NIH Public Access
Author Manuscript
Sci Transl Med. Author manuscript; available in PMC 2014 March 27.

Published in final edited form as:
Sci Transl Med. 2013 March 27; 5(178): 178ra40. doi:10.1126/scitranslmed.3005580.

NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis

Ritika Khandpur[1,†], **Carmelo Carmona-Rivera**[1,†], **Anuradha Vivekanandan-Giri**[2], **Alison Gizinski**[1], **Srilakshmi Yalavarthi**[1], **Jason S. Knight**[1], **Sean Friday**[1], **Sam Li**[1], **Rajiv M. Patel**[3], **Venkataraman Subramanian**[4], **Paul Thompson**[4], **Pojen Chen**[5], **David A. Fox**[1], **Subramaniam Pennathur**[2], and **Mariana J. Kaplan**[1,*]

[1]Division of Rheumatology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI

[2]Division of Nephrology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI

[3]Department of Pathology, University of Michigan, Ann Arbor, MI

[4]Department of Chemistry, Scripps Florida, Jupiter, FL

[5]Division of Rheumatology, UCLA; Los Angeles, CA

Abstract

The early events leading to the development of rheumatoid arthritis (RA) remain unclear but formation of autoantibodies to citrullinated antigens (ACPA) is considered a key pathogenic phenomenon. Neutrophils isolated from patients with various autoimmune diseases display enhanced extracellular trap formation (NETs), a phenomenon that externalizes autoantigens and immunostimulatory molecules. We investigated whether aberrant NETosis occurs in RA, determined its triggers and examined its deleterious inflammatory consequences. Enhanced NETosis was observed in circulating and synovial fluid RA neutrophils, compared to neutrophils from healthy controls and from patients with osteoarthritis. Further, netting neutrophils infiltrated RA synovial tissue, rheumatoid nodules and skin. NETosis correlated with ACPA presence and levels and with systemic inflammatory markers. RA sera and immunoglobulin fractions from RA patients with high levels of ACPA and/or rheumatoid factor significantly enhanced NETosis, and the NETs induced by these autoantibodies displayed distinct protein content. During NETosis, neutrophils externalized citrullinated autoantigens implicated in RA pathogenesis, whereas anti-citrullinated vimentin antibodies potently induced NET formation. The inflammatory cytokines IL-17A and TNF-α induced NETosis in RA neutrophils. In turn, NETs significantly augmented inflammatory responses in RA and OA synovial fibroblasts, including induction of IL-6, IL-8,

*Corresponding author: Mariana J. Kaplan, M.D., Division of Rheumatology, University of Michigan, 1150 W. Medical Center Dr., 5520 MSRBI, Box 5680, Ann Arbor, MI 48109. Phone: 734-936-3257, makaplan@umich.edu.
†These authors equally contributed to this publication and share first authorship.

Author contributions: R. Khandpur designed and performed research, analyzed data and contributed to writing the manuscript; C. Carmona-Rivera designed and performed research, analyzed data and contributed to writing the manuscript; A. Vivekanandan-Giri performed NETs proteomics analysis and contributed to writing the manuscript; A. Gizinski recruited patients and was involved in FLS experiments; S. Yalavarthi quantified inflammatory cytokines and NETosis; JS. Knight performed IHC of biopsies and critically revised the manuscript; S. Friday performed FLS real-time PCR experiments; S.Li performed FLS experiments; RM Patel provided tissue samples and helped in histological scoring; P. Chen isolated and provided IgM RF; V. Subramanian synthesized Cl-amidine; P. Thompson provided PAD4 and Cl-amidine and critically revised the manuscript; D. A. Fox was involved in FLS experiments and critically revised the manuscript, S. Pennathur was involved in analysis of proteomics experiments and critically revised the manuscript, M.J. Kaplan conceived the study, designed experiments, was involved in data analysis, patient recruitment and characterization and wrote the manuscript. All authors read and approved the content of the final version of the manuscript.

Competing interests: Paul Thompson holds the patent for Cl-amidine: Thompson, P.R. and Luo, Y. Synthesis and use of novel inhibitors and inactivators of protein arginine deiminases. Patent number: US 7,964,636 B2.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

chemokines and adhesion molecules. These observations implicate accelerated NETosis in RA pathogenesis, through externalization of citrullinated autoantigens and immunostimulatory molecules that may promote aberrant adaptive and innate immune responses in the joint and in the periphery, and perpetuate pathogenic mechanisms in this disease.

Introduction

Genetic and environmental factors contribute to the development of rheumatoid arthritis (RA), a chronic, systemic inflammatory disease that attacks synovial joints and leads to increased morbidity and mortality. Various cytokines, including TNF-α and IL-17, play fundamental roles in the processes causing inflammation, joint destruction, and various comorbidities in RA(1). RA follows a natural history divided into phases initially characterized by asymptomatic autoimmunity (detection of RA-related autoantibodies (Abs)), then evolving into clinically apparent disease(2). Indeed, RA-related pathogenic autoAbs (those to citrullinated proteins (ACPAs) and rheumatoid factor (RF)) are detected years before clinical diagnosis(2).

AutoAbs to citrullinated antigens (Ags) are highly specific for RA and recognize epitopes centered by citrulline, a postranslationally modified form of arginine(3). Experimental evidence indicates that citrullination is involved in breakdown of immune tolerance and may generate neoAgs that become additional targets during epitope spreading(4). Citrullinated proteins and immune complexes containing various citrullinated Ags have increased immunogenicity and arthritogenicity, and their presence in arthritic joints correlates with disease severity. Some of the candidate citrullinated autoAgs include vimentin, antithrombin, α-enolase and fibrinogen (4–7).

The peptidylarginine deiminase (PAD) enzymes 2 and 4 likely generate these citrullinated Ags because they are expressed in myeloid cells (8) and are detected in the RA synovium closely associated with neutrophilic infiltrates (9). Increased neutrophils in RA synovial fluid (SF), particularly in early disease stages, supports a prominent role for these cells in joint damage(10). Indeed, critical roles for neutrophils in initiating and maintaining joint inflammatory processes have been described in experimental arthritis (10, 11). However, the exact roles that neutrophils play in autoAg modification and disease initiation and perpetuation in RA remain unclear.

Recent evidence suggests that, among the various mechanisms by which neutrophils cause tissue damage and promote autoimmunity, aberrant formation of neutrophil extracellular traps (NETs) could play important roles in the pathogenesis of systemic lupus erythematosus (SLE), psoriasis, small vessel vasculitis (SVV) and gouty arthropathy (12–15). NETs, released via a novel form of cell death called NETosis, consist of a chromatin meshwork decorated with antimicrobial peptides typically present in neutrophil granules(16). Of potential relevance to RA pathogenesis, NETs have the capacity to externalize proinflammatory, immunostimulatory molecules and various autoAgs (13, 14, 17). Histone citrullination, catalyzed by PAD4, appears to be a critical step in NETosis, and citrullinated histones are externalized in the NETs(18). We hypothesized that enhanced NETosis in peripheral joints, blood or other tissues, could promote initiation and perpetuation of aberrant immune responses and inflammation in RA, by externalizing citrullinated proteins and other immunostimulatory molecules. We also investigated whether autoAbs and inflammatory cytokines elevated in RA patients promote NETosis, thereby perpetuating a cycle of citrullinated autoAg generation and induction of autoimmune responses.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Results

NETosis is enhanced in RA peripheral blood (PB) and SF neutrophils and this correlates with ACPA levels and systemic inflammation

PB and SF neutrophils from RA patients display a significantly increased propensity to form NETs in the absence of added stimuli, when compared to PB control neutrophils or to SF neutrophils isolated from patients with osteoarthritis (OA) (Figures 1A, 1C). Significantly increased NETosis was observed following LPS stimulation, when compared to baseline levels, in RA and control neutrophils. Upon LPS stimulation, PB and SF RA neutrophils displayed significantly enhanced capacity to form NETs, when compared to control and OA neutrophils (Figure 1B–D). Furthermore, netting neutrophils were detected as infiltrating cells in RA synovial tissue, rheumatoid nodules and skin from RA patients affected by neutrophilic dermatoses (Figures 1E–F, S1 and S2). These observations suggest that RA neutrophils are primed to undergo NETosis in the joints and in the periphery. Evidence of enhanced NET formation was observed in unstimulated RA neutrophils within 1 h of culture, and continued to increase by 2–3 h in culture (Figure S3). A significant correlation was found between percentage of PB netting neutrophils and serum levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), ACPA (Figure S3) and IL-17. In contrast, RA disease duration, RF titers and counts of painful and swollen joints did not correlate with NETosis. There were no associations between use of various RA medications (disease-modifying antirheumatic drugs (DMARDs), corticosteroids and/or biologics) and percentage of neutrophils undergoing NETosis (Tables S1 and S2). These results suggest that enhanced and accelerated NET formation occurs in RA neutrophils, and is associated with presence and levels of ACPA Abs and with systemic markers of inflammation.

RA autoAbs and inflammatory cytokines induce NETosis

Some autoAbs present in the serum of patients with various autoimmune diseases, including anti-neutrophil cytoplasmic (ANCA) and anti-RNP Abs, stimulate NETosis upon neutrophil priming with inflammatory cytokines (12, 19). To test if RA-specific autoAbs similarly enhanced NETosis, we first compared the effect of RA serum and SF with control serum or OA SF, respectively. Both serum and SF from RA patients with high levels of ACPA and/or RF significantly induced NETosis in control and RA neutrophils, when compared to control sera or OA SF (Figure 2A–C). Similarly, when compared with IgG purified from healthy control sera or osteoarthritis SF, IgG fractions obtained from sera or SF from RA patients with high titers of ACPA and/or RF significantly increased NETosis in control or RA neutrophils, in the absence of additional cytokine priming (Figure 2D–E). Purified IgM RFs also potently induced NETs in control and RA neutrophils, compared to control IgM (Figure 2F). IgG isolated from ACPA-positive and/or RF-positive RA patients could bind to control and RA NETs, while IgG isolated from controls did not (Figure 2G). These observations suggest that RA autoAbs can mediate NETosis, and that autoAbs against NET components are generated in RA patients.

