NETs Are a Source of Citrullinated Autoantigens and Stimulate Inflammatory Responses in Rheumatoid Arthritis

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The early events leading to the development of rheumatoid arthritis (RA) remain unclear, but formation of autoantibodies to citrullinated protein antigens (ACPAs) is considered a key pathogenic event. Neutrophils isolated from patients with various autoimmune diseases display enhanced neutrophil extracellular trap (NET) formation, a phenomenon that exposes autoantigens in the context of 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 RA synovial fluid neutrophils compared to neutrophils from healthy controls and from patients with osteoarthritis (OA). 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. Indeed, during NETosis, neutrophils externalized the citrullinated autoantigens implicated in RA pathogenesis, and anti–citrullinated vimentin antibodies potently induced NET formation. Moreover, the inflammatory cytokines interleukin-17A (IL-17A) and tumor necrosis factor–α (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, 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 tumor necrosis factor–α (TNF-α) and interleukin-17 (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), then evolving into clinically apparent disease (2). Indeed, RA-related pathogenic autoantibodies [autoantibodies to citrullinated protein antigens (ACPAs) and rheumatoid factor (RF)] are detected years before clinical diagnosis (2).

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

Peptidylarginine deiminase 2 (PAD2) and PAD4 likely generate these citrullinated antigens 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 autoantigen 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 autoantigens (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 autoantibodies and inflammatory cytokines elevated in RA patients promote NETosis, thereby perpetuating a cycle of citrullinated autoantigen generation and induction of autoimmune responses.

RESULTS

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

Peripheral blood (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) (Fig. 1, A and C). Significantly increased NETosis was observed after lipopolysaccharide (LPS) stimulation, when compared to baseline levels, in RA and control neutrophils. Upon LPS stimulation, PB and RA SF neutrophils displayed significantly enhanced capacity to form NETs when compared to control and OA neutrophils (Fig. 1, B to D). Furthermore, netting neutrophils were detected as infiltrating cells in RA synovial tissue, rheumatoid nodules, and skin from RA patients affected by neutrophilic dermatoses (Fig. 1, E and F, and figs. 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 hour of culture and continued to increase by 2 to 3 hours in culture (fig. 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 (fig. 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 anti-rheumatic 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 the presence and levels of ACPA antibodies and with systemic markers of inflammation.

RA autoantibodies and inflammatory cytokines induce NETosis

Some autoantibodies present in the serum of patients with various autoimmune diseases, including anti-neutrophil cytoplasmic (ANCA) and anti-ribonucleoprotein (RNP) antibodies, stimulate NETosis upon neutrophil priming with inflammatory cytokines (12, 19). To test whether

![Fig. 1. Enhanced NETosis in RA neutrophils. (A and B) PB RA neutrophils undergo increased NETosis in the absence (A) or presence (B) of added stimulation (LPS for 1 hour) (control, n = 7; RA, n = 13 to 14). (C) RA SF neutrophils undergo increased NETosis in the absence (unstimulated) or presence of 1-hour LPS stimulation (n = 5 per group). *P < 0.05. Results represent means ± SEM. (D) Representative microphotographs display enhanced LPS-induced NETosis in RA (bottom) versus control PB neutrophils (top). NETs were visualized as structures costaining for neutrophil elastase (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue). Magnification, ×40. (E and F) Multiloblate inflammatory cells consistent with netting neutrophils are present in RA synovial tissue. (E) Hematoxylin and eosin staining (×400) of RA synovial tissue. (F) Same area stained with anti-MPO (red) and DAPI (blue). Squares represent areas where netting neutrophils were observed. Images are representative of three RA patients. Statistical analysis was performed with two-tailed unpaired t tests, except for SF experiments where paired t test was used. Scale bars, 200 μm.](www.ScienceTranslationalMedicine.org 27 March 2013 Vol 5 Issue 178 178ra40)
RA-specific autoantibodies 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 (Fig. 2A to C). Similarly, when compared with immunoglobulin G (IgG) purified from healthy control sera or OA 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 (Fig. 2, D and E). Purified IgM RFs also potently induced NETs in control and RA neutrophils compared to control IgM (Fig. 2F). IgG isolated from ACPA- and/or RF-positive RA patients could bind to control and RA NETs, whereas IgG isolated from controls did not (Fig. 2G). These observations suggest that RA autoantibodies can mediate NETosis and that autoantibodies 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, although 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 (Fig. 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 reported (21), control and RA neutrophils exposed to GM-CSF without other priming stimuli did not display increased NET formation (Fig. 3B).

