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Association between levels of synovial anti-citrullinated peptide antibodies and neutrophil response in patients with rheumatoid arthritis

Carolina V. Gorlino[1,2], Mabel N. Dave[1,2], Rodrigo Blas[3], María Inés Crespo[4,5], Alicia Lavanchy[6], HéctorTamashiro[7], Rodolfo Pardo-Hildalgo[8], María C. Pistoresi-Palencia[4,5], María S. Di Genaro[1,2]

[1]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto Multidisciplinario de Investigaciones Biológicas (IMIBIO-SL), San Luis, Argentina; [2]Universidad Nacional de San Luis, Facultad de Química, Bioquímica y Farmacia, San Luis, Argentina; [3]Centro Médico MEDICI, San Luis, Argentina; [4]Universidad Nacional de Córdoba, Facultad de Ciencias Químicas, Departamento de Bioquímica Clínica, Córdoba, Argentina; [5]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI)-Córdoba. Argentina; [6]Centro Médico CENYR, San Luis, Argentina; [7]Clínica Bolívar, San Luis, Argentina; [8]Centro de Rehabilitación Médica CER, San Juan, Argentina.

Corresponding author: Dra. María Silvia Di Genaro, Laboratorio de Inmunopatología y Citometría de Flujo, Universidad Nacional de San Luis, Ejército de los Andes 950, 5700 San Luis, Argentina. E-mail address: sdigena@unsl.edu.ar. Phone: (0054)-266-44520300, Fax: (0054)-266-4422644.

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Abbreviations: RA, Rheumatoid arthritis; SF, synovial fluid samples; ACPAs, anti-citrullinated peptide antibodies; ROS, Reactive oxygen species; NET, neutrophil extracellular traps; RF, rheumatoid factor; Ig, Immunoglobulin; DAS-28, Disease Activity Score; IL, interleukin; TNF, tumor necrosis factor; ICAM-1, intracellular cell adhesion molecule-1;CXCR, chemokine receptor; DMARD, disease-modifying antirheumatic drugs.

Abstract:

Rheumatoid arthritis (RA) is characterized by the presence of anti-citrullinated peptide antibodies (ACPAs) and neutrophils infiltrating the synovial fluid (SF) of the affected joints. The aim of this work was to analyze whether the presence of ACPAs in SF is associated with neutrophil infiltration and with their phenotype in the inflamed joints of RA patients. We found that in the presence of ACPAs, the number of synovial neutrophils correlated with severe disease activity. The SF were divided according to synovial ACPA levels in negative-(<25 U/ml), low-(25-200 U/ml) and high level (>200 U/ml; ACPA^{high}). We observed that IL-6, IL-17 and IL-8 were significantly elevated in ACPA^{high}SF and that IL-8 levels correlated positively with neutrophil counts and with worse clinical manifestations. Additionally, *in vitro* incubation of neutrophils with ACPA^{high} SF resulted in an increased ROS production and extracellular DNA release compared to neutrophils incubated with ACPA-negative SF. These exacerbated effector functions were associated with a fraction of ICAM-1-positive neutrophils, which were induced by ACPA^{high} SF. Likewise, in *in vivo*, we could also detect this subset among neutrophils present in ACPA^{high} SF. In conclusion, the data presented here

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shed light on the role of SF-ACPAs as inductors of a pro-inflammatory profile in neutrophils.

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Introduction:

Rheumatoid arthritis (RA) is a systemic inflammatory disease that manifests itself in persistent synovial inflammation, leukocyte infiltration and immune activation, which can cause cartilage and bone damage and long term disability [1]. Although the pathogenetic events initiating and mediating chronicity of synovitis are not yet fully understood, there are many of inflammatory cells accumulated in the synovial fluid (SF) and involved in its pathogenesis [1]. In the specific case of neutrophils, they can be found at large numbers either in the SF of the affected joints or in the pannus/cartilage interface and they have the greatest potential to cause damage [2-4].

Patients with RA are characterized by presenting some circulating autoantibodies in their serum. Among the numerous autoantibodies associated with RA, anti-cyclic citrullinated peptide antibodies (ACPAs) are now recognized as the most disease-specific [5]. The presence of these antibodies, together with the autoantibodies rheumatoid factor (RF), permits the disease classification into seropositive or seronegative forms. The two RA subsets have different disease manifestations, with the seropositive status associated with poor outcomes, such as increased disease activity, radiographic progression and disability [6]. Although the presence of ACPAs is associated with an aggressive disease course and, thus, can guide clinical practice, the specific role of autoantibody levels is still unclear [5-6]. Neutrophils are now recognized as major orchestrators of inflammation and as deeply influencing other cells from the innate and adaptive immune system [4]. Excessive neutrophil activation can result in severe tissue damage and chronic inflammation through the release of cytotoxic reactive oxygen species (ROS), tissue-damaging molecules and proteases,

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neutrophil extracellular traps (NETs), and inflammatory cytokines and chemokines. It is well established that neutrophils play a relevant role in RA [2]. NETs were identified as a source of peptidyl arginine deiminases (PAD) as well as citrullinated proteins [7-8]. In addition, neutrophils are one of the few cells expressing high levels of PAD4, which is the main enzyme responsible for the citrullination of peptides [10]. Thus, neutrophils have a central role in the production of autoantigens, this phenomenon being a key stage in the autoimmune response in ACPA-positive patients with RA [9-10].

