

Citrullination enhances the activity, stability and autoantigenicity of serine proteases in Rheumatoid Arthritis

Liam O'Neil

liam.oneil@umanitoba.ca

University of Manitoba <https://orcid.org/0000-0001-5580-6472>

Mario Navarrete

University of Manitoba <https://orcid.org/0000-0003-2464-9157>

Ramiza Zaman

University of Manitoba

Jun Kim

University of Manitoba

Clarissa Klenke

University of Manitoba

Neil Mertin

University of Manitoba

Obinna Okeke

University of Manitoba

Jeba Maisha

University of Manitoba

Alina Semenenko

University of Manitoba

Khushali Trivedi

University of Manitoba

Xiaobo Meng

University of Manitoba

Ying Lao

University of Manitoba

Deanna Santer

University of Manitoba <https://orcid.org/0000-0001-6169-7735>

Valerie Minarchick

University of Colorado Anschutz

Kevin Deane

University of Colorado Anschutz Medical Campus <https://orcid.org/0000-0003-2211-4861>

Michael Holers

University of Colorado School of Medicine

Oleg Krokhin

University of Manitoba <https://orcid.org/0000-0002-9989-6593>

Rene Zahedi

University of Manitoba <https://orcid.org/0000-0002-4960-5460>

M Kristen Demoruelle

University of Colorado Denver School of Medicine

Hani El Gabalawy

University of Manitoba

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Abstract

Protein citrullination, catalysed by peptidyl arginine deiminase (PAD) enzymes, is the primary target of autoantibodies in rheumatoid arthritis (RA), yet the functional consequences of citrullination have remained unexplored. Using the serine protease trypsin as a model system, we show that citrullination increases maximum velocity and catalytic rate, stability, while preserving substrate affinity. These findings extend to neutrophil serine proteases (NSP) proteinase-3, neutrophil elastase and cathepsin G, where PAD-mediated citrullination enhances activity and stability across both purified and neutrophil-derived preparations. We also demonstrate that citrullinated neutrophil serine proteases are recognised as autoantigens in RA, forming immune complexes with serum from RA patients. Further elevated NSP activity at mucosal sites is associated with autoantibody production in individuals at-risk to develop future RA. In the joint, citrullinated neutrophil elastase drives enhanced aggrecan degradation, while citrullinated proteinase-3 activates fibroblast-like synoviocytes via protease activated receptor-2 signalling. PAD inhibition significantly attenuates citrullination-mediated NSP activation in recombinant enzymes and human RA synovial fluid, suggesting a potential role for pharmacological targeting of PAD to treat RA. Overall, we demonstrate that PAD-mediated citrullination of serine proteases leads to a gain-of-function phenotype, enhancing catalytic activity and conferring resistance to degradation.

Introduction

Protein citrullination is a post-translational modification of arginine undertaken by a family of enzymes called peptidyl arginine deiminases (PADs)¹. Citrullination is irreversible and leads to loss of a positive charge which can affect hydrogen bond formation. In rheumatoid arthritis (RA), a chronic systemic autoimmune arthritis characterized by synovial inflammation and joint destruction, citrulline containing proteins are the primary target of autoantibodies, called anti-citrullinated protein antibodies (ACPA)². ACPA develop years before clinical disease onset during a pre-clinical phase characterised by progressive autoantibody accrual and epitope expansion, and their presence in at-risk individuals is the strongest known predictor of future clinical RA development³. While studies have identified numerous citrullinated protein targets of ACPA, the functional consequences of citrullination itself are incompletely understood.

Neutrophils contain two isoforms of PAD: PAD2 and PAD4. PAD4 is expressed intracellularly and is involved in the formation of neutrophil extracellular traps (NETs)⁴. PAD2 is found at the cell membrane and intracellularly and is released into the extracellular space upon degranulation⁵. In NET formation, PAD4 is activated and translocated to the nucleus, where it is involved in the citrullination of histone subunits to aid in the unwinding of DNA. It has been previously shown that citrullination of neutrophil serine proteases (NSPs) also occurs during NET formation⁶. In RA, NETs have been implicated as a source of autoantigens and activate fibroblast-like synoviocytes (FLS) in the joint⁶. NSPs, specifically proteinase-3 (PR3), neutrophil elastase (NE) and cathepsin G (CTG), are part of a larger family of enzymes called serine hydrolases⁷. These enzymes share a conserved catalytic triad of serine, histidine

and aspartic acid, in which the catalytic serine acts as the primary nucleophile for peptide bond cleavage. During neutrophil activation, citrullination of NSPs may result in structural alterations due to the loss of positive charge at modified arginine residues⁸, the functional consequences of which have not been explored.

Here, we use the serine protease trypsin as a model to examine whether citrullination alters enzymatic activity. We demonstrate that citrullination of trypsin leads to a gain-of-function phenotype by enhancing catalytic activity despite a preserved affinity constant. Further, citrullination of trypsin leads to increased stability, by limiting autoproteolysis in vitro. We extend this finding to NSPs, and demonstrate that the activity and stability of PR3, NE and CTG are enhanced by citrullination. Importantly we also show that citrullinated NSPs are recognized as target autoantigens across the pre-clinical and clinical spectrum of RA, including at the mucosal site of the lung through sputum studies; and that citrullinated NSPs in the synovial microenvironment can drive joint destruction and activate synovial fibroblasts in RA.

Results

Citrullination enhances the proteolytic activity of trypsin

To study whether citrullination modulates serine protease activity, we used bovine trypsin as a model serine protease, chosen for its well-characterised active site architecture, biochemical tractability and extensive structural data⁹. We performed a Michaelis-Menten kinetic analysis of trypsin following citrullination by PAD2 or PAD4, the two major human isoforms of PAD expressed in neutrophils. Both PAD2 and PAD4 citrullination increased the maximum velocity (V_{max} ; No PAD $1.4 \pm 0.2 \times 10^{-2}$, PAD2 $3.0 \pm 0.1 \times 10^{-2}$, PAD4 $2.3 \pm 0.1 \times 10^{-2}$) and catalytic rate constant (K_{cat} ; No PAD: $5.11 \pm 0.18 \times 10^{-5}$, PAD2: $7.79 \pm 0.08 \times 10^{-5}$, PAD4: $8.80 \pm 0.08 \times 10^{-5}$) while the Michaelis constant (K_m) remained relatively unchanged across conditions (676–788 μ M, Fig. 1A-B). Incubation of trypsin with increasing concentrations of PAD2 (100–2000 mU) resulted in a concentration-dependent increase in proteolytic activity, whereas treatment with PAD4 at equivalent concentrations produced no significant dose-dependent effect after 100 mU (Fig. 1C). To confirm that enhanced activity was directly attributable to citrullination rather than to PAD protein itself, we used a rhodamine-conjugated phenylglyoxal (RhPG) citrulline-specific probe to quantify citrullination of trypsin across the PAD2 and PAD4 concentration series. Citrullination signal increased dose-dependently with PAD2 concentration but not PAD4, and the degree of citrullination correlated directly with proteolytic activity ($R^2 = 0.7$, $p < 0.0001$, Fig. 1D-E), establishing citrullination as the driver of activity enhancement and demonstrating that the two PAD isoforms differ in their capacity to citrullinate bovine trypsin. PAD2 citrullinated trypsin also demonstrated resistance to aprotinin, a competitive serine protease inhibitor, with an IC_{50} increase from 105 nM in untreated trypsin to 265 nM following citrullination (Fig. 1F). We also tested both trypsin and PAD2 citrullinated trypsin across varying pH levels (4.8 to 9), demonstrating enhanced catalytic efficiency above pH 5.8, without altering the K_m (Fig. 1G, Figure S1). Notably, citrullination enhanced the

catalytic efficiency of bovine trypsin by 8.5-fold at pH 9. Collectively, these data suggest that citrullination enhances the activity of bovine trypsin.

Citrullination enhances active site binding and stability of bovine trypsin

We next sought to understand the mechanistic basis for enhanced bovine trypsin activity induced by citrullination. Citrullination alters the electrostatic charge of proteins, which may lead to conformational changes that impacts enzymatic activity¹⁰. To test this, we performed activity-based protein profiling¹¹ (ABPP) using the fluorophosphonate (FP)-TAMRA probe, which binds to the active site of serine proteases. Bovine trypsin was citrullinated with PAD2 (33mU or 66mU) and incubated with FP-TAMRA (0, 10, 20, or 30 minutes) prior to SDS-PAGE. PAD2 citrullination produced a dose-dependent increase in FP-TAMRA labelling of the bovine trypsin, with higher PAD2 concentrations yielding progressively greater probe incorporation (Fig. 2A). This suggests that citrullination allowed for enhanced capacity for binding of substrates to its active site.

Trypsin undergoes autoproteolytic self-digestion under physiological conditions, progressively limiting its functional half-life. To directly test if autoproteolysis of bovine trypsin is altered by citrullination, bovine trypsin was incubated with or without PAD2/PAD4 at 37°C for up to 7 days in vitro. Untreated and PAD4-treated trypsin underwent progressive autoproteolytic degradation, with loss of the intact ~ 25 kDa band apparent by day 3 and near-complete degradation by day 7 as visualized using SDS-PAGE (Fig. 2B). This was also accompanied by a corresponding decline in proteolytic activity (Fig. 2C) in both untreated and PAD4 treated bovine trypsin. Conversely, PAD2-citrullinated bovine trypsin retained an intact protein band throughout the full 7-day incubation, with substantially preserved proteolytic activity ($p < 0.0001$ for all time points), with 20-fold greater activity at day 5. These data indicate that citrullination stabilises bovine trypsin against autoproteolytic degradation, extending its functional half-life, possibly due to modification of trypsin-prone arginine sites. Trypsin is used extensively in mass spectrometry to digest proteins into peptides for downstream analysis¹². To determine whether enhanced catalytic activity translates to increased degradation of a protein substrate, bovine serum albumin (BSA) was incubated with PAD2-citrullinated or untreated bovine trypsin and digestion products assessed by SDS-PAGE. PAD2-citrullinated trypsin produced accelerated and more extensive BSA degradation relative to untreated trypsin at each timepoint (Fig. 2D). Mass spectrometric analysis of digestion products confirmed this at the peptide level with the intensity of five representative BSA-derived peptides: QNCDQFEK, LKECCDKPLLEK, ETYGDMADCCEK, SHIAEVEK, LVNELTEFAK that were consistently elevated in 2 and 4h of digestion time in PAD2-citrullinated trypsin digests ($p = 0.002$, Figure 2E). We also tested the ability of PAD to enhance the activity of sequence-grade trypsin (SGT, porcine). Both PAD2 and PAD4 enhanced the activity of SGT compared to the untreated control (Fig. 2F, Figure S2). SGT is modified commercially via methylation to enhance its stability and only minor resistance to autoproteolysis was observed (Figure S2).

Mass spectrometric identification of citrullination sites

To identify the specific arginine residues modified by PAD-mediated citrullination, we performed mass spectrometric analysis of native and citrullinated bovine trypsin (PAD2 and PAD4). Citrullination was confidently identified at R72 and R122 (mature form R49 and R99, Fig. 2G and H, Figure S3). AlphaFold3 modeling of native and citrullinated trypsin (Figure S4) revealed minimal backbone conformations after superposition (Ca RMSD = 0.164 Å, Figure S4), indicating no substantial global rearrangement of the tertiary structure after citrullination. Secondary structural analysis (DSSP) revealed minor local changes confined to each citrulline site. For example, at cit-49, residues 44 to 46 reclassified from turns to bends/coil and at cit-99, residues 97 to 100 reclassified from bends to turns/beta-bridges (Figure S4). GNINA molecular docking¹³ with a tetrapeptide substrate revealed no difference between native and citrullinated trypsin, consistent with the unchanged K_m observed kinetically (Figure S4). Together, these data indicate that citrullination at R49 and R99 may not alter substrate binding geometry, possibly consistent with a shift in conformational equilibrium of the active site to a more accessible state.