Whereas the molecular mechanisms implicated in NETosis remain incompletely characterized, the NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) 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 DPI or scavenging of ROS with NAC decreased NETosis in RA and control neutrophils stimulated with IL-17 or with IL-17 + TNF. Similarly, the PAD inhibitor CI-amidine (23) significantly inhibited NETosis (Fig. 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 antibodies to TNF-α or to IL-17 receptor (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 antibody. A nonsignificant similar trend was observed in control neutrophils exposed to RA serum plus neutralizing anti–TNF-α (Fig. 3B).
When neutralizing anti-TNF and IL-17R antibodies 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 than inhibiting TNF alone (Fig. 3B). Overall, these observations indicate that various factors present in RA serum, including autoantibodies 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 to 1 hour) nonlytic process independent of oxidant production (24). Because significant NET formation occurred by 1 hour in culture, particularly in the RA samples positive for ACPA (Fig. 1 and fig. S4), we tested whether this phenomenon was accompanied by lysis detection. Significant lactate dehydrogenase release was detected in the same samples where NETs were induced in control and RA neutrophils, in response to various RA-related stimuli (fig. S4). This suggests that enhanced NET formation in RA neutrophils and/or upon exposure to RA autoantibodies and inflammatory cytokines within 1 hour in culture is associated to cell lysis, as described in other conditions (22).

**RA autoantibodies 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 (i) IgG fractions isolated from sera of four RA patients with high ACPA and/or RF titers (100 mg/ml), (ii) purified IgM RF from four patients with monoclonal IgM cryoglobulinemia (100 mg/ml), or (iii) recombinant TNF-α (100 mg/ml) for 1 hour. NETs induced by these stimuli were isolated, and their protein cargo was determined by proteomic analysis. Depending on the experimental condition, the number of individual NET-specific proteins identified ranged from 28 to 40 and included various proteases and defensins that are typically present in neutrophil granules, as well as histones, cytoskeleton-related proteins, and cytosolic proteins (Fig. 4 and table S3). Stimulation with IgM RF and stimulation with RA IgG enriched in ACPA led to the highest number of proteins identified in the NETs (n = 36 for each), whereas TNF-α stimulation led to the identification of 28 proteins. Whereas most NET proteins were conserved upon different types of stimuli (fig. S5), some were only detected following specific stimulation. For example, matrix metalloproteinase-8 (MMP-8), histone 3, and vasodilator-stimulated phosphoprotein were only detected in IgM RF–induced NETs, whereas catalase, moesin, transaldolase, 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 using spectral counts as described (26), and statistically significant differences in NET protein levels in response to different types of stimulation were identified (Fig. 4). Indeed, defensin-2 levels were significantly higher in RF-induced NETs than in the TNF-α–induced NETs, whereas 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 used to induce these structures.
Citrullinated autoantigens are externalized during NETosis

The proteomic analysis described above identified several proteins that, when citrullinated, are important RA autoantigens (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 (Fig. 5A). Given the association between autoantibodies 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 (Fig. 5B). These results indicate that NETs externalize citrullinated autoantigens reported to play important roles in RA pathogenesis.

Antibodies 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 autoantibodies 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 antibodies potently induced NETs in control and RA neutrophils when compared to control IgG (Fig. 6A and fig. S6). Furthermore, anti-CV antibodies recognized vimentin
externalized in the NETs (Fig. 6B). These results indicate that autoantibodies directed to specific citrullinated proteins present in the NETs enhance the formation of these lattices.

**NETs activate RA and OA fibroblast-like synoviocytes**

To expand our understanding of the putative pathogenic roles of NETosis in the joints, we investigated whether NETs can stimulate fibroblast-like synoviocytes (FLS), which are the cells that invade cartilage in RA. On the basis of established kinetics of cytokine upregulation at the mRNA and protein level in these cells, we exposed FLS obtained from RA or OA patients to purified RA NETs for 24 to 48 hours and quantified the synthesis of IL-6, IL-8, CCL20, and ICAM-1 by enzyme-linked immunosorbent assay (ELISA) and/or real-time quantitative polymerase chain reaction (PCR). Treatment of RA and OA FLS with RA NETs significantly up-regulated IL-6 and IL-18 mRNA and protein levels, with responses being more dramatic in RA FLS (Fig. 7, A and B, and fig. S7). Neither IL-6 nor IL-8 was detected in the NETs alone condition. RA NETs also significantly upregulated mRNA levels of CCL20 and/or ICAM-1 in RA and OA FLS (Fig. 7B). In additional experiments, when the NETs’ architecture was disrupted with deoxyribonuclease (DNase), their stimulatory effect on FLS decreased (42 to 44% reduction in induction of IL-6 secretion by OA and RA FLS when stimulated with nuclease-treated versus untreated NETs; 50 to 74% and 43 to 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 = not significant when comparing FLS with FLS exposed to DNase-treated NETs). These results indicate that NETs have a robust capacity as strong stimulants of FLS expression of proinflammatory genes, a phenomenon that may amplify deleterious inflammatory responses in the RA synovium.