Patients with RA have enhanced levels of circulating inflammatory cytokines, including TNF-α and IL-17A. Members of the IL-17 family modulate various proinflammatory effects of neutrophils, including chemotaxis and bone marrow mobilization. However, while TNF-α can induce NETosis in other conditions(20), it is unknown whether IL-17A also promotes NET formation. Both recombinant TNF-α and IL-17A significantly induced NETosis in RA neutrophils (Figure 3A). The degree of NET induction by IL-17 in RA neutrophils was comparable to induction observed by positive control (PMA). In control neutrophils, IL-17A by itself did not significantly enhance NETosis. However, upon priming with TNF-α, recombinant IL-17A led to significant increases in NETosis. In contrast, as previously

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

reported(21), control and RA neutrophils exposed to GM-CSF without other priming stimuli, did not display increased NET formation (Figure 3B).

While the molecular mechanisms implicated in NETosis remain incompletely characterized, the NADPH oxidase (NOX) pathway has been implicated as a critical step (22). Further, an important role for PAD4 in NETosis has been proposed, potentially due to its role in histone deimination (18). To gain insights into which of these signaling pathways control IL-17A–induced NETosis, we took a pharmacological approach and investigated the effects of inhibiting PAD4 and the generation of reactive oxygen species (ROS). NOX inhibition with diphenylene iodonium (DPI) or scavenging of ROS with N-acetylcysteine (NAC) decreased NETosis in RA and control neutrophils stimulated with IL-17 or with IL-17+TNF. Similarly, the PAD inhibitor Cl-amidine (23) significantly inhibited NETosis (Figure 3A). These results implicate both the ROS/NOX pathways and PAD activity as important players controlling NETosis induced by inflammatory cytokines characteristic of RA. Conversely, incubating RA neutrophils with RA serum in the presence of neutralizing Abs to TNF-α or to IL-17R significantly decreased NETosis, when compared to RA serum alone. A significant effect was also observed in control neutrophils exposed to RA serum in the presence of neutralizing anti-IL-17R Ab. A non-significant similar trend was observed in control neutrophils exposed to RA serum plus neutralizing anti-TNF-α (Figure 3B). When neutralizing anti-TNF and IL-17R Abs were added in tandem, significant NETosis inhibition was observed in control and RA neutrophils, which was comparable to inhibiting IL-17R alone but more significant that inhibiting TNF alone (Figure 3B). Overall, these observations indicate that various factors present in RA serum, including autoAbs and inflammatory cytokines promote NETosis. The results also suggest that, in comparison to control neutrophils, RA neutrophils are primed to undergo NETosis upon exposure to IL-17A and TNF-α.

NET formation may also occur through a rapid (10 min-1 h) non-lytic process independent of oxidant production (24). As significant NET formation occurred by 1 h in culture, particularly in the RA samples positive for ACPA (Figures 1 and S4), we tested whether this phenomenon was accompanied by lysis detection. Significant LDH release was detected in the same samples where NETs were induced in control and RA neutrophils, in response to various RA-related stimuli (Figure S4). This suggests that enhanced NET formation in RA neutrophils and/or upon exposure to RA autoAbs and inflammatory cytokines within 1 h in culture is associated to cell lysis, as described in other conditions(22).

RA autoAbs and cytokines induce distinct protein cargo in the NETs

Previous work identified 24 NET-associated proteins released from control neutrophils in response to PMA (25). However, it is unclear whether different stimuli trigger the release of a different subset of proteins into the NETs. We exposed control neutrophils to: a) IgG fractions isolated from sera of 4 RA patients with high ACPA and/or RF titers (100 mg/mL); b) purified IgM RF from 4 patients with monoclonal IgM cryoglobulinemia (100 mg/mL), or c) recombinant TNF-α (100 ng/mL) for 1 h. NETs induced by these stimuli were isolated and their protein cargo was determined by proteomics analysis. Depending on the experimental condition, the number of individual NET-specific proteins identified ranged between 28–40, and included various proteases and defensins that are typically present in neutrophil granules, as well as histones, cytoskeleton-related proteins and cytosolic proteins (Figure 4 and Table S3). IgM RF-stimulation and RA IgG enriched in ACPA-stimulation led to the highest number of proteins identified in the NETs (n=36 for each), while TNF-α stimulation led to the identification of 28 proteins. While most NET proteins were conserved upon different types of stimuli (Figure S5), some were only detected following specific stimulation. For example, MMP-8, histone 3 and vasodilator-stimulated phosphoprotein were only detected in RA IgG-induced NETs, whereas catalase, moesin, transaldolase,

NIH-PA Author Manuscript

phosphoglycerate mutase and olfactomedin-4 were only found in IgM RF-induced NETs. For the TNF-induced NETs, calmodulin, tropomyosin-3 and actin-related protein complex-3 were uniquely present in these samples (Table S3). Relative quantification of protein abundance was performed utilizing spectral counts as described(26), and statistically significant differences in NET protein levels in response to different types of stimulation were identified (Figure 4). Indeed, defensin-2 levels were significantly higher in RF-induced NETs than in the TNF-α-induced NETs, while MPO levels were significantly higher in RF-induced NETs than in those induced by RA IgG. Neutrophil gelatinase associated lipocalin and protein S-100A9 levels were significantly higher in the RF-induced NETs than in the other conditions. Overall, these results suggest that the protein cargo of the NETs varies depending on the specific stimulus utilized to induce these structures.

Citrullinated autoAgs are externalized during NETosis

The proteomic analysis described above identified several proteins that, when citrullinated, are important RA autoAgs(5, 7), including vimentin and α-enolase. We subsequently confirmed that these two proteins were present in the NETs formed by stimulation of healthy control neutrophils and in spontaneously formed or stimulated RA neutrophils (Figure 5A). Given the association between autoAbs targeting citrullinated vimentin (CV) and RA pathogenesis and inflammation(27, 28), we assessed the citrullination status of the vimentin in these NETs and found that this protein is indeed citrullinated (Figure 5B). These results indicate that NETs externalize citrullinated autoAgs reported to play important roles in RA pathogenesis.

Abs to CV stimulate NETosis

Given their high specificity for RA, and because CV is present in the RA-derived NETs, we hypothesized that anti-CV autoAbs can directly stimulate NETosis. To test this hypothesis, we purified human ACPAs with specificity to CV and determined whether they could stimulate NETosis. Indeed, anti-CV Abs potently induced NETs in control and RA neutrophils, when compared to control IgG (Figures 6A and S6). Furthermore, anti-CV Abs recognized vimentin externalized in the NETs (Figure 6B). These results indicate that autoAbs directed to specific citrullinated proteins present in the NETs enhance the formation of these lattices.

NETs activate RA and OA fibroblast-like synoviocytes (FLS)

To expand our understanding of the putative pathogenic roles of NETosis in the joints, we investigated whether NETs can stimulate FLS, which are the cells that invade cartilage in RA. Based on established kinetics of cytokine upregulation at the mRNA and protein level in these cells(29), we exposed FLS obtained from RA or OA patients to purified RA NETs for 24–48 h, and quantified the synthesis of IL-6, IL-8, CCL20 and ICAM-1 by ELISA and/or real-time quantitative PCR. Treatment of RA and OA FLS with RA NETs significantly upregulated IL-6 and IL-18 mRNA and protein levels, with responses being more dramatic in RA FLS (Figure 7A–B and S7). Neither IL-6 nor IL-8 were detected in the NETs alone condition. RA NETs also significantly upregulated mRNA levels of CCL20 and/or ICAM-1 in RA and OA FLS (Figure 7B). In additional experiments, when the NETs' architecture was disrupted with DNase, their stimulatory effect on FLS decreased (42–44% reduction in induction of IL-6 secretion by OA and RA FLS when stimulated with nuclease-treated versus untreated NETs; 50–74% and 43–58% decrease in IL-8 and CCL20 mRNA fold-induction, respectively in OA and RA FLS; $p<0.05$ when comparing NET-exposed versus unstimulated FLS and p=NS when comparing FLS with FLS exposed to DNase-treated NETs). These results indicate that NETs possess a robust capacity as strong stimulants of FLS expression of proinflammatory genes, a phenomenon that may amplify deleterious inflammatory responses in the RA synovium.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Discussion

Experimental evidence suggests that NETosis plays an important role in modification of autoAgs, their exposure to the immune system, and the induction of tissue damage in conditions like SLE, SVV and psoriasis(*12–14*). As activated neutrophils and anti-granulocyte Abs occur in RA, and there is a prominence of neutrophil recruitment in arthritis animal models (10, 11), we hypothesized that NETs may also play a pathogenic role in RA. Here we show that the RA pro-inflammatory milieu, characterized by specific autoAbs and increased proinflammatory cytokines, is highly conducive for the induction of NETosis in the absence of microbial stimuli. In turn, NETs externalize various immunostimulatory molecules and citrullinated autoAgs that, in predisposed individuals, may perpetuate a vicious cycle leading to generation of specific autoAbs and inflammatory responses (Figure 8).

NETs may provide the immune system with access to enhanced sources of citrullinated proteins, and thereby represent an early event preceding epitope spreading. In addition, RA NETs induce proinflammatory responses in FLS, revealing an additional amplifying mechanism of joint damage. Indeed, the secretion of proinflammatory cytokines, such as IL-8, by FLS upon exposure to extracellular traps, may further enhance NETosis (30), amplify citrullinated autoAg exposure and promote autoAb generation in predisposed hosts. Recent observations indicate that CV, one of the autoAgs we identified in the NETs, induces proinflammatory cytokine secretion, PAD4 and RANKL expression in RA FLS (31). This supports that CV and other autoAgs present in NETs may be crucial in inflammatory response activation in the joint.