Citrullination enhances the proteolytic activity of neutrophil serine proteases

Having established that citrullination of trypsin confers a gain-of-function phenotype characterized by enhanced catalytic activity and stability, we next sought to determine whether these effects extend to neutrophil serine proteases including proteinase-3 (PR3), neutrophil elastase (NE) and cathepsin G (CTG). Neutrophils contain both PAD2 and PAD4, and PAD activation occurs during degranulation and NET formation, creating conditions in which NSPs and PAD enzymes are co-localised in the extracellular environment, raising the question of whether citrullination of NSPs confers equivalent functional consequences to those observed in trypsin. Further, NSPs are disease relevant members of the serine protease family implicated in RA pathogenesis, a disease in which citrullination plays a pathogenic role. To determine whether PAD-mediated citrullination enhances NSP proteolytic activity, recombinant PR3, NE, and CTG were citrullinated with PAD2 or PAD4 and activity measured across a range of substrate concentrations. PAD2 citrullination produced a marked increase in V_{max} for all three NSPs relative to untreated controls, with the enhancement most pronounced for PR3 and NE (Fig. 3A, upper row). PAD4 citrullination also enhanced activity across all three proteases, though to a lesser degree than PAD2 for PR3 and NE. Notably, PAD4 citrullination of CTG produced an enhancement of proteolytic activity exceeding that of PAD2. Kinetic analysis confirmed increased V_{max} and K_{cat} with preservation of K_m across all three citrullinated NSPs (Table S1). Using neutrophil supernatants after degranulation (PMA stimulated) we also profiled protease activity with and without citrullination. PAD2 and PAD4 citrullination of neutrophil supernatant produced enhancement of PR3, NE, and CTG activity relative to untreated supernatant, with Michaelis-Menten curves demonstrating increased V_{max} across all three NSPs (Fig. 3A, lower row).

PAD2 citrullination protects against autoproteolysis and enhances active site binding

NSPs are also subject to autoproteolytic degradation under physiological conditions, which progressively limits their functional half-life in the extracellular environment¹⁴. To assess whether citrullination alters this degradation, recombinant PR3, NE, and CTG were incubated with PAD2 or PAD4 at 37°C for up to 72 hours. All three NSPs underwent progressive autoproteolytic degradation under Ca²⁺/DTT control conditions, with loss of intact protein bands and corresponding decline in activity evident by 24–72 hours (Fig. 3B–C). PAD2 citrullination markedly reduced autoproteolytic degradation across all three proteases, with intact protein bands retained at 72 hours and substantially preserved proteolytic activity relative to controls throughout the time course. To test whether protection extended to degradation by exogenous trypsin, we treated PR3 and PAD2 cit-PR3 with trypsin for 2 and 21 hours and found that PAD2 cit-PR3 was protected from trypsin degradation (Fig. 3D). To confirm that the functional effects observed reflect PAD-mediated citrullination of NSPs rather than non-specific effects of PAD, citrullination of PR3 was assessed directly using a citrulline-specific fluorescent probe. Dose-dependent incorporation of citrulline into PR3 was detected following treatment with increasing volumes of both PAD2 and PAD4, with the citrullinated PR3 band (cit-PR3) detected at ~ 29 kDa and absent in HEPES and Ca²⁺ control conditions (Fig. 3E). Further, we observed a strong correlation ($R^2 = 0.945$, $p < 0.0001$) between PAD2-citrullination and proteolytic activity of PR3 (Figure S5). To confirm that PAD enzymatic activity is required for the observed enhancement of NSP proteolytic activity, PR3 was citrullinated with PAD2 in the presence or absence of the PAD inhibitor Cl-amidine (720 μ M). PAD2-induced enhancement of PR3 proteolytic activity was significantly reduced by Cl-amidine pre-treatment ($p = 0.0286$), confirming that the activity enhancement is dependent on PAD (Fig. 3F). Finally, to determine whether citrullination-mediated enhancement of NSP activity in neutrophil-derived material is associated with increased active site accessibility, we performed ABPP using the FP-TAMRA on PAD2-citrullinated neutrophil supernatant and NETs (generated with 100 nM of PMA). Both PAD2 and PAD4 citrullination produced an increase in FP-TAMRA labelling intensity in neutrophil supernatant and NET preparations relative to control, indicating enhanced active site probe binding to NSPs generated from neutrophils (Fig. 3G). Finally, we sought to recapitulate the effects of citrullination using PAD enzymes from human sera. Using antibodies, we immunoprecipitated PAD2 or PAD4 using serum from RA patients. These serum-derived PADs were active and reproduced enhanced NSP activity (Figure S5). Overall, these data suggest that citrullination enhances NSP activity by enhancing substrate binding and stability.

Citrullinated NSPs are recognised as autoantigens across the pre-clinical disease spectrum

We next sought to determine whether citrullinated NSPs are detectable in serum from RA patients, and further, recognised as autoantigens. To determine whether circulating PR3 activity and citrullination are elevated in established RA, we measured PR3 proteolytic activity and circulating cit-PR3 levels in serum from RA patients and healthy controls. PR3 proteolytic activity was significantly elevated in RA serum

compared to healthy controls ($p = 0.0319$; Fig. 4A). Circulating cit-PR3 levels were similarly elevated in RA serum relative to controls ($p = 0.0060$; Fig. 4B), confirming that citrullination of PR3 occurs in vivo and that citrullinated PR3 is detectable in the peripheral circulation of established RA patients. To determine whether citrullinated NSPs serve as autoantigens, we measured IgG reactivity to PAD2- and PAD4-citrullinated NE, PR3, and CTG in a cohort of RA patients their First-Degree Relatives (FDR)³. Antibodies (IgG) to citrullinated NSPs showed a consistent and statistically significant increase across all six antigen-isoform combinations, from ACPA- FDR, ACPA + FDR to established RA (Figs. 4C–H, all $p < 0.0001$ for ACPA + RA vs ACPA- FDR). Antibody reactivity to non-citrullinated NSPs was not different between groups (Figure S5). This indicates that autoantibody responses to citrullinated NSPs are detectable in RA and ACPA+ individuals without RA.

Citrullinated NSPs released from activated neutrophils form immunoglobulin-containing complexes with RA sera

Next, we tested whether neutrophils release citrullinated proteases upon stimulation. After 15 min of treatment with calcium ionophore (A23187), we observed that healthy donor neutrophils release citrullinated proteins into the cell culture supernatant via degranulation (Fig. 4I). At ~ 25 kDa we observed a high citrullination signal, along with active serine hydrolase activity confirmed by FP-TAMRA labelling (Fig. 4I). To test if NSPs released from neutrophils interact with RA sera, we used TAMRA-labelled ionophore-stimulated neutrophil supernatant and incubated this with RA or HC serum and resolved by native PAGE. We observed a discrete high molecular weight band at (~ 720 kDa) which was detected by FP-TAMRA signal specifically in degranulation samples incubated with RA serum and not in the control (Fig. 4I, right panel), suggestive of protein complexes containing neutrophil NSPs and serum proteins. Mass spectrometric analysis of the excised ~ 720 kDa band confirmed immunoglobulins (IgG, IgA and IgM heavy and light chains) as the largest protein group (710 unique peptides and 17.6% of total spectral counts, Figure S5, Table S2). Alpha-2-macroglobulin (A2M), a pan-protease trap that forms covalent high molecular weight complexes with target serine proteases, was the second most abundant component (203 unique peptides, 16.0% of spectral counts). Cathepsin G and Proteinase-3 were also identified. To confirm that immunoglobulins are sufficient to drive complex formation, the experiment was repeated using purified IgG isolated from RA serum mixed with neutrophil degranulation supernatant and found that the ~ 720 kDa band was reproducible (Fig. 4J). Overall, these data suggest that RA IgG binds efficiently to neutrophil NSPs following neutrophil degranulation.

Emerging evidence implicates the lung as a site of early autoimmune events in RA, with mucosal citrullination and local autoantibody generation proposed as initiating mechanisms preceding joint involvement^{15,16}. To further investigate the relationship between NSP activity and autoantibody responses in RA, we sought to detect NSP activity in the mucosal compartment of individuals, some of whom were at-risk for RA based on being an FDR or serum ACPA positive, RA patients and healthy controls (see Methods). NSP activity (PR3, NE, CTG) was measured, and detectable in sputum samples using a fluorogenic substrate. Hierarchical clustering of sputum NSP activity revealed a distinct activity

profile in RA and sputum antibody (ACPA/RF) positive at-risk individuals relative to HC and sputum antibody negative at-risk (Figure S5). Sputum citrullination at ~ 25 kDa was strongly correlated (Fig. 4K, $R = 0.71$, $p = 0.01$) with PR3 activity, suggesting that citrullination may drive NSP activity in sputum. NSP activity was significantly elevated (NE activity, $p = 0.0376$ and CTG, $p = 0.0035$) in sputum antibody positive at-risk individuals (ACPA or RF) compared to sputum antibody negative (Fig. 4L). Similar trends were observed with PR3 activity ($p = 0.1010$). There was also a positive correlation between NE ($R = 0.51$, $p = 0.027$) and CTG ($R = 0.53$, $p = 0.021$) and the number of sputum autoantibodies in the at-risk cohort, which was not apparent in the RA cohort (Fig. 4M). Overall, these findings suggest that NSP activity is associated with RA-specific antibody responses in the sputum, suggesting that citrullinated NSPs in the lungs could serve as autoantigens in RA.

NSP activity is elevated in RA synovial fluid with citrullination enhancing the degradation of aggrecan

Within the RA synovial joint, infiltration of activated neutrophils leads to the release of serine proteases which may orchestrate important aspects of RA pathogenesis⁴. We measured NSP activity in synovial fluid from RA and OA samples, and found elevated activity of PR3, NE, and CTG in synovial fluid in RA (all $p < 0.0068$; Fig. 5A). Next, we performed phenylglyoxal citrulline-enrichment mass spectrometry on RA synovial fluid ($n = 3$). A total of 490 citrullinated proteins were identified (Table S3). Amongst serine proteases identified, NSPs CTG and NE were identified as citrullinated proteins, alongside several serine proteases involved in coagulation and complement cascade (Fig. 5B). Joint damage remains a clinically important outcome for RA patients, and it has been previously shown that NSPs may be involved in the degradation of joint proteoglycans, such as aggrecan¹⁷. After 5 minutes *in vitro*, we found that PAD2 citrullinated NE degraded aggrecan more rapidly compared to native NE (Fig. 5C, $p < 0.0001$). This was observed primarily with PAD2 citrullination and not with PAD4, consistent with the enhanced activity and stability of cit-NE specific to PAD2 (Fig. 5D). PR3 also successfully degraded aggrecan, while CTG did not (Figure S6). We also tested aggrecan degradation using neutrophil supernatant and NETs, with and without PAD citrullination. PAD2 citrullination of neutrophil supernatant and NETs produced consistently enhanced aggrecan degradation compared with untreated and PAD4-treated preparations (Fig. 5D) and a corresponding increase in NSP activity in NETs after PAD citrullination (Figure S6). To identify the relative enzyme contribution in NET-mediated aggrecan degradation, NETs were pre-incubated with a pan-serine hydrolase (Figure S6, Serine Hydrolase (SH) inhibitor, Desthiobiotin FP, 2.5 μM) or a PR3-specific inhibitory probe (10 μM). We found a reduction in aggrecan degradation using the pan-serine hydrolase FP probe, with minimal effect of the PR3 inhibitor (Fig. 5E). Taken together, these data suggest that serine hydrolase activity, specifically NE, drives the degradation of aggrecan by NETs which is enhanced by PAD2 citrullination.