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**Fig. 5.** (A) Vimentin and α-enolase decorate control and RA NETs. Red represents vimentin or α-enolase, and blue is Hoechst. Total magnification, ×63. (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 (kD) are indicated. Cit Vim, citrullinated vimentin; Hc, heavy chain; Lc, light chain; Vim, vimentin; IP, immunoprecipitate; IB, immunoblot. Results are representative of three independent experiments. Scale bars, 10 μm.
DISCUSSION

Experimental evidence suggests that NETosis plays an important role in modification of autoantigens, their exposure to the immune system, and the induction of tissue damage in conditions like SLE, SVV, and psoriasis (12–14). Because activated neutrophils and anti-granulocyte antibodies 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 proinflammatory milieu, characterized by specific autoantibodies 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 autoantigens that, in predisposed individuals, may perpetuate a vicious cycle leading to generation of specific autoantibodies and inflammatory responses (Fig. 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 autoantigen exposure, and promote autoantibody generation in predisposed hosts. Recent observations indicate that CV, one of the autoantigens we identified in the NETs, induces proinflammatory cytokine secretion and PAD4 and

Fig. 6. (A) Anti-CV antibodies isolated from RA sera significantly enhance control (Ctrl) neutrophil NETosis. Results represent the means ± SEM of three independent experiments, each performed in duplicate. Units are expressed as RFUs of DNA fluorescence using SYTOX Green assay. *P < 0.05, ***P < 0.001, two-tailed unpaired t test. (B) Anti-CV antibodies bind to vimentin externalized in the NETs. Original magnification, ×63. Results are representative of two independent experiments. Scale bars, 10 μm.
RANKL expression in RA FLS (31). This supports that CV and other autoantigens 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 autoantibodies and cytokines was observed as early as 1 hour and was accompanied by evidence of cell lysis. The observation that RA autoantibodies (including anti-CV and RF) potently induce NETosis is similar to what has been reported for other autoantibodies (ANCA for SVV and anti-RNP for SLE) (12, 19). We also found that RA sera, but not control, can bind to NETs. Circulating autoantibodies present in serum of patients with Felty’s syndrome, a severe variant of RA, can bind to deiminated histones and NETs (32). Although 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 autoantibodies can recognize other citrullinated epitopes present in netting neutrophils. In support of this hypothesis, anti-CV antibodies bound to vimentin present in the NETs.

Periodontitis has emerged as a risk factor for RA (33). Infection with Porphyromonas 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 ortholog, and immunization with Pg enolase induces autoimmunity to mammalian α-enolase and arthritis in DR4-IE–transgenic mice (36). 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 autoantigens and autoantibodies 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 citrullinate the immunostimulatory peptide LL37. This renders the protein more chemotactic and alters its overall activity (40). Because 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). Although IL-17 is known to potently recruit neutrophils, a role for this cytokine in the induction of NETs had not been previously described.

**Fig. 7.** (A) RA and OA FLS up-regulate inflammatory cytokine synthesis upon exposure to RA NETs for 48 hours. Results represent the means ± SEM of two independent experiments, each performed in triplicate-quadruplicate. *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed unpaired t tests. (B) RA and OA FLS up-regulate 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 hours, when compared to untreated FLS at 24 hours of culture, adjusted for housekeeping gene (GAPDH). Error bars represent maximum RQ value for each sample and represent means ± SEM of two to three independent experiments. *P < 0.05, comparing change in mean ΔCt compared with untreated samples at the same time point, using two-tailed unpaired t tests. For untreated OA FLS, there was no amplification of CCL20, suggesting that the basal expression was below the level of detection.
Our study also supports the concept that not all NETs are created equal, because the 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 autoantibodies, 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. Although we did not observe any correlation between the use of these medications and the ability of RA neutrophils to form NETs, studies in therapy-naive 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 proinflammatory 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), whereas 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 collagen-induced arthritis (23) further supports this hypothesis, because 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 autoantigens. Furthermore, this phenomenon correlates with the presence and levels of ACPA antibodies and with systemic inflammation. ACPAs and RF may perpetuate a vicious circle of NET production that maintains the delivery of modified autoantigens 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.

**MATERIALS AND METHODS**

**Human subjects**

This study was approved by the University of Michigan Medical Institutional Review Board. 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 on 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 the demographic and clinical characteristics of the patients included in the study.