The contribution of neutrophils to the pathology of RA has not been completely elucidated. The aim of this work was to analyze whether the presence of ACPAs in SF is associated with neutrophil infiltration as well as with the phenotype of these cells in the inflamed joints of RA patients. We present data showing the effect of SF-ACPA on neutrophil recruitment *in vivo* and also the effect of different levels of these autoantibodies in SF on neutrophil response and phenotype *in vitro*.

Results

Relationship between neutrophil counts and disease activity in synovial ACPA-positive patients

ACPAs were found in the serumof most patients with established RA. It is believed that ACPA positive and ACPA negative RA may define two different disease entities [5]. Several studies have shown that ACPA-positive RA patients develop earlier and more widely spread erosions than consistently ACPA-negative patients. For this reason, we first sought to determine whether the presence of synovial ACPAs is associated with neutrophilic infiltrates in the SF of RA patients. Although neutrophil counts did not differ significantly between synovial ACPA-positive and ACPA-negative patients (p=0.11), it was observed that in the

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presence of SF-ACPAsthe number of neutrophils infiltrating inflamed joints correlated with severe disease activity score (DAS-28) ($p=0.02$; $n=42$). Surprisingly, this was not the case for synovial ACPA-negative patients ($p=0.36$; $n=21$) (Figure 1A). Differences were not found either in clinical characteristics or in treatments between both groups of patients(Supplemental Table 1). Therefore, this did not influence neutrophilic infiltrates. Next, the association of the clinical parameter DAS-28 with ACPA titers and total Immunoglobulin G (IgG) levels in the SF of synovial ACPA-positive patients was examined. As shown in Figure 1B, total IgG levelsnegatively correlated with DAS-28. A direct relationship between SF-ACPA titers and DAS-28 score was not found (data not shown). However, the correction of ACPA concentration in SF by the ratio ACPA/total IgG showed a positive correlation with disease activity ($p=0.03$), indicating that ACPA levels are increased in relation to total IgG in SF from patients with more serious clinical manifestations.

Differential levels of pro-inflammatory cytokines according to SF-ACPA concentrations

It is well established that cytokines are directly implicated in many of the immune processes associated with the pathogenesis of RA [1]. Among pro-inflammatory cytokines, IL-1β, TNF-α, IL-6, IL-8, IL-23 and IL-17 can be detected in SF of RA patients and they might promote neutrophil activation [2, 11]. Additionally, it has been shown that elevated plasma levels of pro-inflammatory cytokines strongly correlate with ACPA titers in plasma of RA patients [12]. Therefore the pro-inflammatory cytokine environment which is related with neutrophil activity in the SF of our cohort of RA patients was investigated and compared with the different synoviallevels of ACPAs. Thus, the SF samples were divided into three groups according to ACPA levels in SF: <25 U/ml (negative; $ACPA^{negative}$), 25-200 U/ml (low to moderate; $ACPA^{low}$) and >200 U/ml (high; $ACPA^{high}$). We observed that IL-6, IL-17 and IL-8 were significantly elevated in SF of patients with $ACPA^{high}$ SF, which are key cytokines

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implicated in neutrophil activity (Figure 2A). Nevertheless, differences were not found regarding the amount of IL-23, TNF-α and IL-1β among ACPA subgroups (Supplementary Figure 1).

Levels of IL-8 associated with severe disease activity in SFwith high ACPA titers

In RA, neutrophils are recruited into the affected joints by chemoattractants and other molecules generated during inflammation [2]. IL-8/CXCL8 is a potent chemokine for neutrophils and plays a critical role in the recruitment and activation of these cells [13]. Considering our previous results in which IL-8 significantly increased in ACPA^{high} SF, we next studied the association of this cytokine with neutrophils. Figure 2B shows that in SF with high-ACPA titers, neutrophil counts correlated positively with IL-8 levels ($p=0.05$) whereas higher levels of this cytokine were related with more serious clinical manifestations ($p=0.04$).

Increased ROS production and DNA extrusion after stimulation with ACPA^{high} SF

Release of proteases and ROS have been connected with joint damage via direct oxidation of joint components [3]. Given that high levels of ACPAs in SF were related to worse clinical manifestations, we examined thepotential contribution of neutrophils to inflammation by measuring in vitro ROS production. In order to investigate this phenomenon, the ability of neutrophils to release ROS in response to phorbol-12-myristate-13-acetate (PMA) stimulation was tested. Peripheral-blood neutrophils from healthy donors were incubated in the presence of SF from RA patients with negative, low or high levels of synovial-ACPAs for 3 hours and ROS production was assessed by measuring DihidroRhodamine 123 (DHR) fluorescence using flow cytometry. Minimal basal ROS production was observed in the absence of PMA, although SF samples from RA patients had been previously added (Figure 3A). However, in