Within the RA synovium, there is an abundance of extracellular PADs and serine proteases that may interact, leading to citrullination of NSPs. To explore this, we treated RA SF with Ca^{2+} /DTT (endogenous PAD activation) or with the addition of exogenous PAD2 and found enhanced citrullination in each, and a

reduction in citrullination with a PAD2-specific inhibitor (CAY10723, Fig. 5F). Endogenous PAD activation significantly enhanced total NE proteolytic activity in RA synovial fluid relative to control (HEPES, $p = 0.033$; Fig. 5G). To determine whether PAD2 citrullination of SF NSPs could be blocked pharmacologically, RA synovial fluid was treated with exogenous PAD2 with or without CAY10723. PAD2-enhanced SF NE activity was significantly reduced by CAY10723 treatment ($p = 0.0025$; Fig. 5H), as was PAD2-enhanced PR3 activity ($p = 0.0024$; Fig. 5I). These findings provide evidence that PAD-mediated citrullination drives enhanced NSP activity in the RA joint, and that this pathogenic mechanism may be pharmacologically targeted through the inhibition of PAD2.

Citrullinated NSPs activate fibroblast-like synoviocytes via PAR2 signalling

Having established that citrullinated NSPs are elevated and active in RA synovial fluid, we next sought to determine whether citrullinated NSPs can directly activate synovial fibroblasts (FLS), the stromal effector cell in RA. To determine whether cit-PR3 activates FLS, we measured secretion of various inflammatory cytokines from FLS treated for 12 hours. LPS served as a positive control. Treatment with cit-PR3 led to the release of IL-6 ($p = 0.0004$), IL-1 β ($p = 0.0103$), MMP-9 ($p = 0.0025$), and TNF- α ($p = 0.0005$) relative to native PR3 (Figs. 6A–D), suggesting that citrullination of PR3 can activate FLS. Time course analysis of IL-6 secretion demonstrated that cit-PR3 induced significantly earlier onset of IL-6 production relative to native PR3, with significant differences apparent beginning at 4h (Fig. 6E). Protease-activated receptor 2 (PAR2) is a G protein-coupled receptor activated by serine proteases through cleavage of its extracellular N-terminal domain¹⁸. PAR2, as opposed to other PAR receptors, is activated by serine proteases and has been implicated in synovial fibroblast activation in inflammatory arthritis¹⁹. We hypothesised that cit-PR3 activates FLS through enhanced PAR2 cleavage, related to its enhanced proteolytic activity and stability. FLS stimulated with cit-PR3 in the presence of the PAR2 antagonist AZ3451 (0.5 μ M) displayed significantly reduced IL-6 ($p = 0.0072$), IL-1 β ($p < 0.0001$), and MMP-9 ($p = 0.0035$) secretion (Fig. 6F). We observed similar cytokine release profiles with FLS treated with citrullinated and native NE (Figure S7).

To characterise the breadth of the FLS response to cit-PR3, we measured the FLS secretome using Olink Target 48 in cells stimulated with native PR3 or cit-PR3. Principal component analysis demonstrated clear separation of cit-PR3 and native PR3 secretome profiles along PC1 (14.9% variance explained), confirming that citrullination of PR3 induces a distinct activation profile in FLS (Fig. 6G) marked by the release of inflammatory mediators (Fig. 6H). Hierarchical clustering of differentially secreted proteins revealed broad upregulation of chemokines, cytokines, and matrix remodelling proteins by cit-PR3 (Fig. 6I, Table S4). Gene ontology enrichment analysis of the cit-PR3 versus native PR3 secretome identified significant enrichment of *humoral immune response* and *granulocyte chemotaxis* (Fig. 6J), which included networks of various chemokines (CCL2, CXCL8, CXCL9) and inflammatory mediators (IL-6, CSF, Fig. 6K). Finally, to determine the intracellular signalling pathway activated downstream of PAR2 by cit-PR3, we assessed ERK1/2 phosphorylation in FLS stimulated with native PR3 or cit-PR3 by immunofluorescence (Fig. 6L), Western blotting of cell lysate (Figure S7) and intracellular flow cytometry

(Figure S7) and found that cit-PR3 increases phosphorylation of ERK. Together these data demonstrate that cit-PR3 activates FLS through a PAR2-ERK signalling axis, with the enhanced proteolytic activity of citrullinated PR3 driving an inflammatory response consistent with the pathogenesis of RA.

Discussion

Protein citrullination has been studied as a mechanism of autoantigen generation in RA, yet its functional consequences have remained relatively unexplored. Here, we demonstrate that PAD-mediated citrullination of serine proteases fundamentally alters their enzymatic behaviour by enhancing catalytic activity and conferring resistance to autoproteolytic degradation. We observed this phenomenon across several serine proteases, including trypsin and neutrophil serine proteases PR3, NE and CTG, and demonstrated that the primary functional change relates to enhanced catalytic activity which is unrelated to substrate affinity. We show that citrullinated NSPs are recognised as autoantigens across the pre-clinical spectrum of RA, forming immune complexes with RA IgG, and that NSP activity is associated with autoimmunity at mucosal sites. In the joint, citrullinated NSPs drive enhanced degradation of the cartilage proteoglycan aggrecan and activate fibroblast-like synoviocytes through PAR2. Collectively, these findings identify a previously unrecognised functional consequence of protein citrullination in RA, one that simultaneously licences proteases to degrade joint cartilage, drive synovial inflammation and generate autoantibodies, while extending their half-life by conferring resistance to autoproteolysis (Figure 7).

A consistent observation throughout this study is that PAD2 and PAD4 differ in their capacity to citrullinate and modify enzymatic function. The mechanistic basis of this isoform asymmetry remains to be fully determined, but may reflect differences in the substrate preferences of PAD2 and PAD4²⁰, or alternatively, susceptibility of PAD itself to serine protease-mediated degradation, rendering it inactive. Trypsin cleaves at arginine and lysine, and it remains likely that citrullination at susceptible arginine sites protects from direct cleavage by trypsin *in-trans*. However, NSPs vary in their substrate specificity, and do not generally rely on arginine as a site for cleavage⁷. Hence, enhanced NSP stability is not likely to be related to specific modifications, but possibly relates to conformational changes driven by the introduction of citrulline. Regardless, the observation that serine proteases are both more active and stable likely has a compounding effect, and we observed that the relative difference in activity between native and cit-proteases was more pronounced, the longer the enzymes were incubated *in vitro*. For example, PAD2 citrullinated trypsin had 20-fold greater activity compared to untreated trypsin at day 5. Engineering new or redesigning existing enzymes such as proteases to enhance their function remains a fundamental challenge²¹. We show proof of concept data to suggest that citrullinated trypsin may be utilized for proteomics workflows to enhance sample processing and peptide identification. Future work should verify the utility of citrullination of serine proteases for various applications.

A defining feature of serine proteases is their conformational dynamism, where the active site exists in equilibrium between open and closed states⁹. The relative abundance of each conformation directly determines substrate accessibility and catalytic efficiency. In trypsin, this equilibrium is regulated by

multiple factors including electrostatic interactions, pH, and the local protein folding environment²². Our data suggest that arginine residues contribute to this electrostatic landscape through their positively charged guanidinium groups, and citrullination may shift the conformational equilibrium toward the more accessible state. Consistent with this model, we demonstrate that citrullination of serine proteases generally produces a kinetic profile with increased Vmax and preserved Km, and increased FP-TAMRA labelling of the active site.

The demonstration that citrullinated NSPs are recognised as autoantigens across the pre-clinical spectrum of RA extends the functional significance of our mechanistic findings into the clinical domain. We observed a stepwise increase in IgG reactivity to all six citrullinated NSP antigen-isoform combinations, from ACPA-negative FDR, ACPA-positive FDR to established RA. The formation of stable immune complexes between RA IgG and citrullinated NSPs released from activated neutrophils further demonstrates that the autoantibody response to citrullinated NSPs is functionally significant, capable of capturing active NSPs. In RA, immune complexes play a pivotal role in the activation of immune cells, fixing complement and facilitating bone erosion^{23,24}. These observations also extend to mucosal surfaces, where NSP activity was associated with local autoimmunity in an at-risk population. Emerging evidence suggests the lung as a site of early autoimmune events in RA, with local citrullination, autoantibody generation, and immune activation proposed as initiating mechanisms for RA development^{15,16,25}. Previously it has been shown that NETs, a potential source of citrullinated proteases, are also associated with autoimmune responses¹⁵. Whether dysregulation of neutrophil inflammation is the primary driver or helps promote the generation of citrullinated epitopes that serve as the initial breach of immune tolerance in preclinical RA remains an unanswered question.

Within the RA synovial joint, the consequences of PAD-mediated NSP activation are potentially far-reaching. We show that citrullinated NE and NETs degrade aggrecan significantly more efficiently than the native form. Aggrecan is the principal load-bearing proteoglycan of articular cartilage²⁶, and its degradation impacts joint integrity, suggesting that PAD-mediated citrullination of NE within NETs represents a previously unrecognised mechanism of cartilage destruction in RA. We also show that cit-PR3 activates FLS through PAR2 signalling²⁷, leading to the release of proinflammatory cytokines known to play a key role in RA. We demonstrate that PAD inhibition significantly attenuates enhanced NSP activity in human RA synovial fluid, providing evidence that this pathogenic mechanism may be targeted pharmacologically, adding to the growing rationale for PAD inhibitors as therapeutic candidates in RA^{1,28}. Our data suggest that PAD isoform selectivity may need to be considered when designing drugs or antibodies as therapeutics in RA, given the differential effects of PAD2 and PAD4 in our study.

Several limitations of the current study warrant consideration. Although we show that trypsin is citrullinated at R49 and R99, substantial global conformational changes were not observed with in-silico modeling. While the in-silico analysis supported no change in the substrate affinity upon citrullination, it remains unclear precisely how citrullination enhances the catalytic efficiency of serine proteases, specifically whether this reflects dynamic structural alterations not captured by static models. In-silico

molecular dynamics simulations alongside crystallographic or cryo-EM structural analysis of citrullinated NSPs may provide definitive confirmation of the proposed active site changes and represent an important future direction. Longitudinal studies with larger at-risk cohorts will be needed to determine whether mucosal NSP activity predicts disease progression and autoantibody development in preclinical RA. Future studies will explore the role of mucosal NSPs and RA development using longitudinal samples. Finally, although the role of PAD2 in murine arthritis has been explored, preclinical arthritis models that explore the role of PAD isoforms in RA are needed.

In conclusion, we show that citrullination of serine proteases leads to a gain-of-function phenotype with extended half-life and enhanced activity. We implicate citrullination of NSPs in the pathogenesis of RA, suggesting a potential role for autoantibody development, inflammation and joint damage.

Methods

Ethics

All human sample collection was conducted in accordance with the Declaration of Helsinki and approved by the University of Manitoba Health Research Ethics Board (HS2005:093 and HS25252). Informed consent was obtained for all participants involved in this study.

Patient samples and ethics

Details for the at-risk cohort from Manitoba can be found in past publications³. For antibody studies FDR ACPA-, FDR ACPA+ and RA ACPA+ stored serum were used. See Table S5 for demographics; ACPA- FDR (n=34), ACPA+ FDR (n=17) and RA (n=16) groups had similar age (43, 47 and 46 years respectively) and sex distribution (56%, 82% and 75% female). A separate at-risk cohort from the United States was studied to determine the relationship between sputum autoantibody reactivity and NSP activity; details can be found in past publications^{29,30}. In total, healthy controls (n=11), at-risk (FDR 5/19, ACPA+ 14/19) and RA (8/11 ACPA+) sputum samples were used.