The increase in NET formation in RA neutrophils and/or upon exposure to RA-associated autoAbs and cytokines was observed as early as 1 h and was accompanied by evidence of cell lysis. The observation that RA autoAbs (including anti-CV and RF) potently induce NETosis is similar to what has been reported for other autoAbs (ANCA for SVV and anti-RNP for SLE) (12, 19). We also found that RA sera, but not control, can bind to NETs. Circulating autoAbs present in serum of patients with Felty's syndrome, a severe variant of RA, can bind to deiminated histones and NETs (32). While in that study only a small percentage of RA serum samples showed preferential binding to deiminated histones(32), it is possible that RA serum or autoAbs can recognize other citrullinated epitopes present in netting neutrophils. In support of this hypothesis, anti-CV Abs bound to vimentin present in the NETs.

Periodontitis has emerged as a risk factor for RA(33). Infection with *P. gingivalis (Pg)*, the anaerobic pathogen primarily responsible for periodontal disease and also associated with chronic bacteremia(34), may play a central role in the early loss of tolerance to self-antigens in RA(35). Indeed, this microbe is the only identified prokaryote that expresses a PAD orthologue, and immunization with *Pg* enolase induces autoimmunity to mammalian α-enolase and arthritis in DR4-IE-transgenic mice (36). Importantly, increased NETosis occurs in gingival crevicular fluid from patients with periodontitis (37) and *Pg* can induce NET formation(38). One could speculate that enhanced citrullination and NETosis induced in the oral cavity in patients with *Pg*-induced periodontitis could be an initial event leading to generation of citrullinated autoAgs and autoAbs that could further promote NET enhancement and exacerbate autoimmune responses.

Smoking is also considered to be an important risk factor for RA. One of the proposed mechanisms is that tobacco smoke enhances pulmonary PAD expression, leading to generation of citrullinated proteins in this organ (39). Neutrophil derived PADs, present in the airways of smokers and those suffering other inflammatory conditions, can locally

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

citrullinate the immunostimulatory peptide LL37. This renders the protein more chemotactic and alters its overall activity (40). Since LL37 is externalized during NETosis(14, 17), it is possible that its citrullination (as well as the citrullination of other targets in the lung parenchyma), could further promote pathogenic responses in RA and other autoimmune diseases.

IL-17 and TNF are increased in RA sera and SF and their elevated levels predict joint damage progression. IL-17 has widespread inflammatory effects on the joint, orchestrates bone and cartilage damage and induces recruitment of proinflammatory mediators to the synovium(41). While IL-17 is known to potently recruit neutrophils, a role for this cytokine in the induction of NETs had not been previously described.

Our study also supports the concept that not all NETs are created equal, as NET protein content of healthy control neutrophils varied depending on the source of stimulation. This supports the need to better understand how NET composition is regulated in the healthy individual and in disease states, and may allow the development of therapeutic compounds to selectively target some of the deleterious aspects triggered by these traps. Indeed, it will be important to further explore the relevance of the differential protein expression in the NETs triggered by autoAbs, cytokines and other "sterile" conditions.

One limitation of this study pertains to the use of samples from patients that were already receiving DMARDs and/or biologics. While we did not observe any correlation between the use of these medications and the ability of RA neutrophils to form NETs, studies in therapy-naïve patients and longitudinal assessments of the impact of medications on the regulated development and severity of NETosis will be needed. In addition, the proteomic analysis might have failed to comprehensively identify all proteins with potential relevance to induction of pro inflammatory responses in RA, revealing a need to focus efforts on the identification of molecules of low abundance but high biological activity present in the NETs. As an example, LL37 has not been identified in the NETs by proteomic analyses by us and other groups (25), while it is readily identified in these structures by other techniques(14).

Identifying the role of aberrant NET formation in animal models of RA, as well as further characterizing the in vivo responses of FLS in response to netting neutrophils in the specific joint milieu, will clarify the rationale for testing NETosis inhibitors in future clinical trials in this and, potentially, other chronic inflammatory conditions. The observation that the PAD inhibitor Cl-amidine is effective in CIA(23) further supports this hypothesis, as this compound decreased IL-17A–induced NET formation in neutrophils. It will also be important to investigate the role that NETs play in the development of extra-articular manifestations of RA, including lung involvement and cardiovascular disease. Indeed, various studies in other patient populations and murine systems are supporting a role for NETs in both vascular damage and inflammatory conditions of the lung (14, 42).

We have shown that NETosis is enhanced in the PB and the synovium of patients with RA and that these structures contain targeted citrullinated autoAgs. Furthermore, this phenomenon correlates with presence and levels of ACPA Abs and with systemic inflammation. ACPAs and RF may perpetuate a vicious circle of NET production that maintains the delivery of modified autoAgs to the immune system. The propensity of neutrophils to form NETs in individuals with RA may be further enhanced by microbes or their products. Indeed, RA is known to frequently flare following bacterial or viral infections(43). Identifying the role of NETosis in RA pathogenesis could provide new potential targets for the treatment of this disease and its associated complications.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Materials and Methods

Human subjects

This study was approved by the University of Michigan Medical IRB. PB, SF and synovial tissue were collected from patients followed at the University of Michigan. RA patients enrolled met the 1987 American College of Rheumatology diagnostic criteria(44). OA diagnosis was based upon clinical and radiographic features, and confirmed by histopathological findings at joint surgery, when applicable. Age- and gender-matched healthy controls were enrolled by advertisement. Counts of swollen and tender joints, CRP, ESR, ACPA and RF were recorded at clinical visit. RF and ACPA were quantified by sheep cell agglutination titer replacement assay and by enzyme immunoassay, respectively. Table S1 displays demographic and clinical characteristics of patients included.

PB and SF neutrophil isolation: Detailed methods are included in supplementary methodsl

Quantification of NETs by fluorescent microscopy and plate assay—NETs in PB or SF were quantified by immunofluorescence microscopy, as described by our group (14) or by plate assay. Detailed methods are included in supplementary methods. NETs were also quantified in RA and OA frozen synovial tissue, rheumatoid nodules and in skin biopsies from patients with dermatologic manifestations of RA, using techniques described by us (13, 14), and detailed in supplementary methods. The percentage of NETs was calculated as an average of 5–10 fields (400x) normalized to total number of neutrophils, and results expressed as mean % ± SEM.

Quantification of serum inflammatory cytokines: Serum IL-6 and IL-17 were quantified by ELISA as previously described by us(45)

IgG purification—IgG was purified from RA or control sera and from RA or OA SF, using a protein G agarose kit and following manufacturer's instructions (Pierce). Serum or SF were diluted in IgG binding buffer and run through a protein G agarose column 5–10 times. Igs were eluted with 0.1M glycine and neutralized with 1M Tris, followed by overnight dialysis at 4°C. A microtiter plate protein assay (Bio-Rad) was used to calculate Ig concentration; isolation of IgG was verified using by Coomassie staining.

Purification of Abs to citrullinated vimentin—Wild type recombinant human PAD4 was purified as previously described (46) and detailed methods are included in supplementary materials.

Purification of monoclonal IgM RF—The purification of IgM RF obtained from plasma or purified proteins from 5 patients with monoclonal IgM cryoglobulinemia was previously described (47). In brief, IgM cryoglobulins were purified by repeated precipitation at 4°C, followed by chromatography on Sephadex G-200 or Ultrogel AcA 22 in 0.2 M sodium acetate (pH 3.5). IgM and IgG peaks were identified by immunodiffusion, and appropriate fractions were pooled and stored at −20°C until used.

NET purification and quantification—NETs were isolated as previously described(25) and details are included in supplementary methods.

Proteomic analysis of NETs' content and LC/ESI MS/MS analysis: Details are included in supplementary methods

Assessment of citrullinated proteins in NETs—Control and RA NETs were incubated overnight at 4°C with G-Sepharose beads and mouse anti-human vimentin mAb. Beads were washed and bound proteins eluted by boiling in Laemmli buffer. Samples were

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

separated by SDS-PAGE, and proteins were transferred onto nitrocellulose membranes. Citrullination was detected using anti-citrulline (modified) detection kit (Millipore). Briefly, membrane was blocked with 10% casein for 15 min at RT and incubated with 2,3-butanedione monoxine and antipyrine in acids overnight at 37°C. Detection of modified citrulline was performed according to manufacturer's protocol using anti-modified Ab and goat anti-rabbit IgG-HRP.

FLS isolation and culture—FLS were isolated as described(48). Cells were harvested by collagenase (Worthington Biochemical) digestion of RA or OA human synovial tissue obtained at arthroplasty or synovectomy. Cells were maintained in CMRL medium (Invitrogen)/10% FCS/2 mM glutamine/50 U/mL penicillin/50 μg/ml streptomycin (Cambrex). FLS were used after passage 4 and cultured for 48 h in 6- or 12-well plates, followed by stimulation for 24–48 h with 150 μg NETs (1μg/1,000 FLS). In some conditions, NETs were treated with 100 U/mL DNase-I for 30 min at 37°C, prior to FLS stimulation. IL-8 and IL-6 secretion by FLS was quantified by ELISA (BD Biosciences).

FLS isolation of RNA and real-time PCR: Detailed methods are included in supplementary materials

Statistical analysis—The difference between means was analyzed using paired or unpaired Student *t* test or ANOVA with post hoc analysis. Univariate linear regression was performed to determine whether treatment with DMARDS, biologics or steroids was associated with NETosis. Pearson or Spearman's rank correlations were used to examine associations between continuous variables. A p value <0.05 was considered significant. Analysis was performed with Prism 5 software (version 5.0a; GraphPad) and with SPSS software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: Supported by *Within Our Reach* grants from the Rheumatology Research Foundation (to MJK and to SP) and, in part, by the National Institutes of Health through PHS grants HL088419, AR007197, AR38477, AR007080, GM079357 and DK89503.