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response to stimulation with PMA, neutrophils incubated with ACPA^{high}SF showed an increase in ROS production when compared with those incubated with ACPA^{negative} SF (Figure 3A). We also tested the effect of autoantibodies and ROS induction. There were no differences in the oxidative burst between SF and the fraction of IgG purified from SF. Nevertheless, the fraction of SF without IgG was not able to induce ROS, suggesting that the presence of autoantibodies on SF is responsible for this effect (Supplementary Figure 2A). As neutrophils from RA patients may differ from those from healthy individuals [2], we measured ROS production on peripheral-blood neutrophils from RA patients. As previously reported [14], we found that RA neutrophils are already primed for oxidative burst and, in consequence, it was not necessary to stimulate them with PMA. On the same line as to what was shown in PMA-primed neutrophils from healthy individuals, RA neutrophils incubated with ACPA^{high} SF showed an increase in ROS production when compared with those incubated with ACPA^{negative} SF (Supplementary Figure 2B).

Given that ROS production is an important step in the formation of NETs, we also measured the potential capacity of NET release of SF-treated neutrophils. Quantification of extracellular DNA, indicative of NET production, was assessed by fluorometric analysis after neutrophil incubation with SF samples. As shown in Figure 3B, incubation of blood neutrophils from healthy donors with ACPA^{high} SF resulted in a 6-fold increase of extracellular DNA release compared to neutrophils stimulated with ACPA^{negative} SF (ACPA^{negative}: 39±14 %, ACPA^{low}: 170±73 %, ACPA^{high}: 226±62 %). Neither the fraction of IgG purified from SF nor the fraction of SF without IgG was able to induce DNA extrusion in neutrophils from healthy individuals or from RA patients (Supplementary Figure 3A). This indicates that the presence of autoantibodies together with other inflammatory components was responsible for the DNA release by neutrophils. Additionally, no differences between neutrophils from RA patients and those from healthy individuals were found as RA

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neutrophils with $ACPA^{high}$SF also showed an 8-fold increase in DNA release when compared with those incubated with $ACPA^{negative}$ SF (Supplementary Figure 3B).

All in all, these data suggest that the presence of high levels of SF-ACPAs induces a higher production of ROS and extracellular DNA release in RA neutrophils.

High synovial levels of ACPAs and the expression of ICAM-1 on neutrophils

The data described above suggest that the presence of high ACPA levels in SF has an impact on neutrophil functions. To gain more insight into the characteristics of neutrophils after exposure of high titres of these autoantibodies in SF, we studied the phenotype of these cells by flow cytometry. As the integrin ICAM-1 can be considered as a reference marker found to increase in migrated neutrophils [15-16], we decided to evaluate changes in the expression of this molecule. We found that incubation of peripheral-blood neutrophils from healthy donors with $ACPA^{high}$ SF induced a significant increase in the percentage of ICAM-1-expressing neutrophils (Figure 4A). Additionally, this fraction of ICAM-1-positive neutrophils showed a decreased expression of the adhesion molecule CD62L and the chemokine receptor CXCR1, which were more pronounced after incubation with $ACPA^{high}$ SF, suggesting an activated-cell phenotype (Figure 4B). It has been shown that certain stimuli, such as LPS, can induce the expression of ICAM-1 on the cell surface of neutrophils [17]. When the induction of ICAM-1 on neutrophils after LPS or $ACPA^{high}$ SF incubation was compared, differences between these stimuli were not found (Supplemental Figure 4A). Moreover, stimulation with IgG purified from SF induced a fraction of ICAM-1-positive neutrophils similar to the one observed after stimulation with $ACPA^{high}$ SF, showing a direct effect of autoantibodies on the induction of the expression of ICAM-1 on neutrophils (Supplemental Figure 4B). Peripheral-blood neutrophils from RA patients showed the same pattern of induction of ICAM-1 identified in blood neutrophils from healthy individuals, which excludes the possibility of

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differences in neutrophil response from RA patients (Supplementary Figure 4C). Considering that neutrophils incubated with SF samples with high-ACPA levels favored the formation of NETs, the association of enhanced NET release and ICAM-1-positive neutrophils in the presence of different SF-ACPA levels was investigated. A correlation between ICAM-1-positive neutrophils and DNA extrusion was noted in SF with high-ACPA levels ($p=0.02$) (Figure 4C). Collectively, these data provide evidence to suggest that the presence of high levels of ACPA in SF induced a fraction of ICAM-1-positive neutrophils which are associated with an increased extracellular DNA release.

ICAM-1-positive neutrophils in synovial fluids of RA patients

Having found that high-ACPA levels in SF induce an up-regulation of ICAM-1 on neutrophils, and having related this phenomenon with enhanced effector functions *in vitro*, we next sought to investigate the presence of ICAM-1-positive neutrophils *in vivo*. To this end, freshly SF samples from RA patients were analyzed by flow cytometry (Supplemental Figure 5). In agreement with the results found *in vitro*, ACPA^{high} SF showed a significant increase in the percentage of ICAM-1-positive neutrophils (Figure 5A). Furthermore, the fraction of ICAM-1-positive neutrophils from patients with high-SF-ACPA levels showed a greater decrease in both CD62L and CXCR1 markers, suggesting that these cells might have an activated phenotype due to the presence of high titres of these autoantibodies (Figure 5B). In conclusion, these results demonstrate that ICAM-1-positive neutrophils are found in SF from patients with high synovial levels of ACPAs.