Reagents and recombinant proteins

Human PR3, NE, and CTG were obtained from Athens Research and Technology (catalogue numbers 16-14-161820, 16-14-051200, 16-14-030107 respectively). Trypsin acetylated from bovine pancreas trypsin was obtained from Sigma (catalogue number T6763). Sequence-grade modified Trypsin (porcine; Promega, catalogue number V511) was used for mass spectrometric citrullination site identification experiments. Recombinant human PAD2 and PAD4 were obtained from Sigma-Aldrich (catalogue numbers SAE0061 and SAE0086 respectively). The PAD inhibitor CAY10723 was obtained from Cayman Chemical (catalogue number 22653). Cl-amidine was obtained from Cayman Chemical (catalogue number 10599) and used at a final concentration of 720 μ M. FP-TAMRA and FP-desthiobiotin activity-based probes were obtained from Thermo Fisher Scientific (catalogue numbers 88318 and 88317 respectively). Rhodamine-phenylglyoxal (Rh-PG) citrulline-specific fluorescent probe was obtained from

Cayman Chemical (catalogue number 16172). Aprotinin was obtained from sigma (catalogue number A1153). Chromogenic substrates for trypsin (Sigma B4875), PR3 (Cayman 9002021), NE (Cayman16467) and CTG (Biosynth SFR-3231-V). Aggrecan was obtained from Biolegend (catalogue number 551404). Bovine serum albumin (BSA) was obtained from Thermo scientific (catalogue number 23209).

Fluorogenic/chromogenic proteolytic activity assays of cit-proteases

Proteins were citrullinated by incubation with recombinant PAD2 or PAD4 in reaction buffer containing 50 mM HEPES pH 7.4, 0.2 mM CaCl_2 and 1 mM DTT at 37°C for 60 minutes unless otherwise stated. PAD enzyme concentrations and incubation times are indicated in the relevant figure legends. Briefly, 2-10 nM of citrullinated or untreated PR3, NE and CTG was incubated with 5 μM fluorogenic/chromogenic substrate in 50 μL of reaction mix containing 750 mM NaCl, 50 mM HEPES pH7.4 at 37°C in a 96-well half-area dark plate (Greiner 675076). Fluorescence was measured at Abs/Em: 320/420 nm using a multi-mode micro plate reader (Agilent Biotek Synergy H1). Trypsin at 25-100 nM was assayed in 96 well plates (Avantor 734-2802) in presence of 50 mM HEPES pH7.4 and Na-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNa, Sigma B4875) that was followed at 405 nm in same plate reader. For determination of bovine trypsin's optimum pH we used 100 mM of the following buffers: MES pH4.8, Sodium acetate pH 5.2 Sodium phosphate pH5.8 and pH6.8, tris pH 8 and sodium borate pH9.0 to dissolve the treated and non-treated enzyme and also to dissolve substrate (BAPNa in serial dilutions from 6 mM to 49 μM). Velocity was calculated as the linear rate of signal increase and expressed as V(RFU/min) for fluorescence assay and in $\text{Abs}_{405\text{nm}}/\text{min}$ for colorimetric assay. For Michaelis-Menten kinetic analysis, substrate concentrations ranges were used as indicated, and V_{max} , K_{m} , K_{cat} and catalytic efficiency values were derived by nonlinear regression curve fitting using GraphPad Prism 11.

Rhodamine-phenylglyoxal probe or Activity-based protein profiling (ABPP)

Citrullination was detected using the rhodamine-phenylglyoxal (Rh-PG) probe (Cayman 16172), which reacts selectively with citrullinated residues³¹. Following citrullination reactions, proteins were incubated with Rh-PG probe at a final concentration of 100 μM in 20% trichloroacetic acid (SigmaT9159) for 30 minutes at 37°C before placing tubes on ice for another 30 minutes. Then samples were washed with cold acetone and pellet resuspended in sample buffer heated at 95 °C for 10 minutes and sonicated for another 15 minutes. Samples were resolved by SDS-PAGE and citrullination signal detected by fluorescence imaging at 520 nm excitation 605 nm emission on a Amersham™ Imager 680 system. Total protein was visualised by Sypro Ruby staining as described below. In brief, gels were stained overnight in Sypro Ruby stain (Invitrogen S21900) and destained in 10% methanol/7% acetic acid for 30 minutes prior to fluorescence imaging 520 nm excitation 605 nm emission on a Amersham™ Imager 680 system. For ABPP using FP-TAMRA, citrullinated or untreated protein was incubated with FP-TAMRA at a final concentration of 1 μM in 50 mM HEPES pH7.4 buffer at 37°C. TAMRA fluorescence was detected by fluorescence imaging at 520 nm excitation 605 nm emission on a Amersham™ Imager 680 system. Total protein loading was confirmed by Coomassie staining (GelCode™ Blue Stain reagent

Thermo scientific 24592). For inhibition experiments in the aggrecan degradation assay, proteins or neutrophil-derived preparations were pre-incubated with FP-desthiobiotin at a final concentration of 2.5 μ M or PR3-specific inhibitory probe at 0.5 μ M for 30 minutes at room temperature prior to substrate addition. For the long-term stability assays, bovine trypsin or recombinant NSPs were incubated with or without PAD2 or PAD4 in citrullination buffer at 37°C for timepoints ranging as indicated. At each timepoint, aliquots were removed and assessed by SDS-PAGE with Sypro Ruby staining and by fluorogenic activity assay for proteolytic activity as described above. Activity is expressed as absolute velocity or as a percentage of initial activity at time zero as indicated.

Degradation of bovine serum albumin

Bovine serum albumin (BSA; 12 μ g) was incubated with PAD2-citrullinated or untreated trypsin at 37°C for 5, 15 or 30 minutes in 50 mM HEPES pH7.4. Reactions were terminated by addition of SDS loading buffer and digestion products resolved by SDS-PAGE with Coomassie staining. A standard in solution digestion with reduction (dithiotrietol) and alkylation (iodoacetamide) was used to estimate trypsin activity upon citrullination. Non-citrullinated or citrullinated enzyme was added to reduced/alkylated bovine serum albumin sample in 1:50 enzyme:substrate ratio. Digestion was terminated at 2 and 4 hrs time points by addition of 1% trifluoroacetic acid. Digests were diluted prior to the LC-MS analysis to provide ~1 pmole of BSA injection on column. Extracted ion chromatograms' signals were monitored for 5 selected BSA tryptic peptides: QNCDQFEK, LKECCDKPLLEK, ETYGDMADCCEK, SHIAEVEK, LVNELTEFAK.

Mass spectrometry

Identification of trypsin cit-sites

Samples were digested using thermolysin as described³². Per sample, 4 pmol were analyzed by nano-LC-MS/MS on an Orbitrap Exploris 480 (Thermo Fisher) coupled to an EvosepOne (Evosep Biosystems) equipped with an EV1137 Performance column (15 cm $\times$ 150 μ m, 1.9 μ m C18) and operated with the manufacturer's preset 30 samples-per-day (30 SPD) method. The mass spectrometer was operated in positive-ion data-dependent acquisition (DDA) mode. Full MS scans were acquired from m/z 375–1500 at 60,000 resolution with a normalized AGC target of 300% and a maximum injection time of 50 ms. Precursors with charge states 1–5 and intensities $\geq 2 \times 10^4$ were selected for fragmentation, with the 15 most intense precursors subjected to HCD MS/MS per cycle. MS/MS spectra were acquired at 22,500 resolution using a 1.6 m/z isolation window, a normalized collision energy of 30, and a lower mass limit of m/z 110. Dynamic exclusion was set to 8 s with a ± 10 ppm mass tolerance, and isotopic peaks were excluded from precursor selection. The acquisition method duration was 44 min.

Raw files were processed in FragPipe v24.0 using MSFragger v4.4.1. Spectra were searched against a custom protein sequence database comprising 154 proteins previously identified in the samples and common contaminants. Restricting the database to proteins known to be present in the experimental system was used to limit the exceptionally large search space generated by non-specific cleavage and

multiple variable modifications – under the applied search settings, 94 million modified peptide candidates were considered in the search. Precursor and fragment mass tolerances for the main search were ± 10 ppm and 7 ppm, respectively. Cleavage specificity was set to non-specific, considering peptides between 5 and 35 amino acids. Carbamidomethylation of Cys (+57.02146 Da) was specified as a fixed modification. Variable modifications included oxidation of Met (+15.9949 Da), deamidation of Asn/Gln (+0.9840 Da), citrullination of Arg (+0.9840 Da), dimethylation of Lys (+28.0313 Da), and sulfation of Tyr (+79.9568 Da), with a maximum of three variable modifications per peptide. MSBooster v1.4.14, Percolator v3.7.1, PTMProphet v6.3.2, ProteinProphet, and Philosopher v5.1.3 were used for validation, PTM localization, protein inference, and FDR filtering. Philosopher sequential filtering was performed at 10% PSM FDR, 10% peptide FDR, and 1% protein FDR. Identification filtering was performed using Philosopher with sequential target-decoy filtering; the final retained PSM, peptide, and protein sets had empirical FDRs below 1%. Label-free MS1 quantification was performed using IonQuant v1.11.20 with a 10 ppm m/z tolerance and 0.4 min retention-time tolerance; match-between-runs was enabled with an ion-level FDR of 1%.

Citrullinated bovine trypsin hits were inspected and annotated, including b- and y-ions, internal ions, and chemically permissible neutral losses of H₂O, NH₃ and HNC=O, allowing a maximum fragment mass deviation of ± 3 ppm. The reported citrullinated peptide-spectrum matches had Arg-site localization probabilities of ≥ 0.969 , and absolute precursor mass deviations of ≤ 1.36 ppm. Citrullinated peptide identifications were observed only after PAD treatment and were not identified by MS/MS in the untreated sample. Extracted-ion chromatograms were generated using a ± 5 ppm mass window. Three-dimensional structural models of native and citrullinated trypsin were generated using AlphaFold3. Citrullination at R49 and R99 was introduced by residue substitution. Secondary structure assignments for native and citrullinated trypsin models were generated using DSSP (Define Secondary Structure of Proteins) via mkdssp wrapper in Python. Molecular docking of a peptide substrate (LLRR) to native and citrullinated trypsin structural models was performed using GNINA. A total of 27 docking poses were generated per condition and CNN affinity scores extracted from GNINA output. CNN affinity scores were compared between native and citrullinated trypsin models by unpaired t-test in GraphPad Prism.

RA synovial fluid identification of cit-proteins

RA synovial fluid samples from four patients were pooled and citrullinated proteins enriched using a phenylglyoxal-based chemical enrichment protocol³³. Briefly, 300 μ g of proteins from RA synovial fluid were labelled with a biotin-phenylglyoxal probe (Cayman 17450). Streptavidin coated beads were used to bind biotinylated proteins that were washed and on bead digested with trypsin. Eluted peptides were analysed by LC-MS/MS as described above. The samples were analyzed on an Orbitrap Exploris 480 instrument (Thermo Fisher Scientific, Bremen, Germany). The digests were introduced using an Easy-nLC 1200 system (Thermo Fisher Scientific) at 1 μ g per injection. Mobile phase A was 0.1% (v/v) formic acid and mobile phase B was 0.1% (v/v) formic acid in 80% acetonitrile (LC-MS grade). Gradient separation of peptides was performed on a C18 [Luna C18(2), 3 μ m particle size (Phenomenex, Torrance, CA)] column packed in-house in Pico-Frit (100 μ m X 30 cm) capillaries (New Objective, Woburn, MA). Peptide

separation was conducted using the following gradients: started with 2% of phase B, 2 – 6% over 5 minutes, 6 – 30% over 62 minutes, 30 – 45% over 7 minutes, 45 – 90% over 1 minute, with final elution of 90% B for 15 minutes at a flow rate of 300 nL/minute. Data acquisition on the Orbitrap Exploris 480 instrument was configured for data-dependent method in a positive mode. Spray voltage was set to 2.6 kV, funnel RF level at 40, and heated capillary at 275°C. Survey scans covering the mass range of 380–1500 m/z were acquired at a resolution of 60,000 (at m/z 200), with a normalized automatic gain control (AGC) target of 300% and an auto maximum ion injection time. This was followed by MS2 acquisition at a resolution of 15,000 with an intensity threshold kept at 2e4. During MS2 acquisition, the 20 most abundant ions were selected for fragmentation at 30% normalized collision energy. AGC target value for fragment spectra was set to standard with an auto maximum ion injection time and an isolation width set at 1.6 m/z. Dynamic exclusion of previously selected masses was enabled for 20 seconds, charge state filtering was limited to 2–6, peptide match was set to preferred, and isotope exclusion was on.