References and Notes

1. Tarner IH, Muller-Ladner U, Gay S. Emerging targets of biologic therapies for rheumatoid arthritis. Nat Clin Pract Rheumatol. 2007; 3:336–345. [PubMed: 17538565]
2. Deane KD, Norris JM, Holers VM. Preclinical rheumatoid arthritis: identification evaluation and future directions for investigation. Rheum Dis Clin North Am. 2010; 36:213–241. [PubMed: 20510231]
3. Makrygiannakis D, af Klint E, Lundberg IE, Lofberg R, Ulfgren AK, Klareskog L, Catrina AI. Citrullination is an inflammation-dependent process. Ann Rheum Dis. 2006; 65:1219–1222. [PubMed: 16540548]
4. Kidd BA, Ho PP, Sharpe O, Zhao X, Tomooka BH, Kanter JL, Steinman L, Robinson WH. Epitope spreading to citrullinated antigens in mouse models of autoimmune arthritis and demyelination. Arthritis Res Ther. 2008; 10:R119. [PubMed: 18826638]
5. Lundberg K, Nijenhuis S, Vossenaar ER, Palmblad K, van Venrooij WJ, Klareskog L, Zendman AJ, Harris HE. Citrullinated proteins have increased immunogenicity and arthritogenicity and their presence in arthritic joints correlates with disease severity. Arthritis Res Ther. 2005; 7:R458–R467. [PubMed: 15899032]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

6. Sokolove J, Zhao X, Chandra PE, Robinson WH. Immune complexes containing citrullinated fibrinogen costimulate macrophages via Toll-like receptor 4 and Fcgamma receptor. Arthritis Rheum. 2011; 63:53–62. [PubMed: 20954191]
7. Kinloch A, Tatzer V, Wait R, Peston D, Lundberg K, Donatien P, Moyes D, Taylor PC, Venables PJ. Identification of citrullinated alpha-enolase as a candidate autoantigen in rheumatoid arthritis. Arthritis Res Ther. 2005; 7:R1421–R1429. [PubMed: 16277695]
8. Jones JE, Causey CP, Knuckley B, Slack-Noyes JL, Thompson PR. Protein arginine deiminase 4 (PAD4): Current understanding and future therapeutic potential. Curr Opin Drug Discov Devel. 2009; 12:616–627.
9. Foulquier C, Sebbag M, Clavel C, Chapuy-Regaud S, Al Badine R, Mechin MC, Vincent C, Nachat R, Yamada M, Takahara H, Simon M, Guerrin M, Serre G. Peptidyl arginine deiminase type 2 (PAD-2), PAD-4 but not PAD-1 PAD-3and PAD-6 are expressed in rheumatoid arthritis synovium in close association with tissue inflammation. Arthritis Rheum. 2007; 56:3541–3553. [PubMed: 17968929]
10. Pillinger MH, Abramson SB. The neutrophil in rheumatoid arthritis. Rheum Dis Clin North Am. 1995; 21:691–714. [PubMed: 8619095]
11. Matsubara S, Yamamoto T, Tsuruta T, Takagi K, Kambara T. Complement C4-derived monocyte-directed chemotaxis-inhibitory factor. A molecular mechanism to cause polymorphonuclear leukocyte-predominant infiltration in rheumatoid arthritis synovial cavities. Am J Pathol. 1991; 138:1279–1291. [PubMed: 2024711]
12. Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, Grone HJ, Brinkmann V, Jenne DE. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med. 2009; 15:623–625. [PubMed: 19448636]
13. Lin AM, Rubin CJ, Khandpur R, Wang JY, Riblett M, Yalavarthi S, Villanueva EC, Shah P, Kaplan MJ, Bruce AT. Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. J Immunol. 2011; 187:490–500. [PubMed: 21606249]
14. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, Rubin CJ, Zhao W, Olsen SH, Klinker M, Shealy D, Denny MF, Plumas J, Chaperot L, Kretzler M, Bruce AT, Kaplan MJ. Netting neutrophils induce endothelial damage infiltrate tissues and expose immunostimulatory molecules in systemic lupus erythematosus. J Immunol. 2011; 187:538–552. [PubMed: 21613614]
15. Mitroulis I, Kambas K, Chrysanthopoulou A, Skendros P, Apostolidou E, Kourtzelis I, Drosos GI, Boumpas DT, Ritis K. Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. PLoS One. 2011; 6:e29318. [PubMed: 22195044]
16. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. Science. 2004; 303:1532–1535. [PubMed: 15001782]
17. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, Meller S, Chamilos G, Sebasigari R, Riccieri V, Bassett R, Amuro H, Fukuhara S, Ito T, Liu YJ, Gilliet M. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Sci Transl Med. 2011; 3:73ra19.
18. Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. J Exp Med. 2010; 207:1853–1862. [PubMed: 20733033]
19. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, Punaro M, Baisch J, Guiducci C, Coffman RL, Barrat FJ, Banchereau J, Pascual V. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med. 2011; 3:73ra20.
20. Gupta AK, Joshi MB, Philippova M, Erne P, Hasler P, Hahn S, Resink TJ. Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death. FEBS Lett. 2010; 584:3193–3197. [PubMed: 20541553]
21. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ. 2009; 16:1438–1444. [PubMed: 19609275]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

22. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol. 2007; 176:231–241. [PubMed: 17210947]
23. Willis VC, Gizinski AM, Banda NK, Causey CP, Knuckley B, Cordova KN, Luo Y, Levitt B, Glogowska M, Chandra P, Kulik L, Robinson WH, Arend WP, Thompson PR, Holers VM. N-alpha-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide a protein arginine deiminase inhibitorreduces the severity of murine collagen-induced arthritis. J Immunol. 2011; 186:4396–4404. [PubMed: 21346230]
24. Pilsczek FH, Salina D, Poon KK, Fahey C, Yipp BG, Sibley CD, Robbins SM, Green FH, Surette MG, Sugai M, Bowden MG, Hussain M, Zhang K, Kubes P. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. J Immunol. 2010; 185:7413–7425. [PubMed: 21098229]
25. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, Brinkmann V, Jungblut PR, Zychlinsky A. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog. 2009; 2009; 5:e1000639.
26. Vivekanandan-Giri A, Slocum JL, Buller CL, Basrur V, Ju W, Pop-Busui R, Lubman DM, Kretzler M, Pennathur S. Urine glycoprotein profile reveals novel markers for chronic kidney disease. Int J Proteomics. 2011; 2011:214715. [PubMed: 22091387]
27. Harre U, Georgess D, Bang H, Bozec A, Axmann R, Ossipova E, Jakobsson PJ, Baum W, Nimmerjahn F, Szarka E, Sarmay G, Krumbholz G, Neumann E, Toes R, Scherer HU, Catrina AI, Klareskog L, Jurdic P, Schett G. Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. J Clin Invest. 2012; 122:1791–1802. [PubMed: 22505457]
28. Montes A, Perez-Pampin E, Calaza M, Gomez-Reino JJ, Gonzalez A. Association of anti-citrullinated vimentin and anti-citrullinated alpha-enolase antibodies with subsets of rheumatoid arthritis patients. Arthritis Rheum. 2012; 64:3102–3110. [PubMed: 22674012]
29. Katz Y, Nadiv O, Beer Y. Interleukin-17 enhances tumor necrosis factor alpha-induced synthesis of interleukins 1 ,6 and 8 in skin and synovial fibroblasts: a possible role as a “fine-tuning cytokine” in inflammation processes. Arthritis Rheum. 2001; 44:2176–2184. [PubMed: 11592383]
30. Gupta AK, Hasler P, Holzgreve W, Gebhardt S, Hahn S. Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. Hum Immunol. 2005; 66:1146–1154. [PubMed: 16571415]
31. Fan L, He D, Wang Q, Zong M, Zhang H, Yang L, Sun L. Citrullinated vimentin stimulates proliferation pro-inflammatory cytokine secretion and PADI4 and RANKL expression of fibroblast-like synoviocytes in rheumatoid arthritis. Scand J Rheumatol. 2012; 41:354–358. [PubMed: 22765310]
32. Dwivedi N, Upadhyay J, Neeli I, Khan S, Pattanaik D, Myers L, Kirou KA, Hellmich B, Knuckley B, Thompson PR, Crow MK, Mikuls TR, Csernok E, Radic M. Felty's syndrome autoantibodies bind to deiminated histones and neutrophil extracellular traps. Arthritis Rheum. 2012; 64:982–992. [PubMed: 22034172]
33. Scher JU, Ubeda C, Equinda M, Khanin R, Buischi Y, Viale A, Lipuma L, Attur M, Pillinger MH, Weissmann G, Littman DR, Pamer EG, Bretz WA, Abramson SB. Periodontal disease and the oral microbiota in new-onset rheumatoid arthritis. Arthritis Rheum. 2012; 64:3083–3094. [PubMed: 22576262]
34. Perez-Chaparro PJ, Gracieux P, Lafaurie GI, Donnio PY, Bonnaure-Mallet M. Genotypic characterization of Porphyromonas gingivalis isolated from subgingival plaque and blood sample in positive bacteremia subjects with periodontitis. J Clin Periodontol. 2008; 35:748–753. [PubMed: 18662301]
35. Mikuls TR, Thiele GM, Deane KD, Payne JB, O'Dell JR, Yu F, Sayles H, Weisman MH, Gregersen PK, Buckner JH, Keating RM, Derber LA, Robinson WH, Michael Holers V, Norris JM. Porphyromonas gingivalis and disease-related autoantibodies in individuals at increased risk for future rheumatoid arthritis. Arthritis Rheum. 2012; 64:3522–3530. [PubMed: 22736291]
36. Kinloch AJ, Alzabin S, Brintnell W, Wilson E, Barra L, Wegner N, Bell DA, Cairns E, Venables PJ. Immunization with Porphyromonas gingivalis enolase induces autoimmunity to mammalian

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

alpha-enolase and arthritis in DR4-IE-transgenic mice. Arthritis Rheum. 2011; 63:3818–3823. [PubMed: 21953289]