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Discussion

There is growing evidence indicating that neutrophils have an active role in the pathogenesis of RA [2]. Inflammatory joint effusions of RA patients are characterized by a high number of neutrophils in the SF where these cells mediate tissue destruction after improper activation [2-4]. The aim of the present study was to analyze the characteristics of neutrophils infiltrating the inflamed joints in the presence of ACPA autoantibodies in SF of RA patients. We observed that in synovial ACPA-positive patients, the number of SF-neutrophils correlates with disease activity and also with IL-8 concentrations in the case of high levels of SF-ACPAs. Furthermore, the levels of synovial ACPAs have an impact on ROS production, on DNA extracellular release and on the induction of ICAM-1 expression on neutrophils. Additionally, our data were further supported by *in vivo* studies, demonstrating that ICAM-1-positive neutrophils can be found in the SF of RA patients.

One of the characteristics of RA is the presence of autoantibodies in the serum of patients [1]. Anti-citrullinated protein antibodies comprise a group of highly-specific RA autoantibodies. Although the clinical features of ACPA-positive and ACPA-negative patients with RA are similar at baseline, the presence of ACPAs is associated with greater radiological joint damage [5]. Several studies have focused on synovial differences in RA patients according to ACPA status [5], but only few reported the effect of these autoantibodies on the role of synovial-neutrophils [7, 18]. In accordance with a previous report, we could not find differences in the numbers of synovial-neutrophils in any group of patients [19]. However, we observed that neutrophil counts correlated with disease activity in the SF of synovial ACPA-positive but not in ACPA-negative patients. Even though ACPA titers in their sera were not included in this study since the focus was on ACPA levels present in SF of our cohort of RA patients, we demonstrated a higher synovialIgG-ACPA/total IgG ratio. This

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was associated with a worse DAS-28 score, supporting the notion that ACPA production and/or retention at the site of inflammation have an impact on clinical manifestations [20]. The role of pro-inflammatory cytokines has extensively been described in RA [11, 21-22]. In our cohort of patients, we found a greater amount of IL-6 and IL-17 in the ACPA^{high} SF. IL-17 is a well-known cytokine associated with the pathogenesis of RA [21], with higher levels in SF and also an increase prevalence of IL17^{+} cells in plasma of RA patients [23]. IL-17 is a strong inducer of inflammatory mediators in endothelial cells and fibroblasts, resulting in neutrophil recruitment [24]. Additionally, IL-6 is one of the key cytokine effectors of the tissue response in RA [21], also having important effects on the production of antibodies by B cells [25]. It was showed that IL-6 inhibition in ACPA-seropositive patients reduced bone loss, demonstrating that IL-6 have a negative impact on bone metabolism in the presence of ACPAs [26].

IL-8/CXCL8, considered an important inflammatory chemokine associated with arthritis, chemoattract neutrophils and it is produced by macrophages, synovial lining cells, fibroblasts and endothelial cells [27]. It was shown that elevated IL-8 levels are present in the serum and SF of RA patients [27-28]. It was also reported that IL-8 levels are associated with clinical signs and symptoms of arthritis in synovial tissue of RA patients [29]; however, this relationship has not been tested according to the ACPA status. We demonstrated that synovial IL-8 levels showed a positive correlation with disease activity and also with SF-neutrophil counts when ACPAs were present in SF of RA patients. Although we did not test a relationship between IL-8 and neutrophil infiltration, we might speculate that this cytokine is partly responsible for the accumulation of neutrophils. Indeed, it was demonstrated that IL-8 is involved in the migration of neutrophils into the inflamed joints [30]. Additionally, the association observed between IL-8 levels and disease severity could be related to increased

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DNA extrusion. In fact, it was demonstrated that NET production induces increased production of IL-8 and IL-6 by synovial fibroblasts in RA [7].

In recent years, particular attention has been to the contribution of NET formation in the pathogenesis of RA disease [4, 7-8]. NET production results in exposure of intracellular self-molecules that can serve as autoantigens. In fact, the generation of proteins modified by citrullination in the joint by activation of PADs is a key stage to trigger anticitrulline immunity in ACPA-positive patients [31]. Thus, NET formation is likely to play a pathogenic role in RA as NETosis may serve as an important source of citrullinated autoantigens[4]. Although this study involved a relatively low number of SF samples, we observed that *in vitro* incubation of neutrophils with ACPA^{high} SF resulted in an increase of ROS production in PMA-primed cells, and also in extracellular DNA release. It is important to notice that we only quantified extracellular DNA release, as this can be an indicative of NETosis. However, this could also be related to other neutrophil functions such as leukotoxic hypercitrullination [32]. Indeed, stimulation of neutrophils from healthy individuals with ACPA^{high}SF but without PMA-priming showed an increased extracellular DNA release in the absence of ROS production. Nevertheless, this was not the case for neutrophils from RA patients, in which stimulation with ACPA^{high} SF showed an enhancement in DNA extrusion together with an increase in ROS production, but without the requirement of PMA-priming. While the process observed in neutrophils from healthy individuals would be indicative of leukotoxic hypercitrullination, in RA neutrophils DNA extrusion can be related to NETosis, because an increase in ROS was also observed [32]. It was reported that both circulating and SF-neutrophils from RA patients show enhanced NET release, and this increase correlates with the presence and levels of ACPAs and with systemic inflammation [7]. An increased extrusion of DNA was only observed in RA neutrophils after incubation with ACPA^{high} SF but not in the case of stimulation with purified IgG from SF or with the fraction of SF without