Identification of immunocomplexes

The ~720 kDa band excised from native PAGE gel pieces were destained three times with 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v), dehydrated with 100% acetonitrile, and lyophilized. Proteins were reduced with 10 mM dithiothreitol at 57°C for 30 min, alkylated with 55 mM iodoacetamide for 30 min at room temperature in the dark, and residual iodoacetamide was quenched with 25 mM dithiothreitol for 10 min at room temperature, with dehydration performed between steps to remove excess reagent. Gel pieces were rehydrated in 50 mM ammonium bicarbonate containing 12.5 ng/μL trypsin and digested for 15 h at 37°C. Peptides were collected from the digestion supernatant, further extracted once with water and three times with 5% formic acid in 50% acetonitrile, pooled, lyophilized, resuspended in 0.5% formic acid, and loaded onto Evotip Pure tips according to the manufacturer's protocol for LC-MS/MS analysis. Peptide samples were analyzed by nanoflow LC-MS/MS using an Evosep One coupled to a timsTOF Pro2 operating in PASEF (parallel accumulation-serial fragmentation) mode. An Evosep EV1137 column was used to separate peptides (1.5 μm particle, 150 μm ID x 15 cm, Evosep) with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) using the preset 30 samples per day gradient and flowrate. Mass and inverse reduced ion mobility (1/K0) values were calibrated using a doped filter prepared from commercially obtained calibrants (m/z 622, 922, 1222) according to the manufacturer's instructions. The 20 μm nanoflow emitter was charged at 1600V and the TOF analyzer is set to a scan range of 100 – 1700 m/z. The overall acquisition cycle of 0.74s comprised one full TIMS-MS scan and six PASEF MS/MS scans (singly charged ions were excluded via standard polygon filter). The ion mobility was scanned from 0.7 to 1.5 V*s/cm² at a ramp time of 100ms while ramp and accumulation times are locked to 100% duty cycle. Precursor ions were selected with an intensity threshold of 1000 counts and dynamic exclusion was set to 0.4 minutes except for low-abundance precursors were repeatedly targeted for fragmentation until a cumulative intensity of 12500 counts was achieved. The collision energy was ramped linearly as a function of the mobility from 59eV at 1/K0 = 1.6 Vs cm⁻² to 20eV at 1/K0 = 0.6 Vs cm⁻².

Neutrophil isolation and stimulation

Human peripheral blood neutrophils were isolated from healthy donor blood by ficoll density gradient centrifugation. After removal of plasma, PBMCs and residual Ficoll solution, RBCs were removed from granulocytes using EasySep RBC depletion kit (#18170). Neutrophil purity was assessed by flow cytometry and was consistently > 95%. For degranulation experiments, isolated neutrophils at 1.0×10^5 cells/mL were stimulated with A23187 ionophore at 1 μ M for 60 minutes at 37°C. Cells were centrifuged at 1500 rpm for 5 minutes and supernatants collected and stored at -80°C until use. For NET induction, neutrophils were stimulated with PMA at 100 nM for 4 hours at 37°C. NETs were harvested using our previous protocols²⁴.

Native PAGE immune complex separation

Neutrophil supernatant was labelled with FP-TAMRA at 1 μ M for 30 minutes at 37°C. Then free non-reactive probe was removed by washing with PBS in a 15 ml centrifugal filter (Amicon Ultra-15 UFC903024) prior to incubation RA serum or purified RA IgG, at a ratio of 100/1 at 37°C overnight in constant shaking. IgG was purified from RA serum by protein G affinity chromatography using Nab TM protein A plus spin columns according to manufacturer instructions (Thermo scientific 89952). Purity of isolated IgG was confirmed by SDS-PAGE. Mixtures were resolved by Blue-native gel electrophoresis (native PAGE) under non-denaturing conditions on 4-12% polyacrylamide gels at 150 mV for 120 minutes at 20°C. TAMRA fluorescence was detected by fluorescence imaging and total protein confirmed by Coomassie staining.

Aggrecan degradation assay

Aggrecan (500 ng) was incubated with PAD-citrullinated or untreated recombinant NE, PR3 or CTG, or with PAD-citrullinated neutrophil supernatant or NETs at 37°C for 15 or 30 minutes in 20 mM HEPES pH7. For inhibition experiments, proteases or neutrophil-derived preparations were pre-incubated with FP-desthiobiotin (2.5 μ M) or PR3-specific inhibitory probe (0.5 μ M) for 30 minutes at room temperature prior to aggrecan addition. Reactions were terminated by addition of SDS loading buffer and degradation products resolved by SDS-PAGE and revealed with Sypro Ruby staining. Band density of aggrecan bands at ~98 kDa was quantified.

ELISA; cit-PR3 quantification and autoantibody measurement

Circulating cit-PR3 levels in serum were measured by ELISA using a custom sandwich ELISA using anti-citrulline (ab100932, 1:100) and anti-PR3 (1:500, PA5-85928). For autoantibody measurement, 100 μ L of PAD2- or PAD4-citrullinated NE, PR3 or CTG were coated onto 96-well plates at 1 μ g/mL in overnight at 4°C. Plates were blocked with 1% BSA for 2 hours and incubated with serum samples diluted 1:100 for 1 hour at room temperature. Detection was performed using anti-human IgG-HRP conjugate (1:10000) and TMB substrate. Results were expressed as OD index normalised to control samples. N = 15 samples

were used per group, selected from the larger characterized cohort (Table S5) based on sample availability.

Fibroblast-like synoviocyte culture and stimulation

Human FLS were obtained from human donors and cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin, and 1% MEM Non-Essential Amino Acids at 37°C in 10% CO₂. Cells were used between passages P2 and P4. For stimulation experiments, FLS were seeded at 1.0×10^5 cells per well in 12-well plates and stimulated with native PR3 or cit-PR3 at 0.2 µg/mL, LPS at 0.1 µg/mL, or left untreated at various timepoints. For PAR2 inhibition experiments, AZ3451 (Cayman, Catalogue number- 29671) was added at 0.5 µM 30-60 minutes prior to cit-PR3 stimulation.

Cytokine measurement

IL-6, IL-1β, MMP-9 and TNF-α in FLS supernatants were measured by ELISA using commercial kits (R&D systems) according to manufacturer instructions. FLS supernatants were collected after 8 hours of stimulation with native PR3 or cit-PR3 and analysed using the Olink Target 48 proximity extension assay panel (Olink Proteomics, Uppsala, Sweden) according to manufacturer instructions. Normalised protein expression (NPX) values were used for all downstream analyses. Differential expression between cit-PR3 and native PR3 conditions was assessed using a paired t-test in R. Principal component analysis and hierarchical clustering were performed and GO enrichment analysis of the cit-PR3 versus native PR3 FLS secretome was performed using clusterprofiler. Enrichment significance threshold was set at adjusted $p < 0.05$. Network visualisation was generated using cnetplot.

Western blotting

FLS were lysed in cell lysis buffer (Thermofisher #87787). Protein concentration was determined by BCA assay. Equal quantities of protein (10 µg) were resolved by SDS-PAGE on 4-12% Bolt Bis-Tris Plus gels, transferred to nitrocellulose membrane, blocked with 5% BSA for 60 minutes, and incubated with primary antibodies against pERK1/2 (Biotechne R&D systems, catalogue number MAB1018, dilution 1:100) and total ERK1/2 (Biotechne R&D systems, catalogue number AF-1576-SP, dilution 1:100) overnight at 4°C. HRP-conjugated secondary antibody (dilution 1:10000) was applied for 1 hour at room temperature, and the signal was detected by enhanced chemiluminescence using Amersham ECL prime reagent (Cytiva, Catalogue number- RPN2322) on an Amersham Imager 680 imaging system.

Immunofluorescence

FLS were seeded on glass coverslips at 50,000 cells per coverslip, stimulated as described, fixed in 4% paraformaldehyde for 10 minutes at room temperature, permeabilized with 0.1% Triton X-100 in PBS permeabilization buffer, and blocked with 0.2% gelatin for 60 minutes. Cells were incubated with anti-pERK1/2 antibody (Biotechne, R&D systems, catalogue number MAB1018, dilution 1:100) at room temperature for 60 minutes, followed by fluorescent secondary antibody conjugate (Cy3, dilution 1:100)

(Jackson ImmunoResearch) for 30 minutes at room temperature protected from light. F-actin was labelled with phalloidin-Alexa Fluor 633 (dilution 1:100) (Invitrogen, Catalogue number-A22284) and nuclei counterstained with Hoescht 33258 (10 µg/mL). FLS were also stimulated as described, fixed with BD Phosflow™ Lyse/Fix buffer for 10 minutes at 37°C permeabilised with BD Phosflow™ Perm/Wash Buffer I for 20 minutes on ice, and stained with anti-pERK1/2 antibody (Biolegend, catalogue number 369505, dilution 1:100) in BD Phosflow™ Perm/Wash Buffer I as staining buffer for 45 minutes at room temperature. Data were acquired on a BD Fortessa flow cytometer and analysed using FlowJo software (version 10).

Statistical analysis

Statistical analyses were performed using GraphPad Prism. Data are presented as mean ± SEM unless otherwise stated. Comparisons between two groups were performed by Mann-Whitney U test for non-parametric data. Comparisons across three or more groups were performed by Kruskal-Wallis test with Dunn's post-hoc correction or one-way ANOVA with Tukey post-hoc test as indicated in the figure legends. Pearson correlation coefficient was used to assess correlations between citrullination and proteolytic activity. A p-value of < 0.05 was considered statistically significant throughout.

Declarations

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Author Contributions

M.N. led the project, conducted or supervised most of the experiments, and contributed to data analysis. J.K., Ra.Z., C.K., N.M., O.I.O., J.A.M., A.S., K.T., X.M., and Y.L. contributed to experimental work and data collection. Y.L. performed mass spectrometric analyses under the supervision of R.Z and O.K. D.M.S. provided resources and intellectual input. V.M., K.D.D., V.M.H., and M.K.D. provided clinical samples and contributed to study design. O.K. contributed to data collection and analysis. H.E.G. contributed to study design and patient recruitment. L.J.O. conceived and designed the study, supervised all experimental work, and wrote the manuscript. All authors reviewed and approved the final manuscript.

Data Availability

The mass spectrometry proteomics data generated in this study have been deposited in the MassIVE repository (Center for Computational Mass Spectrometry, University of California San Diego) under accession code MSV000103321 (<https://doi.org/10.25345/C5JD4Q42K>), released under a CC0 1.0 licence, and will be made publicly available upon publication. Raw Olink Target 48 secretome data are provided in Table S6.

Competing Interests

The authors declare no competing interests.

