blocking FcαRI using anti-FcαRI monoclonal antibody therapy, which may help to alleviate disease in RA patients.

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References
Neutrophils activate Neutrophil Netting

Definition of FcalphaRI-

Enhanced Neutrophil Mast cells and Netting

Chapter 3


Lande, R., et al. Neutrophils activate plasmacytoid dendritic cells by...


Supplementary Figure 1: Heat-aggregated IgA induces release of neutrophil extracellular traps by PMNs. (A) Quantification of extracellular DNA with sytox green (which reflects NETs) after 3h when PMNs were stimulated with heat-aggregated IgA. *p < 0.05. (B) Lactoferrin release (reflecting degranulation of PMNs) was measured by ELISA in duplicates of supernatants of non-stimulated neutrophils (white bar) or after stimulation of neutrophils with heat-aggregated IgA (black bar). *p < 0.05 (C) ROS production was measured over time (3h) in unstimulated PMNs (○) or after stimulation with heat-aggregated IgA ( blue ■). (D) IgA uptake by neutrophils. PMNs were incubated heat-aggregated IgA at 4°C for 1 hour, followed by incubation at 37°C for 30 minutes. Cytospins were stained for IgA (green), FcαRI (red) and DNA (blue).
**Supplementary Video 1**
Fluorescence time lapse video microscopy of non-stimulated PMNs. PMNs stained with a nuclear dye (Hoechst, blue), which were incubated in culture medium containing sytox green (stains extracellular DNA).

https://www.youtube.com/watch?v=wQzvwHEsn-o

**Supplementary Video 2**
Fluorescence time lapse video microscopy of PMNs stimulated with isolated immune complexes from RA patients’ plasma. PMNs stained with a nuclear dye (Hoechst, blue), which were incubated in culture medium containing sytox green (stains extracellular DNA).

https://www.youtube.com/watch?v=fpeM7NKEenU

**Supplementary Video 3**
Fluorescence time lapse video microscopy of PMNs stimulated with isolated immune complexes from RA patients’ synovial fluid. PMNs stained with a nuclear dye (Hoechst, blue), which were incubated in culture medium containing sytox green (stains extracellular DNA).

https://www.youtube.com/watch?v=RtUIKeck67k