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IgG. This could result from the combination of elevated autoantibodies and IL-17, since this inflammatory cytokine was associated with enhanced NET release in RA [7].

ICAM-1 is a key adhesion molecule that plays an important role in cell migration and intercellular interactions. ICAM-1 is generally absent or expressed at very low levels on circulating neutrophils [33], though elevated levels were reported in numerous infectious and inflammatory clinical settings [15, 33-34]. In a recent study, Woodfin et al. have shown that certain stimuli, such as LPS, can induce the expression of ICAM-1 on the cell surface of murine neutrophils [17]. This study also demonstrated that the expression of ICAM-1 supported enhanced ROS generation and phagocytosis [17].We found that neutrophil incubation with $ACPA^{high}$ SF induced a significant increase in the percentage of ICAM-1-expressing neutrophils. To our knowledge, this is the first time that the ICAM-1 expression on neutrophils has been linked to autoantibodies, since previous reports had associated neutrophil ICAM-1 induction with the stimulants LPS, TNF and bacterial components [17, 34]. It has been demonstrated that the induction of ICAM-1 in infectious settings supports phagocytosis and ROS generation, proposing it as an important molecule for pathogen clearance [17]. Although we did not measure phagocytic capability in our study, we might speculate that, after been recognized by FcγRs, synovial ACPA autoantibodies might stimulate phagocytosis.

In conclusion, the data presented here shed new light on the function of synovial ACPAs and infiltrating neutrophils in perpetuating inflammation in RA. Besides, the subset of ICAM-1positive neutrophils can be considered as a promising candidate biomarker of disease activity. Understanding the role of SF ACPAs on neutrophils in RA may open new perspectives to the treatment of this rheumatic disease.

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Materials and Methods

Ethics statement

Experiments were performed in accordance with an internal review board-approved protocol at Instituto de Biología y Medicina Experimental (IBYME; Buenos Aires, Argentina; CE 003-2/2013). All healthy donors and patients gave their written informed consent.

Participants and synovial fluid samples

Synovial fluid samples (SF) were obtained from 82 patients with RA and blood samples were obtained from 4 patients with RA and from 5 healthy individuals. The patients fulfilled the revised criteria proposed by the American College of Rheumatology/European League Against Rheumatism for RA [35-37]. Demographic and clinical characteristics of RA patients are included in Supplementary Table 1. The mean age of RA patients was 55 years old (range, 28-79 years old), and the mean duration of the disease was 7 years (range, 1–30 years).Seventy-three RA patients were seropositive for Immunoglobulin (Ig) M rheumatoid factor (IgM RF). Patients (65females, 17males) were taking the following medications: non biologicaldisease-modifying antirheumatic drugs (nbDMARDs; n=67), or biological disease-modifying antirheumatic drugs (bDMARDs; n=8).

Synovial fluid was obtained after aspiration of knee joints, collected into heparinized tubes and centrifuged at 800 g for 5 min to remove the cells, which were used in flow cytometric analysis. The cell-free fluid was then centrifuged at 3,000 g for 10 min and the SF supernatant was stored at -20 °C until analysis.

Antibodies and cytokine measurements on SF

Before assay, each SF was treated with 1 mg/ml hyaluronidase (type IV-S, SIGMA-Aldrich, St. Louis, MO) for 30 min at 37°C followed by centrifugation (10,000 *g*, 5 min). IgGACPA levels (units) were determined by enzyme-linked immunosorbent assay (ELISA; Quanta Lite

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CCP3 IgG ELISA, INOVA Diagnostics, San Diego, CA) according to the manufacturer's instructions. SF from patients with other joint inflammation disorders (ankylosing spondylitis: n=3; osteoarthritis: n=9; psoriatic arthritis: n=8)were used to set the cut-off value of ACPA negativity (23 U/ml). Total synovial IgG levels were tested by radial immunodiffusion assay (IAC International SRL, Buenos Aires, Argentina). IgG from 4 $ACPA^{high}$ SF samples was isolated by affinity chromatography on Protein G Sepharose 4 Fast Flow column (GE Healthcare). Briefly, SF samples were diluted to 1:2 in PBS, dialyzed with binding buffer and then loading onto the column. Bound antibodies were eluted with 0.1 M Glycine-HCl and neutralized with Tris-HCl 1M. The isolated IgG and unbound fraction were then dialyzed against PBS. The concentration of interleukin (IL)-6, IL-17 (eBioscience, San Diego, CA), IL-8 (Immuno Tools GbmH, Friesoythe, Germany) was measured by ELISA following manufacturer's instructions. The limits of detection were 4 pg/ml for IL-17, 2 pg/ml for IL-6 and 3 pg/ml for IL-8.