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Figures

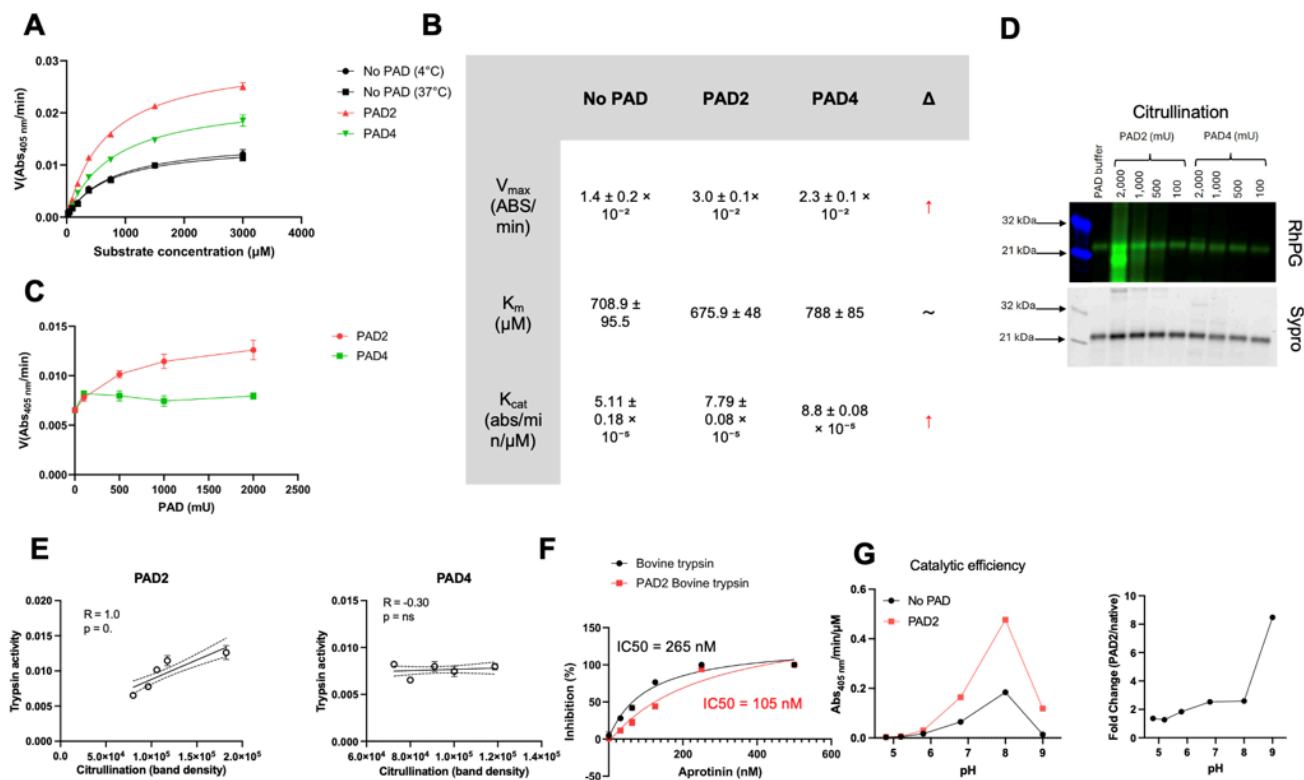


Figure 1

PAD citrullination enhances the proteolytic activity of trypsin (A) Michaelis-Menten kinetic curves for bovine trypsin ± PAD2/PAD4 citrullination and summary of derived kinetic parameters (B). (C) Proteolytic activity of bovine trypsin following incubation with increasing concentrations of PAD2 or PAD4 (100–2000 mU), measured by fluorogenic substrate assay. PAD2 citrullination resulted in a concentration-dependent increase in proteolytic activity; PAD4 produced no significant dose-dependent effect. (D) Citrullination of trypsin across PAD2 and PAD4 concentration series confirmed by rhodamine-conjugated phenylglyoxal (RhPG) citrulline-specific probe (upper panel) with Sypro Ruby total protein staining as loading control (lower panel). (E) Correlation between PAD2-induced citrullination signal (normalised band density) and proteolytic activity across the PAD2 concentration series (100–2000 mU); (F) Inhibition of trypsin proteolytic activity by aprotinin, a competitive serine protease inhibitor. (G) Trypsin

and citrullinated trypsin catalytic efficiency across varying pH. Data shown as mean ± SEM from ≥3 independent experiments.

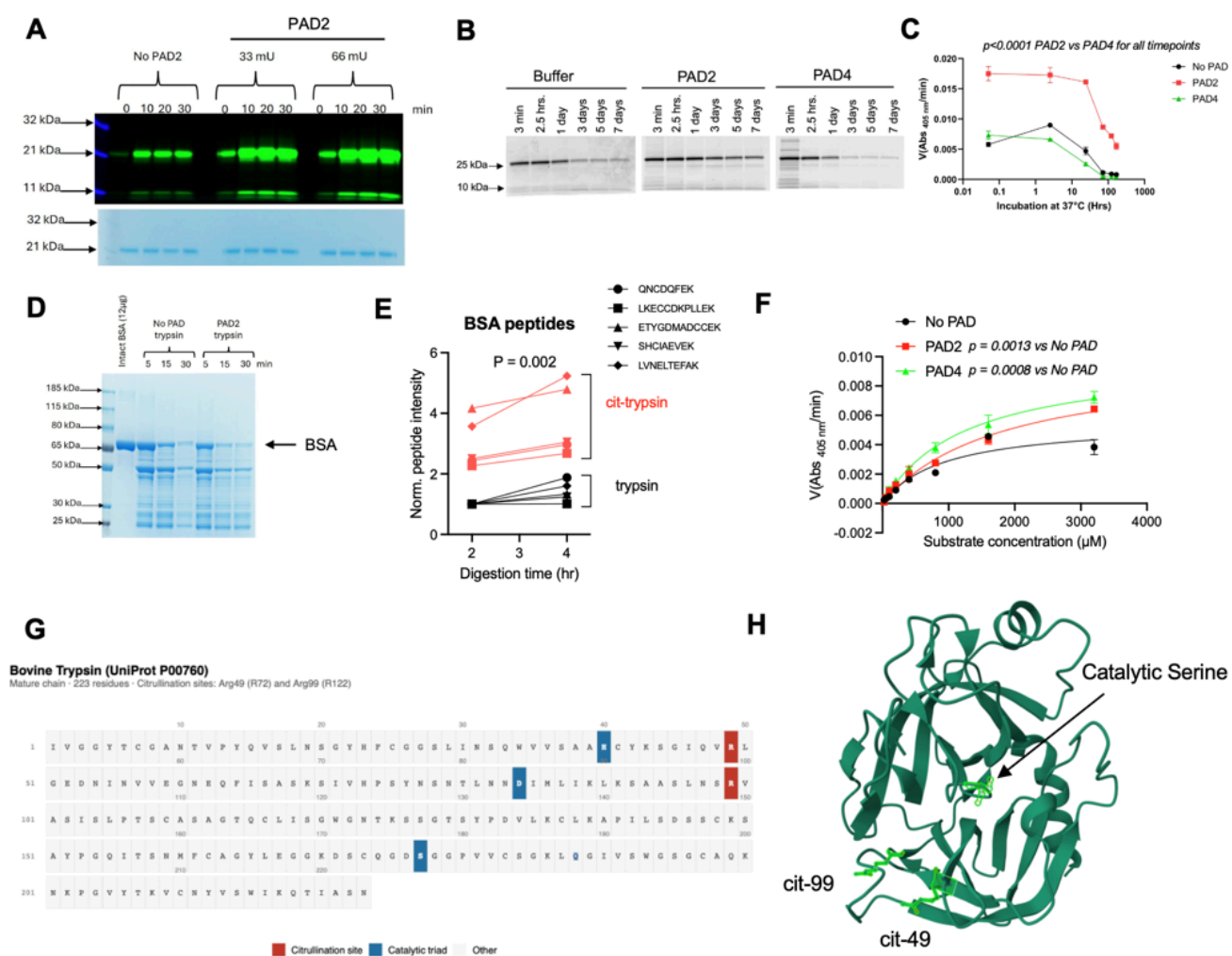


Figure 2

PAD2 citrullination enhances trypsin active site accessibility, structural stability, and proteolytic capacity through modification of residues R49 and R99. (A) Activity-based protein profiling (ABPP) of bovine trypsin using the FP-TAMRA active site probe following citrullination with increasing concentrations of PAD2 (33 and 66 mU) compared to untreated trypsin (No PAD2), assessed at 0, 10, 20, and 30 minutes. Total protein is shown by Coomassie staining (lower panel). (B) Time-lapse stability of bovine trypsin assessed by SDS-PAGE with Sypro Ruby staining following incubation at 37°C for 3 minutes, 2.5 hours, 1, 3, 5, and 7 days under buffer, PAD2, or PAD4 citrullination conditions. Untreated trypsin undergoes progressive autoproteolytic degradation, whereas PAD2-citrullinated trypsin retains an intact band throughout the 7-day incubation. (C) Proteolytic activity of bovine trypsin (No PAD, PAD2, PAD4) measured by fluorogenic substrate assay across the same incubation period, demonstrating sustained activity in PAD2-citrullinated trypsin relative untreated and PAD4-citrullinated preparations.

Analyzed by Mann-Whitney U test. (D) Degradation of bovine serum albumin (BSA) by PAD2-citrullinated versus untreated bovine trypsin at 5, 15, and 30 minutes, assessed by SDS-PAGE with Coomassie staining. (E) Mass spectrometric quantification of BSA-derived peptide intensity from digests generated by PAD2-citrullinated (red) versus untreated trypsin (black). Analyzed by Wilcoxon matched-pairs signed rank test. (F) Michaelis-Menten kinetic curves for sequence-grade trypsin (SGT) $\pm$ PAD2/PAD4 citrullination. (G) Mass spectrometric identification of citrullination sites in bovine trypsin; R49 and R99 are identified as the principal sites of citrullination. (H) Three-dimensional structural representation of trypsin showing the positions of citrullination sites R49 and R99 relative to the catalytic serine, all highlighted in light green.