37. Vitkov L, Klappacher M, Hannig M, Krautgartner WD. Neutrophil fate in gingival crevicular fluid. Ultrastruct Pathol. 2010; 34:25–30. [PubMed: 20070150]
38. Delbosc S, Alsac JM, Journe C, Louedec L, Castier Y, Bonnaure Mallet , Ruimy R, Rossignol P, Bouchard C, Louedec P, Michel JB, Meilhac O. Porphyromonas gingivalis participates in pathogenesis of human abdominal aortic aneurysm by neutrophil activation. Proof of concept in rats. PLoS One. 2011; 6:e18679.
39. Makrygiannakis D, Hermansson M, Ulfgren AK, Nicholas AP, Zendman AJ, Eklund A, Grunewald J, Skold CM, Klareskog L, Catrina AI. Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. Ann Rheum Dis. 2008; 67:1488–1492. [PubMed: 18413445]
40. Kilsgard O, Andersson P, Malmsten M, Nordin SL, Linge HM, Eliasson M, Sorenson E, Erjefalt JS, Bylund J, Olin AI, Sorensen OE, Egesten A. Peptidylarginine deiminases present in the airways during tobacco smoking inflammation can citrullinate the host defense peptide LL-37 resulting in altered activities. Am J Respir Cell Mol Biol. 2012; 46:240–248. [PubMed: 21960546]
41. Maddur MS, Miossec P, Kaveri SV, Bayry J. Th17 cells: biology pathogenesis of autoimmune, inflammatory diseases and therapeutic strategies. Am J Pathol. 2012; 181:8–18. [PubMed: 22640807]
42. Doring Y, Manthey HD, Drechsler M, Lievens D, Megens RT, Soehnlein O, Busch M, Manca M, Koenen RR, Pelisek J, Daemen MJ, Lutgens E, Zenke M, Binder CJ, Weber C, Zernecke A. Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. Circulation. 2012; 125:1673–1683. [PubMed: 22388324]
43. Stojanovich L. Influenza vaccination of patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Clin Dev Immunol. 2006; 13:373–375. [PubMed: 17162380]
44. van der Linden MP, Knevel R, Huizinga TW, van der Helm-van Mil AH. Classification of rheumatoid arthritis: comparison of the 1987 American College of Rheumatology criteria and the 2010 American College of Rheumatology/European League Against Rheumatism criteria. Arthritis Rheum. 2011; 63:37–42. [PubMed: 20967854]
45. Marder W, Khalatbari S, Myles JD, Hench R, Yalavarthi S, Lustig S, Brook R, Kaplan MJ. Interleukin 17 as a novel predictor of vascular function in rheumatoid arthritis. Ann Rheum Dis. 2011; 70:1550–1555. [PubMed: 21727237]
46. Kearney PL, Bhatia M, Jones NG, Yuan L, Glascock MC, Catchings KL, Yamada M, Thompson PR. Kinetic characterization of protein arginine deiminase 4: a transcriptional corepressor implicated in the onset and progression of rheumatoid arthritis. Biochemistry. 2005; 44:10570–10582. [PubMed: 16060666]
47. Chen PP, Houghten RA, Fong S, Rhodes GH, Gilbertson TA, Vaughan JH, Lerner RA, Carson DA. Anti-hypervariable region antibody induced by a defined peptide: an approach for studying the structural correlates of idiotypes. Proc Natl Acad Sci U S A. 1984; 81:1784–1788. [PubMed: 6200884]
48. Tran CN, Thacker SG, Louie DM, Oliver J, White PT, Endres JL, Urquhart AG, Chung KC, Fox DA. Interactions of T cells with fibroblast-like synoviocytes: role of the B7 family costimulatory ligand B7-H3. J Immunol. 2008; 180:2989–2998. [PubMed: 18292521]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

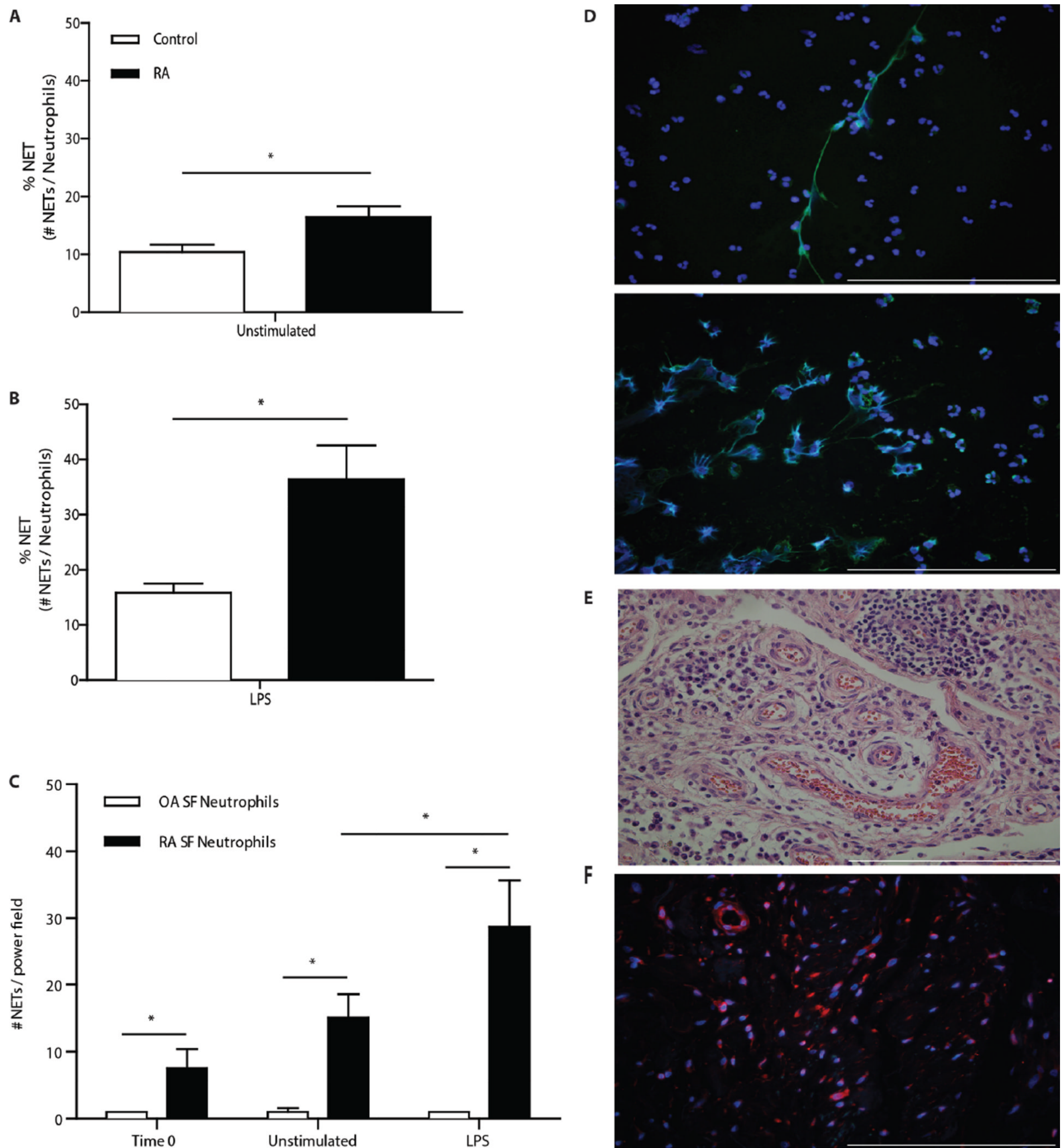

Figure 1. Enhanced NETosis in RA neutrophils

A and **B**. Peripheral blood (PB) RA neutrophils undergo increased NETosis in the absence (**A**) or presence (**B**) of added stimulation (LPS for 1 h); (control, n=7; RA, n=13–14). **C**. SF RA neutrophils undergo increased NETosis in the absence (unstimulated) or presence of 1 h LPS stimulation, n=5/group, *p<0.05. Results represent mean ± SEM **D**. Representative microphotographs display enhanced LPS-induced NETosis in RA (bottom) vs. control PB neutrophils (top). NETs were visualized as structures co-staining for neutrophil elastase (green) and DAPI (blue); 40x magnification. **E**. and **F**. Multilobate inflammatory cells consistent with netting neutrophils are present in RA synovial tissue. **E** represents H&E

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

stain (400X) of RA synovial tissue. **F** represents same area stained with anti-MPO (red) and DAPI (blue). Squares represent areas where netting neutrophils were observed. Images are representative of 3 RA patients. Statistical analysis was with two-tailed unpaired t tests except for SF experiments where paired t-test was used. Bar is 10 μm.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