Cell isolation and stimulation

Neutrophils were obtained from heparinized venous blood cells from healthy donors and were isolated using Percoll (GE Healthcare, Fairfield, CA, USA) density gradient as reported in Buckley et al. [33]. Contaminating erythrocytes were removed by hypotonic lysis. Cell population purity (>85%) was checked by flowcytometry analysis. Purified neutrophils were then incubated in the presence or absence of SF for 3 hours at 37 °C under 5% CO_2 in 96-well culture plates.

Assessment of ROS production

Oxidative stress biomarkers were analyzed in neutrophils using flow cytometry. For the assessment of ROS generation, neutrophils were first stimulated with SF and then incubated with Dihidro Rhodamine 123 (DHR; 1 μM; SIGMA-Aldrich) at 37 °C for 10 min. Next, cells were stimulated with phorbol-12-myristate-13-acetate (PMA; 200 ng/ml; SIGMA-Aldrich)

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for 20 min at 37 °C and after incubation were then washed, re-suspended in PBS, and analyzed by flow cytometry[38]. DHR loaded unstimulated cells were used as control. DHR positive neutrophils were identified as cells with a higher DHR signal than control cells.

Quantitative flourometric analysis of NET release

Neutrophils (1 x 10^5) were incubated in HBSS in 96-well plates with SF with the following reagents: PMA (an activator of NET formation), Triton X-100 (to determine total DNA content), DNase (2 U/µl; Promega), as appropriate, for 3 h at 37 °C. Release of extracellular DNA was detected by adding SYTOX Green Nucleic Acid Stain (2.5 µg/ml; Invitrogen Life Technologies, Carlsbad, CA). Fluorescence was measured using a fluorescence microplate reader (Perkin-Elmer LS 55, Perkin-Elmer, Waltham, MA) at 480 nm excitation, 520 nm emission. The data were normalized as percentage of total DNA, substracting the fluorescence intensity of the DNAse-containing wells from the comparative control, and dividing it by the fluorescence intensity emitted from cells treated with Triton X-100 (total DNA present) [39].

Flow cytometry

Flow cytometric analysis was used to assess surface expression of CD16-FITC (clone B73.1), CD62L-PE (clone DREG-56), CXCR1-PerCp-Cy5 (clone G265-8), and CD54-APC (clone HA58) on *in vitro*-stimulated neutrophils from peripheral blood of healthy individuals and on SF-neutrophils from RA patients. All antibodies were from BD-Biosciences (San Jose, CA). Neutrophils were identified by their light scatter properties and by the expression of CD15-FITC (clone HI98; BioLegend, Munich, Germany). Cell acquisitions were performed on a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using the CellQuest software (BD Biosciences) or the FlowJo software (TreeStar Inc., CA).

Statistical analysis

Nonparametric one-way analysis of variance (Kruskal–Wallis test) followed by Dunn's post-

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hoc test were used to compare multiple groups. The nonparametric Student's t test (Mann Whitney) was performed for two experimental groups. Correlation was assessed by the Spearman's correlation coefficient. Analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, La Jolla, CA, USA). *P*-value less than 0.05 was regarded as significant.

Conflicts of interest

The authors declare no commercial or financial conflict of interest.

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Figure Legends:

Figure 1.Relationship among disease activity, neutrophil counts and ACPA levels in SF of RA patients. SF samples were tested for the presence of ACPA autoantibodies and grouped forACPA-positive or ACPA-negative patients. **A**) The scatter plot shows the median and the interquartile range of neutrophil counts in SF of ACPA-positive (n=42) and ACPA-negative patients (n=21); the correlation analysis between the number of neutrophils in SF and DAS-28 in both groups is also shown. **B**) Graphs show correlation between IgG levels and DAS-28 (n=32) and ACPA-IgG in relation to total IgG (arbitrary units (U)/mg IgG) and DAS-28 in SF samples of ACPA-positive patients (n=42). **A**,**B**) Statistical analysis was performed with Mann Whitney test and Spearman correlation test. Data are pooled from at least 7 independent experiments with minimum 3 RA patients per experiment.