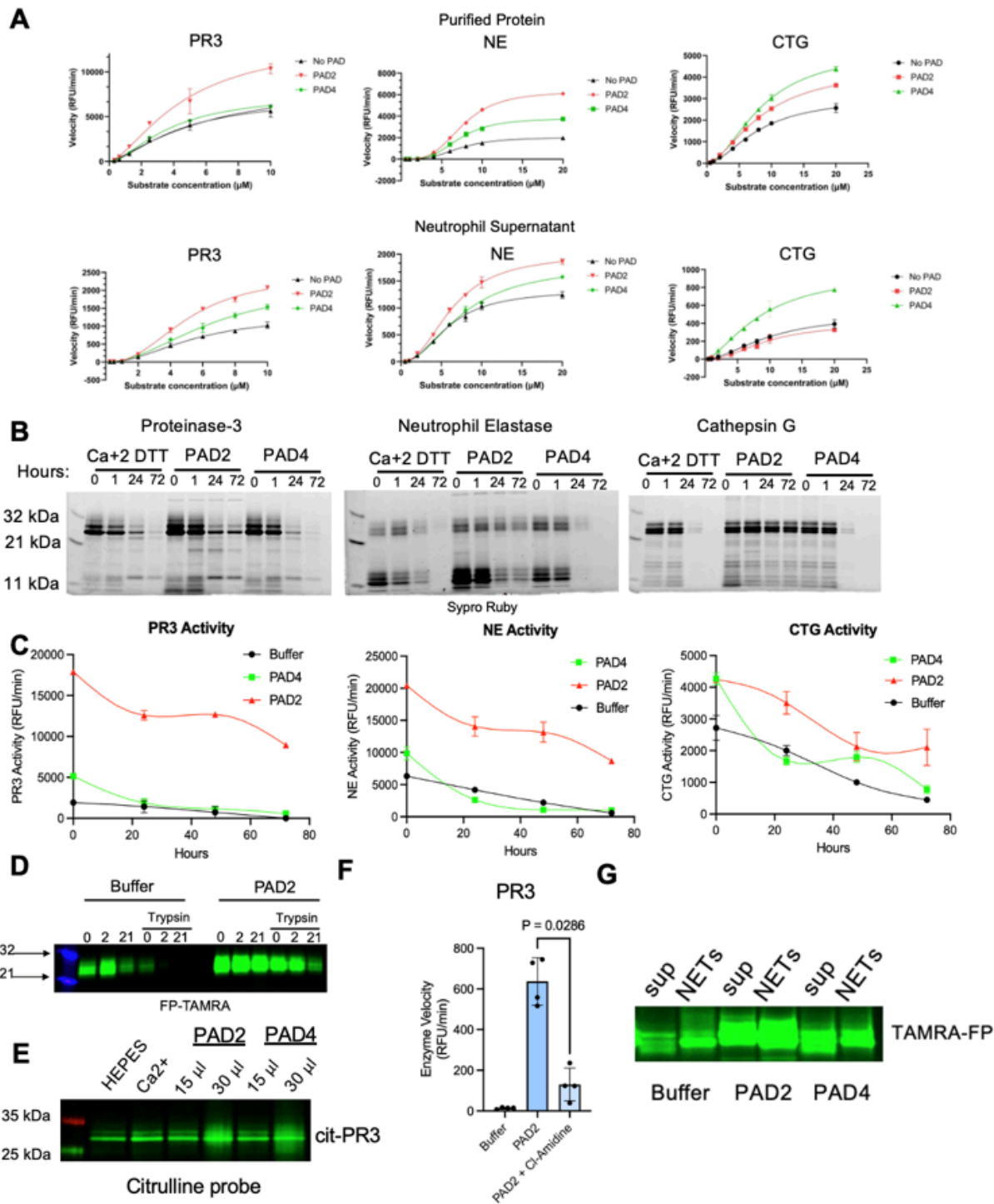


Figure 3

PAD2 citrullination enhances the proteolytic activity and stability of neutrophil serine proteases. (A) Michaelis-Menten kinetic analysis of proteolytic activity of recombinant PR3, NE, and CTG (upper row) and PAD-citrullinated neutrophil supernatant preparations (lower row) following citrullination with PAD2 or PAD4 compared to untreated controls (No PAD). Velocity is expressed as RFU/min across a range of substrate concentrations. (B) Stability of recombinant PR3, NE, and CTG assessed by SDS-PAGE with

Sypro Ruby staining at 0, 1, 24, and 72 hours of incubation at 37°C under Ca²⁺/DTT control, PAD2, or PAD4 citrullination conditions. PAD2 citrullination reduced autoproteolytic degradation across all three NSPs, with intact bands retained at 72 hours. (C) Proteolytic activity of PR3, NE, and CTG measured by fluorogenic substrate assay over 72 hours at 37°C under buffer, PAD2, or PAD4 citrullination conditions. (D) Activity-based protein profiling (ABPP) of PAD2-citrullinated PR3 using the FP-TAMRA active site probe following incubation with or without exogenous trypsin at 0, 2, and 21 hours. (E) Citrullination of PR3 confirmed by citrulline-specific fluorescent probe following treatment with increasing volumes of PAD2 (15 and 30 µl) or PAD4 (15 and 30 µl) compared to HEPES and Ca²⁺ controls. (F) Proteolytic activity of PR3 (RFU/min) following PAD2 citrullination in the presence or absence of the pan-PAD inhibitor Cl-amidine (720 µM); PAD2-induced enhancement of PR3 activity is significantly abrogated by Cl-amidine pre-treatment ($p=0.0286$), confirming that PAD enzymatic activity is required for the observed effect. Analyzed by one-way ANOVA with Tukey post-hoc test (G) Activity-based protein profiling (ABPP) of PAD2- and PAD4-citrullinated neutrophil supernatant and NETs using the FP-TAMRA active site probe. Data shown as mean ± SEM from ≥3 independent experiments.

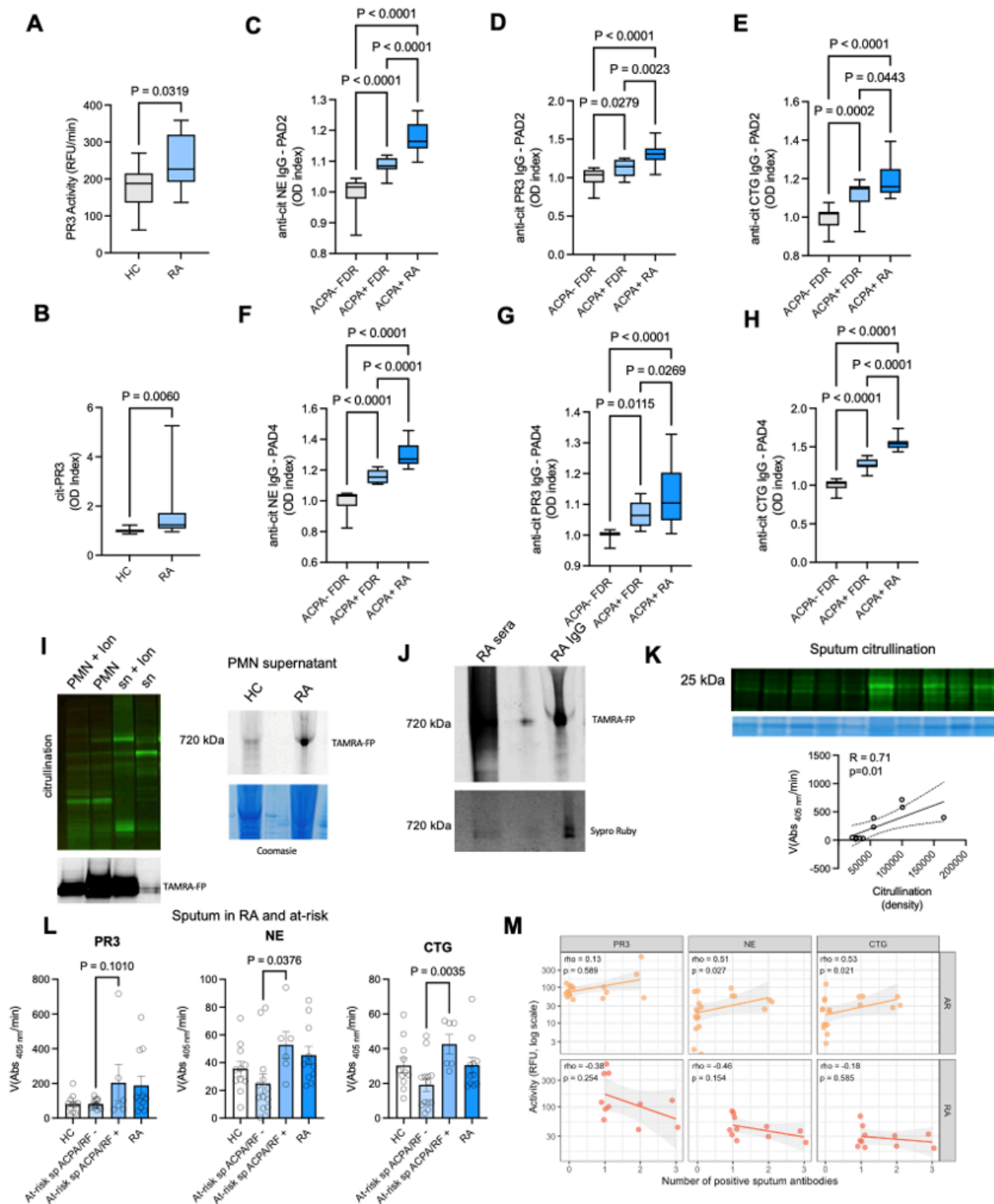


Figure 4

Citrullinated neutrophil serine proteases are recognised as autoantigens in serum and at mucosal sites in RA (A) Proteolytic activity of PR3 in serum from patients with established RA compared to healthy controls (HC), measured by fluorogenic substrate assay (n=11 per group; Mann-Whitney U test, $p=0.0319$). (B) Circulating citrullinated PR3 (cit-PR3) levels quantified by ELISA in RA patients and HC, expressed as OD index (n=35 RA, n=21 HC; Mann-Whitney U test, $p=0.0060$). (C–E) IgG autoantibody

reactivity to PAD2-citrullinated NE (C), PR3 (D), and CTG (E) measured by ELISA across ACPA-negative first-degree relatives (ACPA- FDR), ACPA-positive first-degree relatives (ACPA+ FDR), and ACPA-positive RA patients (ACPA+ RA, n=15 per group). (F–H) IgG autoantibody reactivity to PAD4-citrullinated NE, PR3, and CTG. Statistical comparisons for panels C–H by Kruskal-Wallis with Dunn's post-hoc test. (I) Left panel: citrullination of cultured neutrophils assessed by citrulline-specific fluorescent probe following ionophore (A23187, Ion) stimulation; PMN cell lysate (PMN + Ion, PMN) and supernatant fractions (sn + Ion, sn) are shown. Lower panel: FP-TAMRA active site labelling confirming serine hydrolase activity in the same fractions at 25 kDa. Right panel: TAMRA-labelled neutrophil supernatant incubated with HC or RA serum and resolved by native PAGE; a discrete ~720 kDa band with FP-TAMRA signal is detected specifically in RA sera, consistent with capture of active site-labelled NSPs into a high molecular weight complex by RA serum components. (J) Native PAGE of TAMRA-labelled neutrophil supernatant incubated with purified IgG isolated from RA serum; the ~720 kDa band is reproduced with purified IgG alone. (K) Citrullination in sputum correlates with activity of proteinase-3 ($R=0.71$, $p=0.01$). (L) Proteolytic activity of PR3, NE, and CTG in sputum from HC (n=11), at-risk sputum ACPA/RF- individuals (n=13), at-risk sputum ACPA/RF+ individuals (n=6), and RA patients (n=11), measured by fluorogenic substrate assay. NE ($p=0.0376$) and CTG activity ($p=0.0035$) is significantly elevated in at-risk sputum Ab+ individuals compared to at-risk sputum Ab-. (M) Correlation between PR3, NE and CTG activity and # of sputum autoantibody (range 0 to 3) split by at-risk (AR, top) and RA (bottom). Data shown as mean $\pm$ SEM unless otherwise stated.