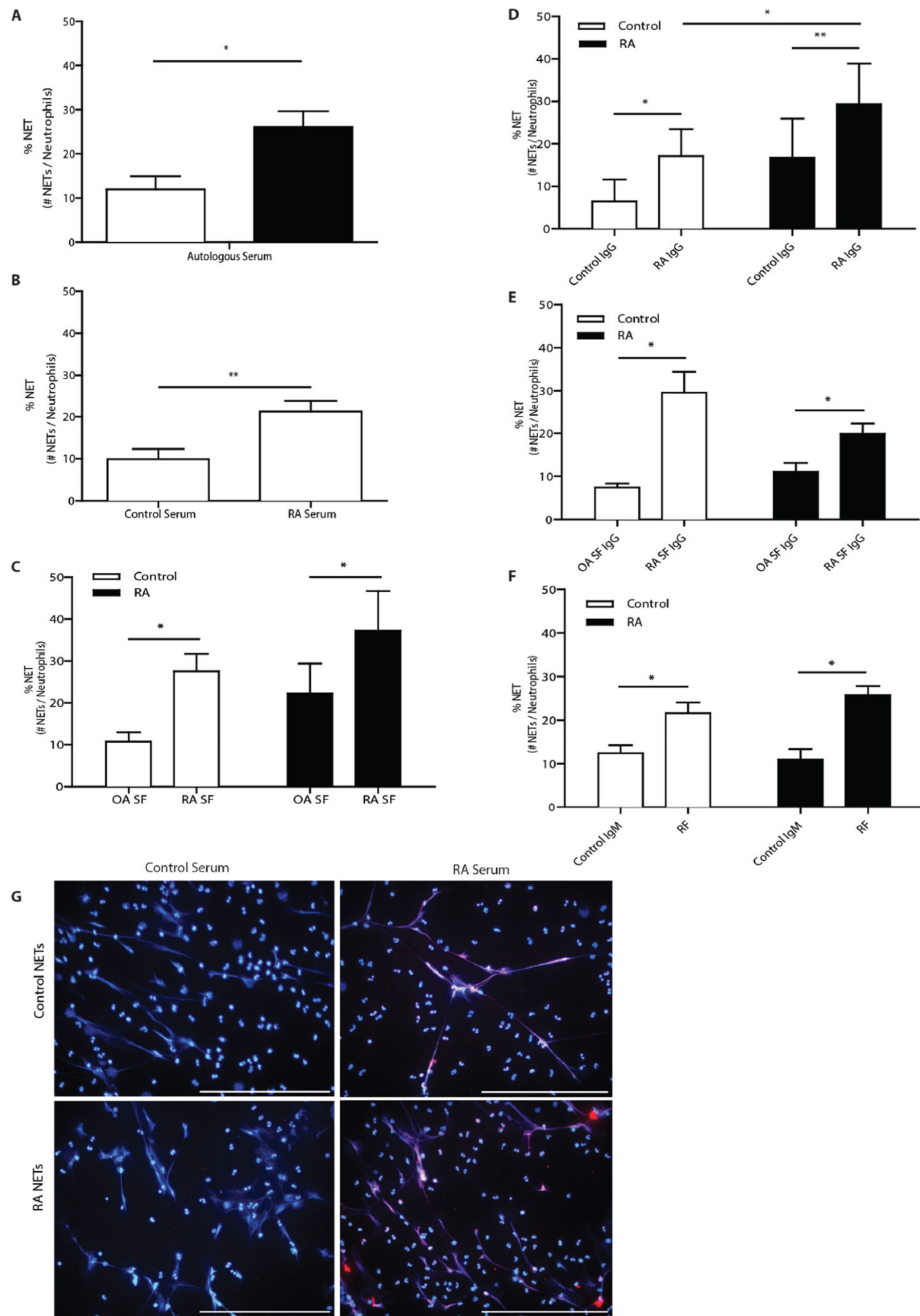

Figure 2. AutoAbs present in RA serum and SF induce NETosis and bind to NETs

A–C: RA serum and SF induce significantly higher NETosis in RA autologous neutrophils (white bar) and control neutrophils (black bar), when compared to the effect of control sera or OA SF on autologous neutrophils or RA neutrophils. **D–E**. Similar effects were observed when comparing IgG purified from RA serum or from SF from patients with seropositive RA, compared to control serum or OA SF. **F**. Purified IgM RF significantly enhances NETs in RA and control neutrophils (n=3–8/group). The % of NETosis (elastase and DAPI labeled neutrophils/total neutrophils) was quantified after 1 h exposure to serum or SF. Results represent mean ± SEM; *p<0.05; **p<0.01 using two-tailed unpaired t tests. **G**. RA IgG

isolated from patients with higher titer ACPA and/or RF binds to RA and control NETs induced by LPS. Results are representative of 3 independent experiments. Bar is 10 μm. NC: normal control; OA: osteoarthritis: RA: rheumatoid arthritis, SF: synovial fluid; RF: rheumatoid factor.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

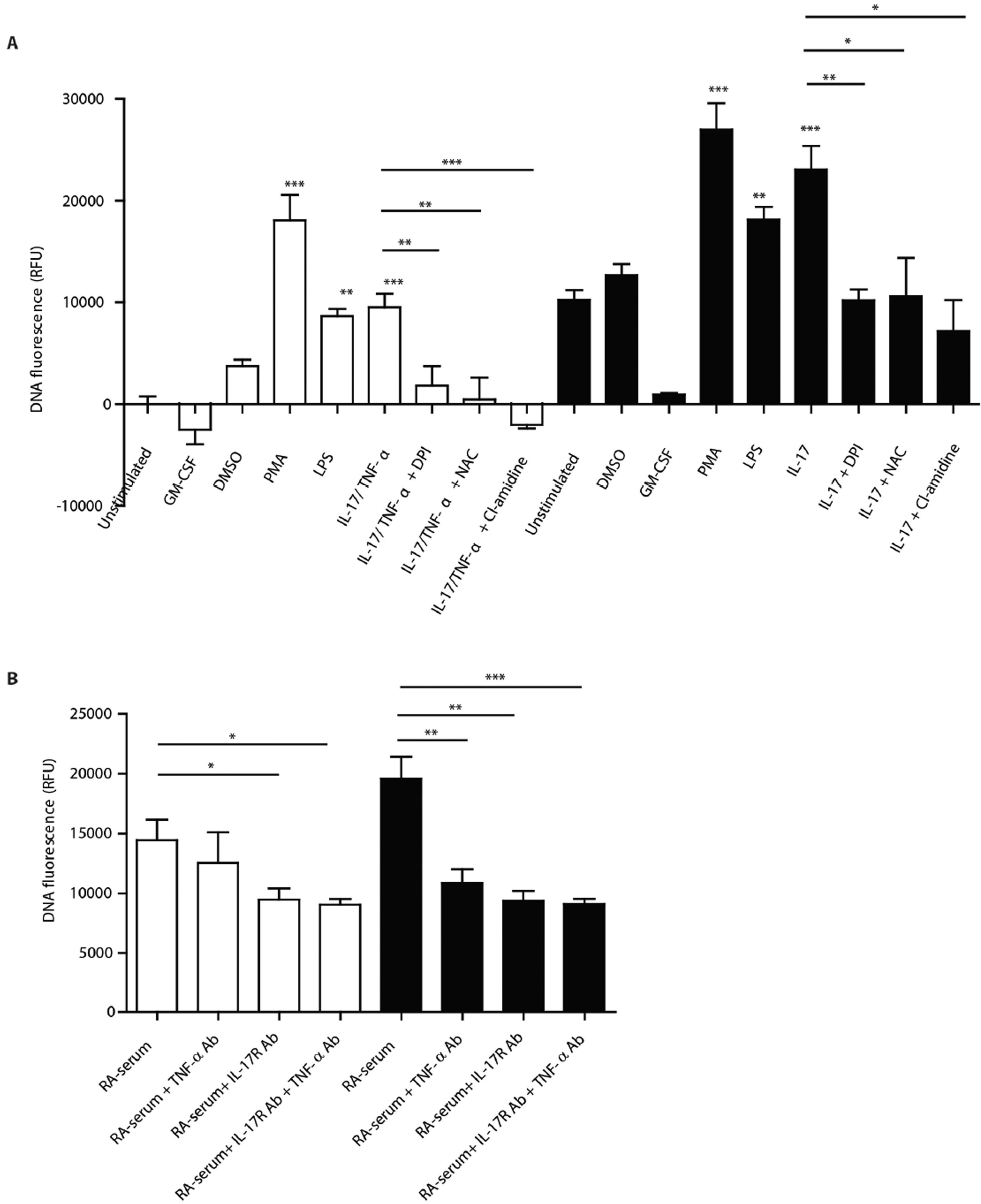

Figure 3. Proinflammatory cytokines induce NETosis and this is enhanced in RA neutrophils

A. Results are adjusted to levels observed in unstimulated control neutrophils. DPI: diphenyleneiodonium chloride; NAC: N-acetyl cysteine; Cl-amidine: PAD inhibitor. **B. Neutralizing Abs to TNF and/or IL-17R decrease NETosis induced by RA sera**. Results are expressed as relative fluorescent units (RFUs) of DNA fluorescence using Sytox green assay. Results represent mean ±SEM of 3 independent experiments, performed in triplicate; * $p<0.05$, ** $p < 0.005$, *** $p < 0.0001$, using two-tailed (A) and one-tailed (B) unpaired t tests.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