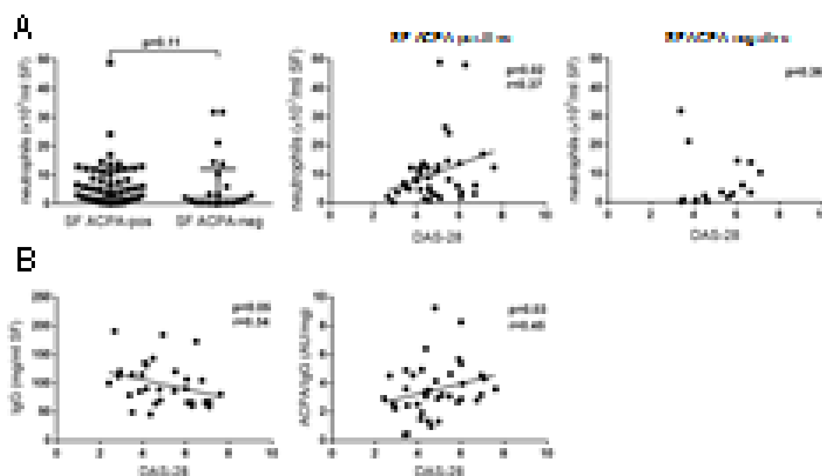

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Figure 2.Increased levels of IL-6, IL-17 and IL-8 in ACPA^{high} SF.**A**) SF samples were grouped according to the levels of ACPA autoantibodies into *negative* (open circles; <25 U/ml), *low* (gray square; 25-200 U/ml) and *high* (filled circle; >200 U/ml). Levels of IL-6 (n=86), IL-17 (n=72) and IL-8 (n=78) were then measured by ELISA. The median concentration of each cytokine is represented by a horizontal bar. Between-group comparisons were performed using the Kruskal Wallis test with Dunn's multiple comparison post hoc test. *$p<0.05$. **B**) Correlation between IL-8 and neutrophil counts, and between DAS-28 and IL-8 levels in patients with high ACPA levels. Spearman correlation test was used to calculate the correlation coefficient (r) and p-value; n=37. **A**, **B**) Data are pooled from at least 4 independent experiments with minimum 9 RA patients per experiment.

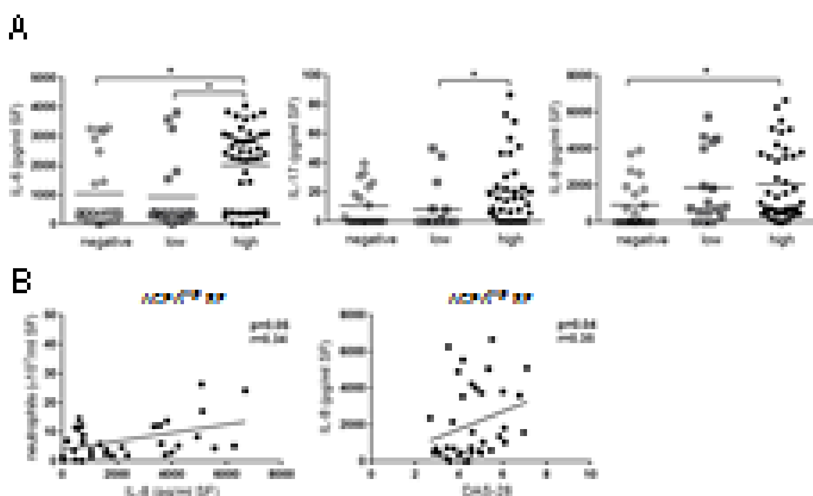

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Figure 3. Neutrophils stimulated with ACPA^{high} SF produced higher ROS and extracellular DNA release. Peripheral-blood neutrophils from healthy donors were incubated in the presence of SF from RA patients with negative-, low- or high-ACPA levels. **A**, **B**) ROS production was assessed by measuring DHR fluorescence in the presence (PMA-stimulated; n=26) or absence (unstimulated; n=21) of PMA using flow cytometry. The graph shows the average DHR fluorescence of neutrophils stimulated with SF with negative-, low- or high-ACPA levels after stimulation or with no stimulation with PMA. Representative histograms of isotype control and each group of SFs are also shown.The percentage of increase in ROS production over control is also shown.**C**) Quantitative fluorometric analysis with SYTOX Green of neutrophil extracellular DNA release after being stimulated by SF from RA patients with negative-, low- or high-ACPA levels for 3 hours. The data were normalized as percentage of total DNA of each condition; n= 39.**A**, **B**, **C**) Data represent mean + SD. At least 3 independent experiments were performedwhereperipheral-blood from 2 healthy individuals and 6-9 RA SF samples were used per experiment. Between-group comparisons were performed using the Kruskal Wallis test with Dunn's multiple comparison post hoc test. *p<0.05; **p<0.01.

Figure 4. The frequency of ICAM-1^{positive} neutrophils is related to levels of synovial ACPAs. **A**) Peripheral-blood neutrophils from healthy donors were incubated or not incubated (basal) with SF from RA patients with negative-, low- or high-ACPA levels, and their phenotype was analyzed by flow cytometry. The graph shows the percentage of ICAM-1-positive neutrophils (as percentage of CD16^{bright}FSC^{high}SSC^{high} cells).Representative histograms of isotype control (shaded) and each group of patients (non-shaded) are also shown. **B**) Flow cytometry analysis of cell-surface expression of CD62L and CXCR1 of basal (peripheral-blood neutrophils) and ICAM-1^{positive} neutrophils from SF of RA patients with *negative*-, *low*- and *high*-ACPA

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levels. **A**, **B**) Between-group comparisons were performed using the Kruskal Wallis test with Dunn's multiple comparison post hoc test. *p<0.05; **p<0.01; ***p<0.001. Data represent mean +SD. n= 16.**C**) Positive correlation between the percentage of ICAM-1^{positive} neutrophils and extracellular DNA release in neutrophils stimulated with SF from patients with high ACPA levels. Spearman correlation test was used to calculate the correlation coefficient (r) and p-value. n= 14.**A**, **B, C**) Data are pooled from at least 4 independent experiments where peripheral-blood from 2 healthy individuals and 6-9 RA SF samples were used per experiment.