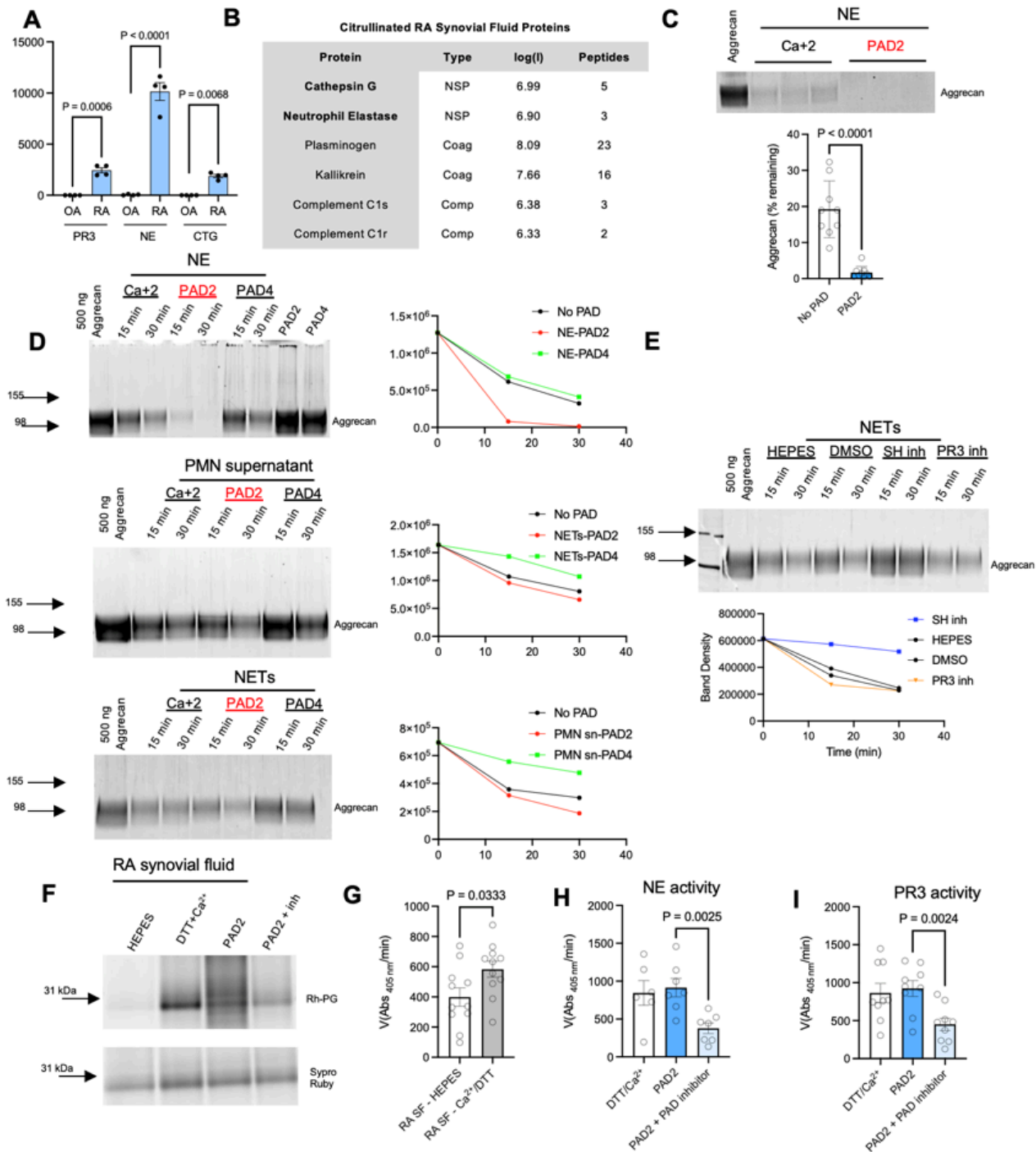


Figure 5

Citrullinated neutrophil serine proteases degrades the proteoglycan aggrecan. (A) Proteolytic activity of PR3, NE, and CTG in synovial fluid from RA and OA patients, measured by fluorogenic substrate assay (n=4 per group). Statistical comparisons by Mann-Whitney U test. (B) Mass spectrometric identification of citrullinated serine proteases in RA synovial fluid following phenylglyoxal citrulline-enrichment pull-down from a combined preparation of RA synovial fluid samples (n=3). (C) Aggrecan degradation by

PAD2 citrullinated NE compared to native NE (5 min, Mann-Whitney $p < 0.0001$). (D) Aggrecan degradation by PAD2- or PAD4-citrullinated recombinant NE, PMN supernatant or NETs with Sypro Ruby staining at 15 and 30 minutes. (E) Aggrecan degradation by NETs in the presence of HEPES buffer, DMSO vehicle, serine hydrolase (SH) probe, or a PR3 probe 15 and 30 minutes. (F) Citrullination of RA synovial fluid proteins assessed by rhodamine-phenylglyoxal (Rh-PG) citrulline-specific probe following incubation with HEPES buffer, DTT+Ca²⁺ (endogenous PAD activation), exogenous PAD2, or PAD2 plus CAY10723 PAD inhibitor. Total protein loading confirmed by Sypro Ruby staining (lower panel). (G) Proteolytic activity of RA synovial fluid under HEPES buffer or endogenous activation of PAD with Ca²⁺/DTT (n=11 per group; Mann-Whitney U test, $p = 0.033$). (H) NE ($p = 0.0025$) or (I) PR3 proteolytic activity ($p = 0.0024$) in RA synovial fluid following incubation with DTT/Ca²⁺, exogenous PAD2, or PAD2 plus CAY PAD inhibitor. Data shown as mean $\pm$ SEM with individual data points.

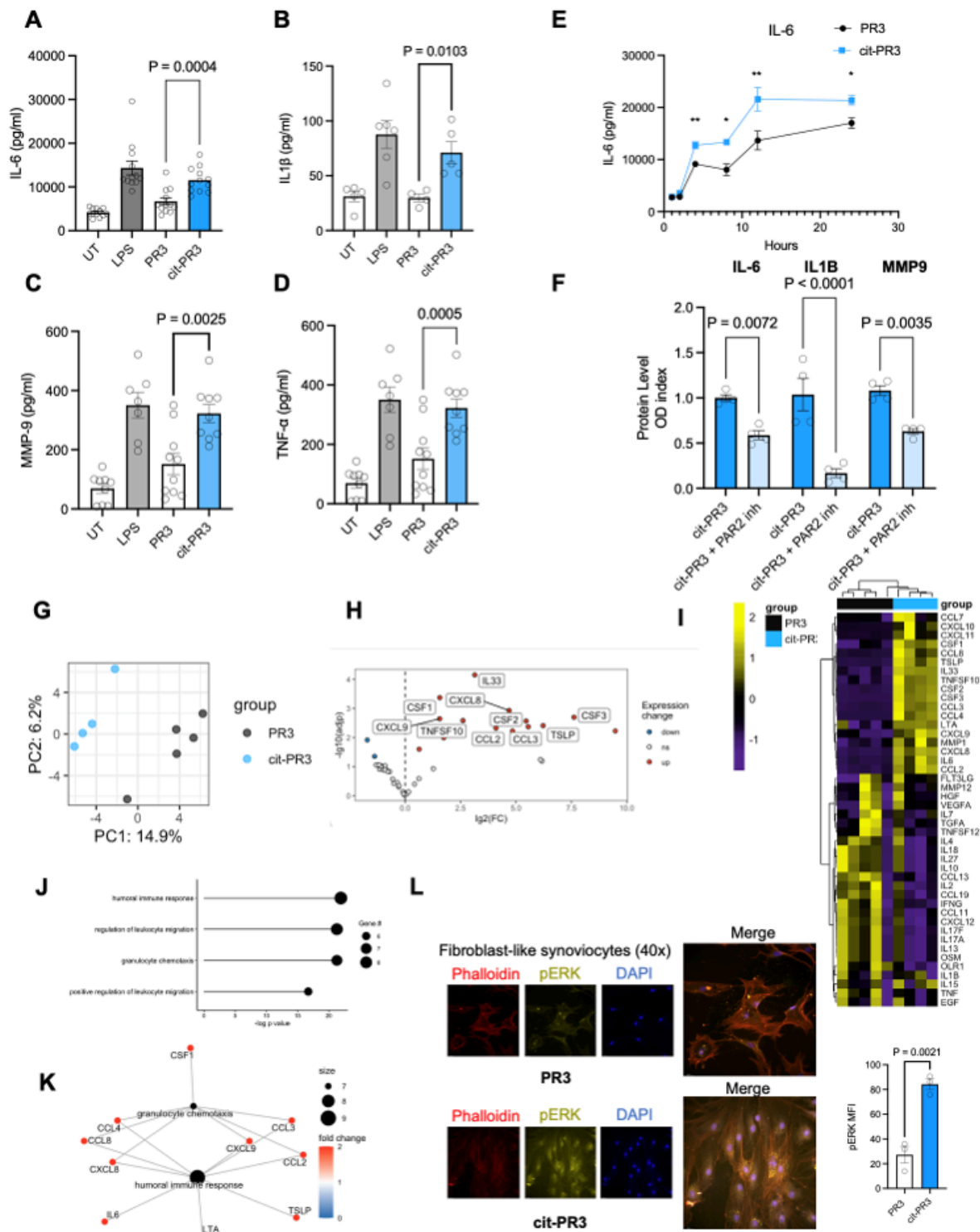


Figure 6

Citrullinated PR3 activates fibroblast-like synoviocytes via PAR2-ERK signalling. (A) IL-6 ($p=0.0004$), (B) IL-1 β ($p=0.0103$), (C) MMP-9 ($p=0.0025$) and (D) TNF- α ($p=0.0005$) secretion from FLS stimulated with untreated control (UT), LPS, native PR3, or PAD2-citrullinated PR3 (cit-PR3) for 12 hours. Analyzed by Mann-Whitney U test. (E) Time course of IL-6 secretion from FLS stimulated with native PR3 or cit-PR3 over 24 hours; ($** p < 0.01$, $* p < 0.05$). (F) IL-6 ($p=0.0072$), IL-1 β ($p < 0.0001$), and MMP-9 ($p=0.0035$)

secretion from FLS stimulated with cit-PR3 or cit-PR3 plus a PAR2 antagonist AZ3451 (0.5 μ M). Analyzed by Mann-Whitney U test. (G) Principal component analysis (PCA) of the FLS secretome (Olink Target 48) following stimulation with native PR3 or cit-PR3. (H) Volcano plot of differentially expressed secreted proteins comparing cit-PR3 and native PR3. (I) Heatmap of secreted proteins from FLS stimulated with native PR3 or cit-PR3. (J) Gene ontology (GO) enrichment analysis of the cit-PR3 versus native PR3 FLS secretome; significantly enriched pathways. (K) GO network visualisation of granulocyte chemotaxis and humoral immune response pathways, showing key secreted proteins contributing to each pathway; node size represents gene count, and colour represents fold change relative to native PR3. (L) Immunofluorescence imaging of FLS stimulated with native PR3 (upper row) or cit-PR3 (lower row), stained for phalloidin (red), pERK1/2 (yellow), and DAPI (blue) with merged images along with quantification by median fluorescence intensity. Data shown as mean $\pm$ SEM from ≥ 3 independent experiments. Analyzed by Mann-Whitney U test.

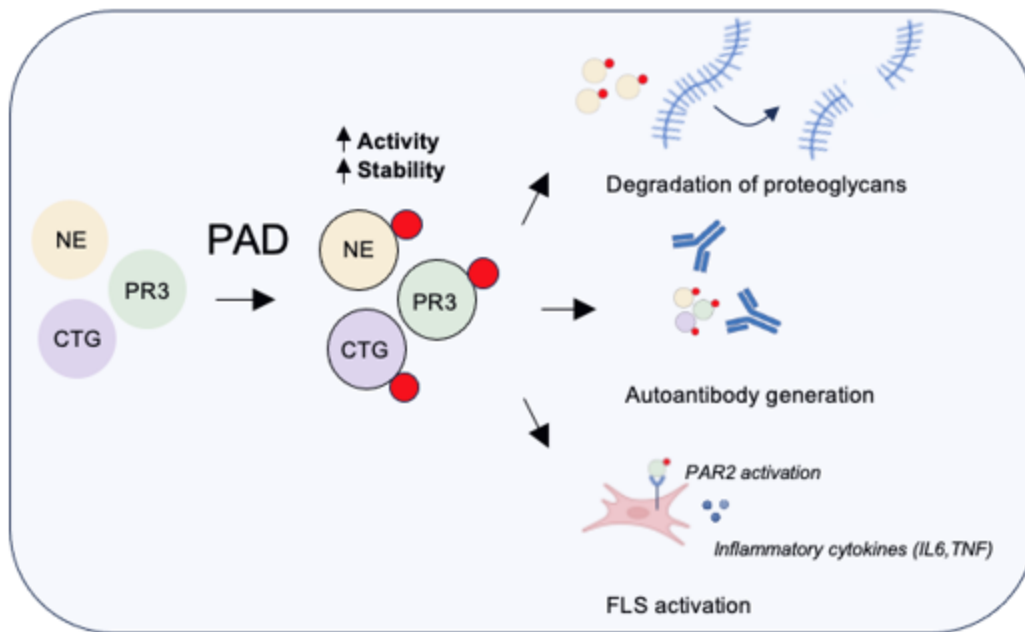


Figure 7

Conceptual framework for the role of citrullinated neutrophil serine proteases in RA pathogenesis.

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