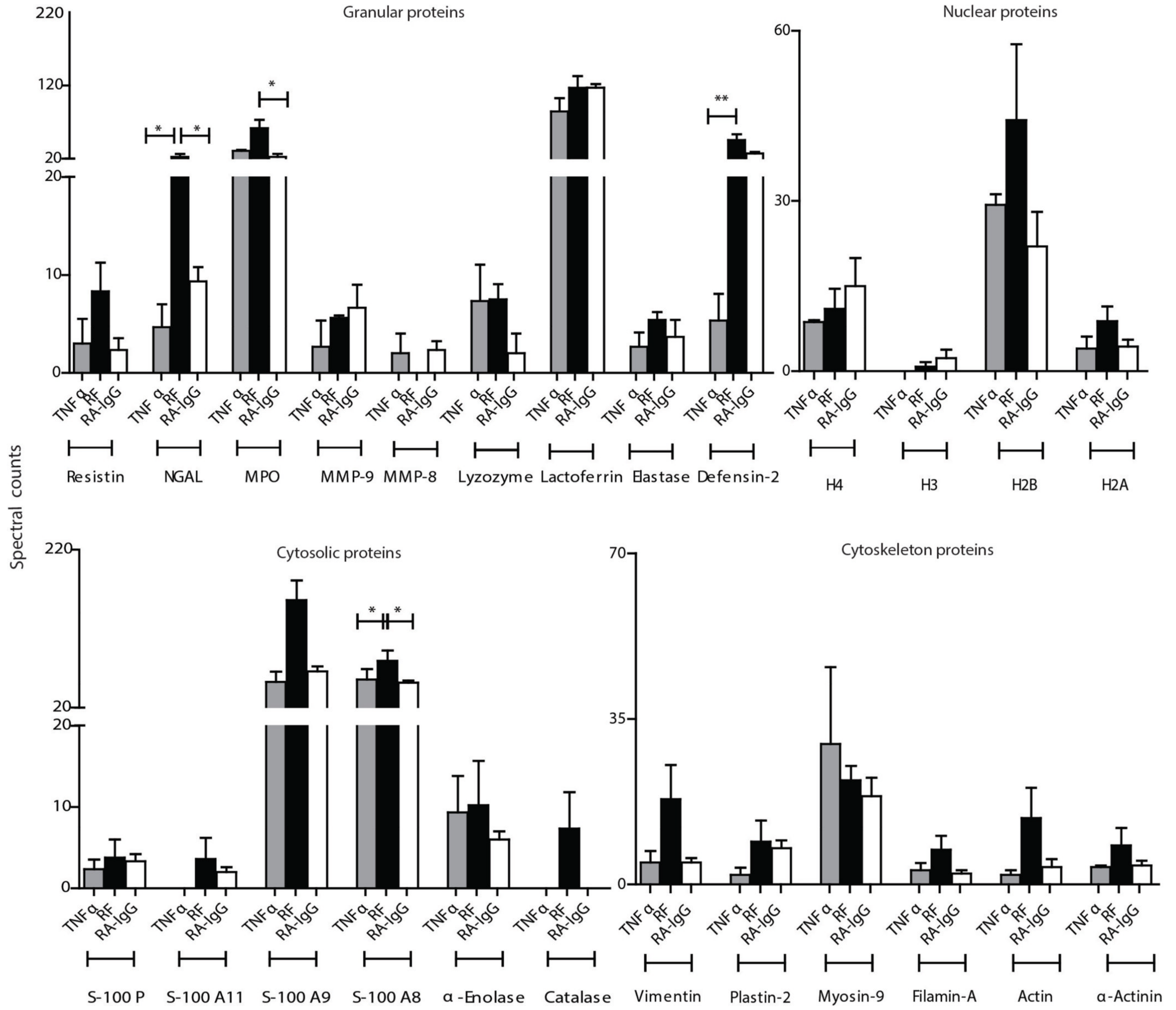

Figure 4. Various stimuli present in RA sera induce distinct protein cargo in control NETs

Bar graphs represent spectral counts from 25 of the proteins identified in the NETs, which were normalized for protein content. RF=rheumatoid factor, RA IgG=IgG isolated from RA patients with high titers of ACPA and/or RF; *p<0.05, ** p<0.01, when comparing proteins expressed in the NETs induced by the 3 conditions. Results represent mean ±SEM of 3–5 independent experiments. Comparisons among groups were done with one-way ANOVA and p values were adjusted for Bonferroni's multiple comparison test.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

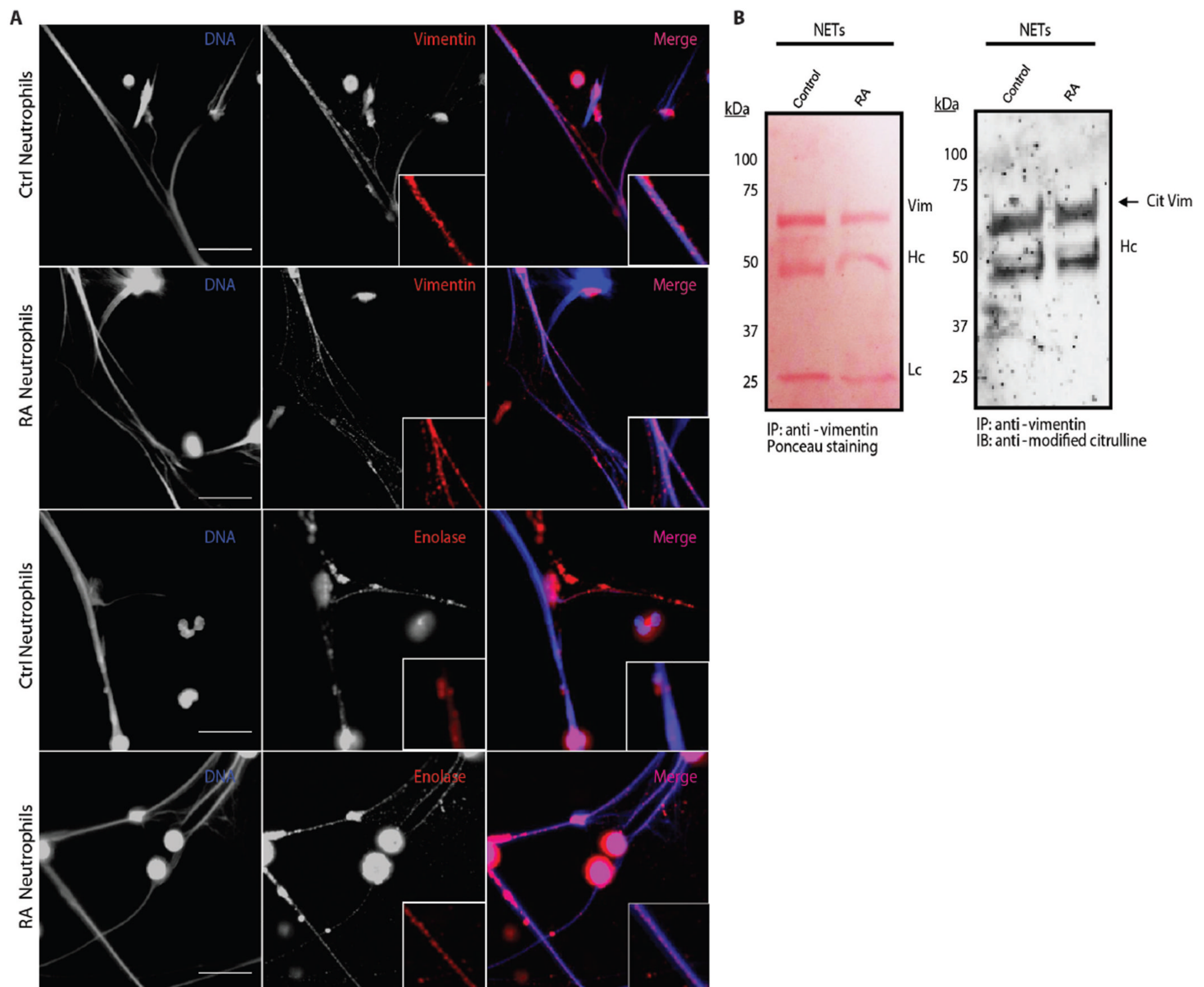

Figure 5.

A. Vimentin and α-enolase decorate control and RA NETs. Red represents vimentin or α-enolase and blue is Hoechst. Total magnification 63X. **B. Vimentin externalized in control and RA NETs is citrullinated**. Left panel is visualization with Ponceau. Right panel depicts immunoblot using anti-modified citrulline and detection by chemiluminescence (right). Molecular weight markers (kDa) are indicated. Cit Vim=citrullinated vimentin; Hc=heavy chain; Lc=light chain; Vim=vimentin; IP: immunoprecipitate; IB: Immunoblot. Results are representative of 3 independent experiments. Bar is 10 μm.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

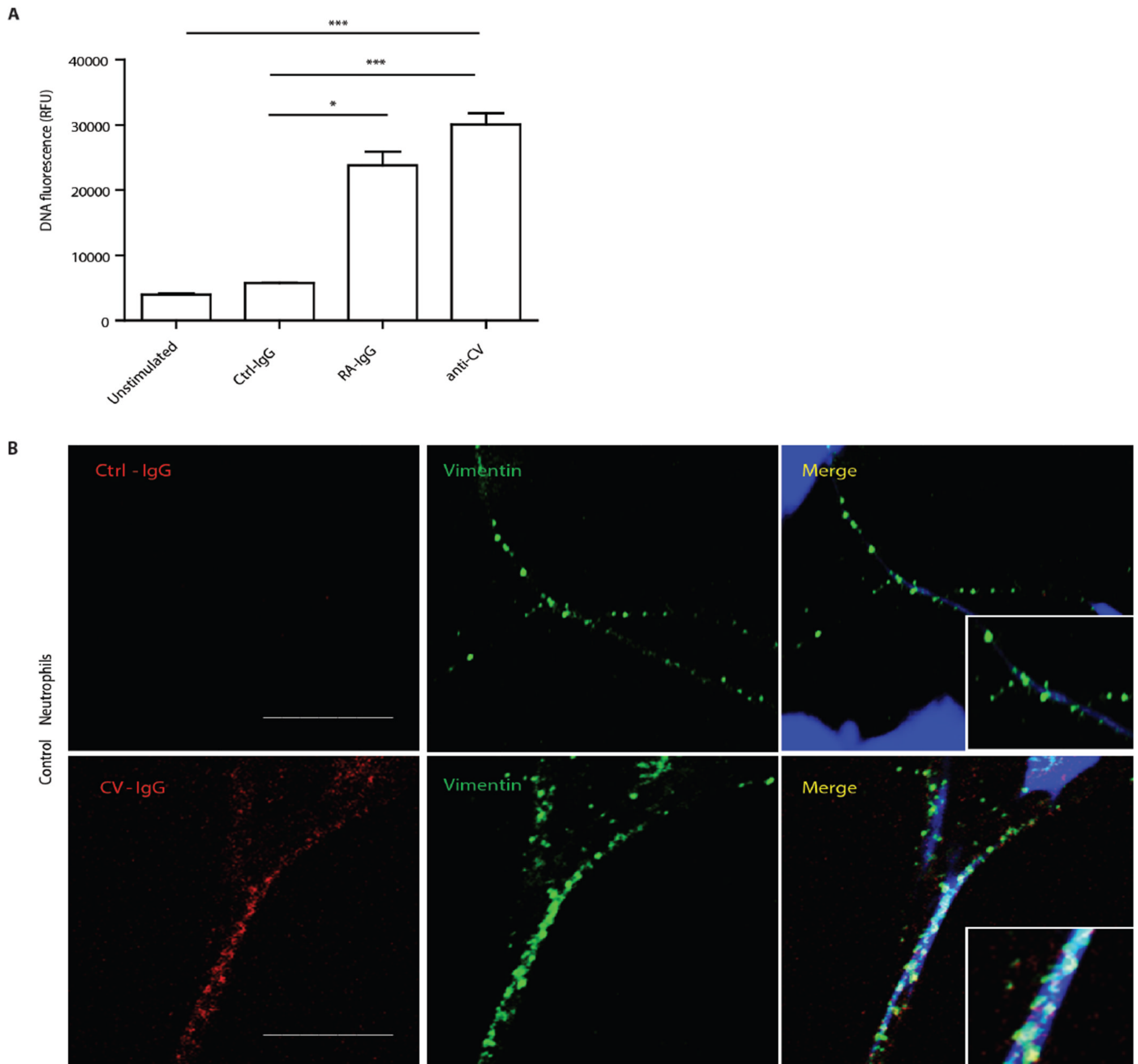

Figure 6.