**PB and SF neutrophil isolation**

Detailed methods are included in the Supplementary Materials and Methods.

**Quantification of NETs by fluorescence 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 the Supplementary Materials and Methods. NETs were also quantified in RA and OA frozen synovial tissue, rheumatoid nodules, and skin biopsies from patients with dermatologic manifestations of RA, using techniques described by us (13, 14), and detailed in the Supplementary Materials and Methods. The percentage of NETs was calculated as the average of 5 to 10 fields (×400) normalized to the total number of neutrophils, and results were 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 with a protein G agarose kit following the manufacturer’s instructions (Pierce). Serum or SF was diluted in IgG binding buffer and run through a protein G agarose column 5 to 10 times. Igs were eluted with 0.1 M glycine and neutralized with 1 M 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 with Coomassie staining.

**Purification of antibodies to CV**

Wild-type recombinant human PAD4 was purified as previously described (46), and detailed methods are included in the Supplementary Materials.

**Purification of monoclonal IgM RF**

The purification of IgM RF obtained from plasma or purified proteins from five 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 Ultragel 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 the Supplementary Materials and Methods.

**Proteomic analysis of NETs’ content and liquid chromatography/electrospray ionization tandem mass spectrometry analysis**

Details are included in the Supplementary Materials and Methods.

**Assessment of citrullinated proteins in NETs**

Control and RA NETs were incubated overnight at 4°C with protein G–Sepharose beads and mouse anti-human vimentin monoclonal antibody. Beads were washed, and bound proteins were eluted by boiling in Laemmli buffer. Samples were separated by SDS–polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membranes. Citrullination was detected with anti-citrulline (modified) detection kit (Millipore). Briefly, membrane was blocked with 10% casein for 15 min at room temperature and incubated with 2,3-butanedione monoxime and antipyrine in acids overnight at 37°C. Detection of modified citrulline was performed according to the manufacturer’s protocol with anti-modified antibody and goat anti-rabbit IgG–horseradish peroxidase.

**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% fetal calf serum/2 mM glutamine/penicillin (50 U/ml)/streptomycin (50 μg/ml) (Cambrex). FLS were used after passage 4 and cultured for 48 hours in 6- or 12-well plates, followed by stimulation for 24 to 48 hours with 150 μg of NETs (1 μg/1000 FLS). In some conditions, NETs were treated with DNase I (100 U/ml) for 30 min at 37°C before 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 the Supplementary Materials.

**Statistical analysis**

The difference between means was analyzed with paired or unpaired Student’s 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 of <0.05 was considered significant. Analysis was performed with Prism 5 software (version 5.0a; GraphPad) and with SPSS software.
SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/5/178/178ra40/DC1

Materials and Methods

Fig. S1. Netting neutrophils are identified in rheumatoid nodules.

Fig. S2. Netting neutrophils are present in skin from RA patients.

Fig. S3. NET formation is accelerated in RA neutrophils and correlates with the presence and titer of ACPA in RA patients.

Fig. S4. ACPA are present in synovial fluid from RA patients.

Fig. S5. ACPA are produced by RA synovial fibroblasts.

Materials and Methods

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Abstracts

One-sentence summary: Neutrophil NETs may contribute to pathogenesis of rheumatoid arthritis.

Editor’s Summary:
Autoantigens Slip Through the NET

Autoimmune diseases are caused when the body’s immune system attacks the very tissues it’s supposed to protect. Yet, what exactly induces this loss of tolerance to self remains murky. For some autoimmune diseases, autoantigens—cellular targets of the immune response—have been identified, although it remains unclear how these normally intracellular proteins are exposed to the immune response. One hypothesis as to how these proteins may be externalized is through the excretion of neutrophil extracellular traps (NETosis). NETosis is thought to be involved in neutrophil response to bacteria, but the secretion of self-antigens in the context of inflammatory stimuli may boost autoimmune response. Now, Khandpur et al. look at the role of NETosis in rheumatoid arthritis.

Autoantibodies to citrullinated antigens (ACPAs) are thought to be pathogenic in rheumatoid arthritis. The authors observed increased NETosis in patients with rheumatoid arthritis compared with both healthy controls and patients with non-autoimmune osteoarthritis. Indeed, NETosis correlated with levels of ACPA, and ACPA actually altered the makeup of the proteins secreted by neutrophils. NETs from rheumatoid arthritis patients contained citrullinated proteins, and these NETs enhanced the inflammatory response in fibroblasts from inflamed joints. Thus, altered NETosis in rheumatoid arthritis patients may contribute to the pathogenesis of disease.