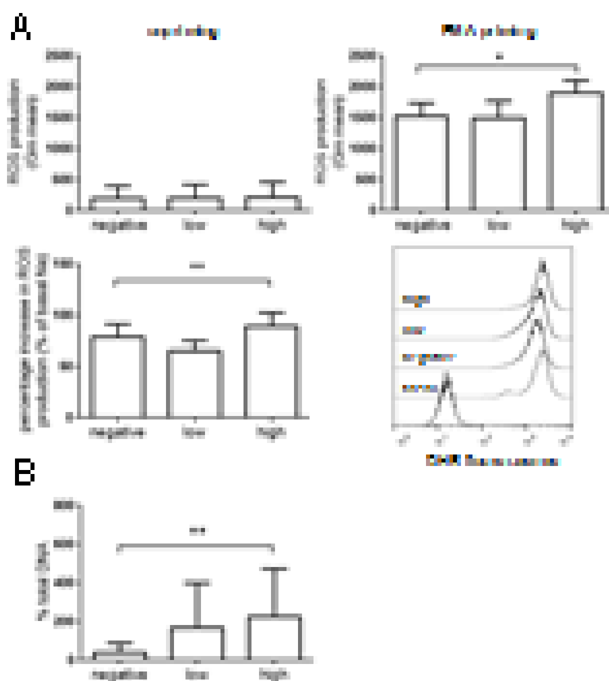

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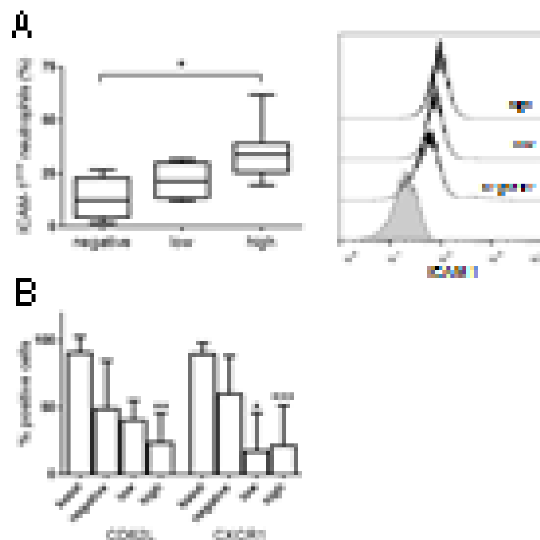
Figure 5.ICAM-1^positive^ neutrophils in vivo. **A**) SF samples of RA patients were analyzed by flow cytometry for the identification of ICAM-1^{positive} neutrophils. The graph shows the percentage, as quantified by flow cytometry, of ICAM-1^{positive} neutrophils (as percentage of CD16^{bright} FSC^{high}SSC^{high} cells) in SF of patients with *negative*-, *low*- and *high*-ACPA levels. Data are represented in a box and whisker diagram which shows the interquartile range, median and minimum and maximum. Representative histograms of isotype control (shaded) and each group of patients (non-shaded) are also shown. **B**) Flow cytometry analysis of cell-surface expression of CD62L and CXCR1 of basal (peripheral-blood neutrophils) and ICAM-1^{positive} neutrophils from SF with *negative*-, *low*- and *high*-ACPA levels. Data represent mean + SD**. A**, **B**) Between-group comparisons were performed using the Kruskal Wallis test with Dunn's multiple comparison post hoc test. *p<0.05; **p<0.01; ***p<0.001. n= 17. Data are pooled from at least 6 independent experiments with 1-3 RA patients per experiment.



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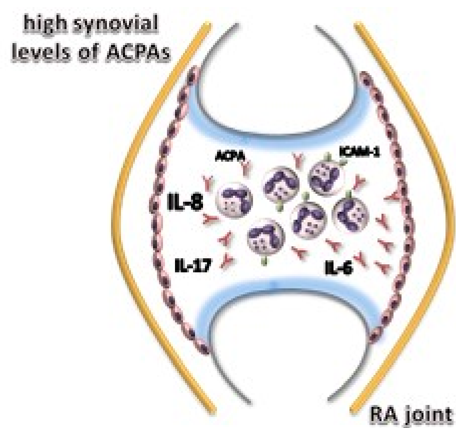

In rheumatoid arthritis (RA), the presence or absence of anti-citrullinated peptide antibodies (ACPAs) can define different disease entities. We analyze the characteristics of synovial neutrophils in the presence of different synovial levels of ACPAs and provide insight into the association of synovial ACPA titers and local inflammation in RA patients.

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