A. Anti-CV Abs isolated from RA sera significantly enhance control neutrophil NETosis. Results represent mean+SEM of 3 independent experiments, each performed in duplicate. Units are expressed as RFUs of DNA fluorescence using Sytox green assay; *$p<0.05$, ***$p<0.001$, using two-tailed unpaired t test. **B. Anti-CV Abs bind to vimentin externalized in the NETs**. Original magnification is 63x; Ctrl=control. Results are representative of 2 independent experiments. Bar is 10 μm.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

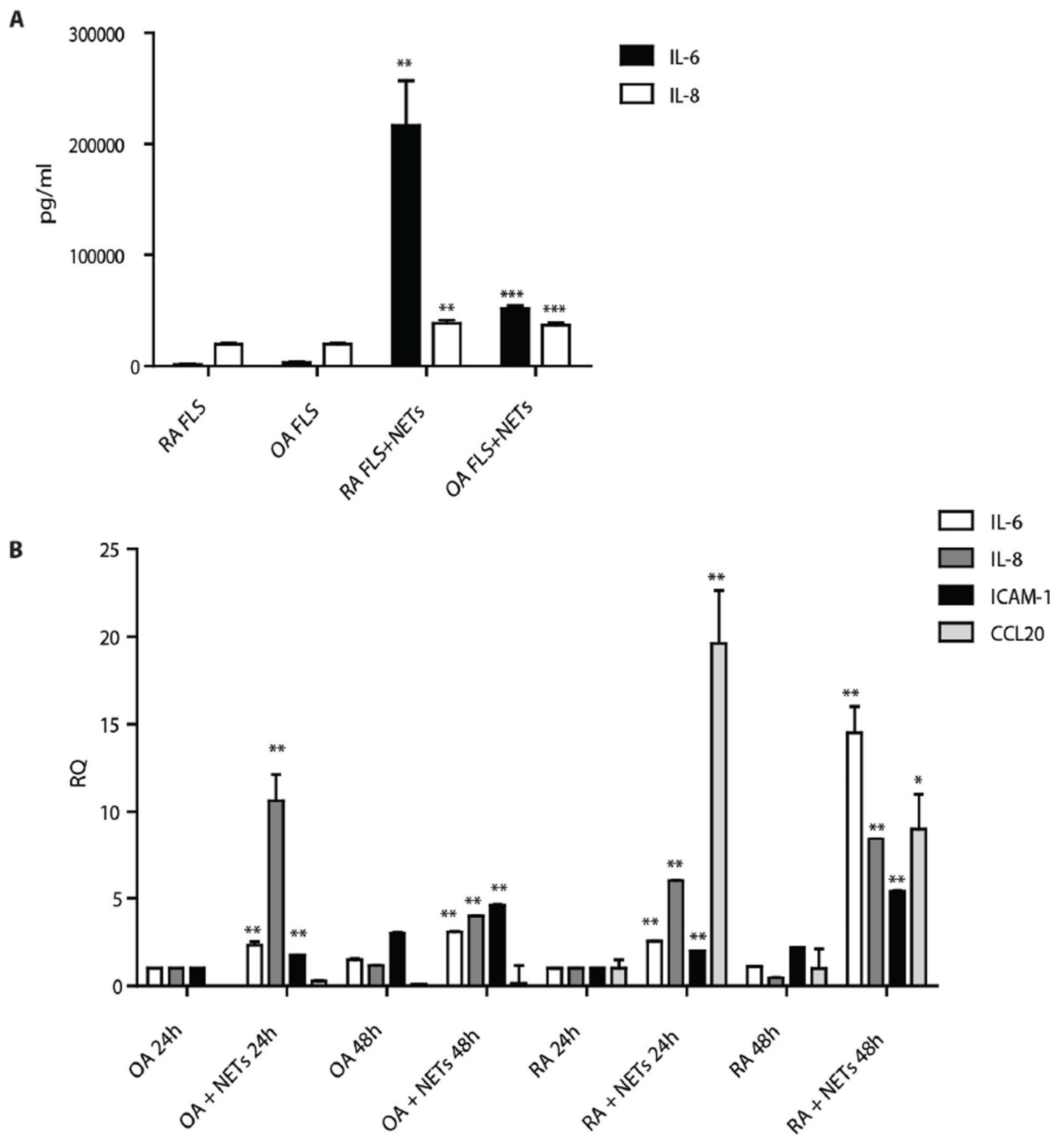

Figure 7.

A. RA and OA FLS upregulate inflammatory cytokine synthesis upon exposure to RA NETs for 48 h. Results represent mean±SEM of 2 independent experiments, each performed in triplicate-quadruplicate; $^*p<0.05$, $^{**} p<0.01$, $^{***}p<0.001$, using two-tailed unpaired t tests. **B. RA and OA FLS upregulate mRNA synthesis of inflammatory cytokines, chemokines and adhesion molecules upon exposure to RA NETs**. Results are expressed as fold induction of the specific gene at 48 h, when compared to untreated FLS at 24 h of culture, adjusted for housekeeping gene (*GAPDH*). Error bars represent maximum RQ value for each sample and represent mean±SEM of 2–3 independent experiments.

*p<0.05 comparing change in mean DCt compared with untreated samples at same time-point, using two-tailed unpaired t tests. For untreated OA FLS, there was no amplification of CCL20, suggesting the basal expression was below the level of detection.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

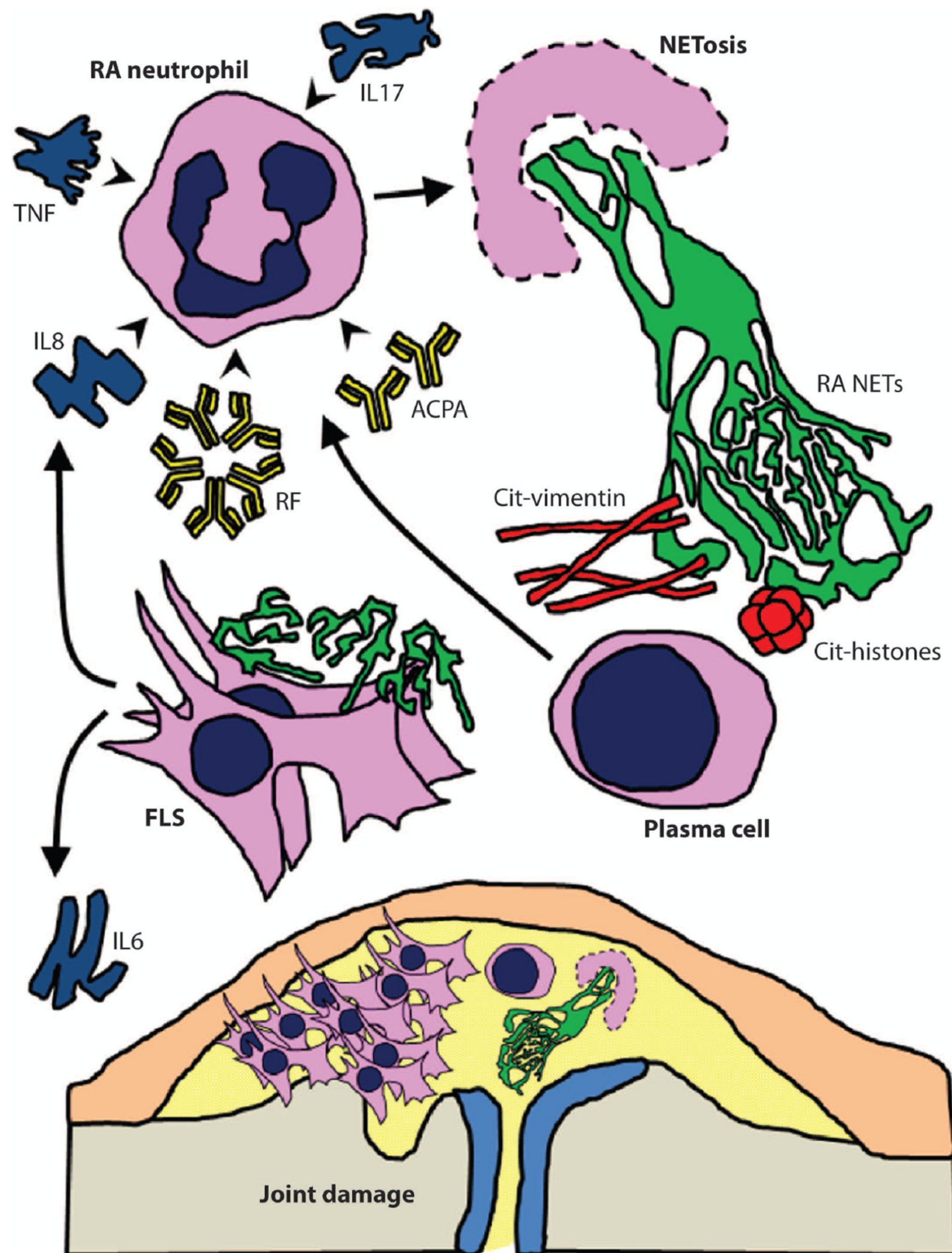

Figure 8. Proposed model of the role of NETosis in RA

RA NETs may provide a source of autoAgs and activate FLS and B cells. AutoAbs (RF, ACPA) and inflammatory cytokines (IL-17A, TNF-α, and IL-8) are all potential stimuli for enhanced NETosis in RA. In turn, RA NETs are a source of citrullinated (Cit-) autoAgs, including vimentin, further triggering production of ACPA. RA NETs also promote activation and cytokine release by FLS, with implications for joint damage and further propagation of a vicious cycle of NET induction and autoAb formation.