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The Mechanisms of SAA/TLR4 Inducing Angiogenesis in Rheumatoid Arthritis through NETs Formation

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Abstract

Objective: Rheumatoid arthritis (RA) is an autoimmune disease in which angiogenesis represents a critical early event of synovial inflammation. The present study aimed to reveal the potential molecular mechanisms of SAA/TLR4 induction of angiogenesis through NETs in RA. **Materials and methods:** Firstly, immunohistochemistry and immunofluorescence were used to determinate TLR4 and NETs expression in synovial tissue, respectively. ELISA was used to detect the content of SAA, MPO and NE in serum and synovial fluid of patients. DNA quantification was done by fluorescence. DNA fluorescence staining was used to compare NETs formation in RA and HC sera, and to investigate the mechanism of NETs formation induced by SAA stimulation. PicoGreen DNA testing was used to characterize the DNA in the supernatants. Also, DNA fluorescence staining to explore whether NETs formation induced by SAA was dependent or independent on NADPH oxidase pathway. MTT assay, Wound healing assay, Tube formation assay were performed to analyze human veins umbilical cells (HUVECs) proliferation, migration, and tube vessels formation, respectively under NETs or NETs + DNase stimulants. **Results:** Firstly, we demonstrated that TLR4 was predominantly and widely expressed in synovial tissues with elevated serum levels of SAA, compared to osteoarthritis (OA) patients, and the similar results were observed for NETs formation. Afterwards, in a series of *in vitro* experiments, we reported an increased MPO and NE levels, and a relatively decreased DNA level in the sera of RA patients. Set apart, the levels of MPO and NE in RA were correlated to the disease activity. Moreover, an increased spontaneous NETs formation was observed in RA patients, enhanced under SAA stimulation and regulated by TLR4 activation. And the total DNA expressed in RA patients

was partly composed of NET-DNA. Also, SAA induced NETs formation dependent on NADPH pathway. Finally, our results indicated that extracted SAA-induced NETs promoted endothelial cells (ECs) migration, proliferation, and vascular tube formation. **Conclusion:** Our current study highlighted the role of SAA/TLR4 interaction in the induction of angiogenesis through formed NETs. Therefore, this study offers new perspectives in the understanding of RA pathogenicity and its management.

Keywords

Angiogenesis, Neutrophil Extracellular Traps, Rheumatoid Arthritis, Serum Amyloid A, Toll-Like Receptor 4

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory systemic autoimmune disorder, in which angiogenesis can foster the infiltration of inflammatory cells into the joints causing synovitis and pannus, both leading to bone and cartilage destruction [1] [2]. The main pathological features of RA are the formation of erosive pannus and inflammation of joint. The formation of erosive pannus is the basis for the destruction of bone and cartilage in RA, while angiogenesis is the basis for the formation of pannus [1] [2]. Angiogenesis is a highly complex sequence of events orchestrating various molecular events involving multiple cell populations, cytokines, and chemokines.

Nowadays, a piece of information regarding the potential role of serum amyloid A (SAA) in the pathogenesis of RA has emerged [3] [4]. SAA is a major acute-phase protein of the apolipoprotein family, primarily secreted by hepatocytes in response to trauma, inflammation, or infection. Its normal concentration is 20 - 50 μg/ml, and it has a striking acute phase response with serum levels rising as much as 1000-fold in 24 hours [5]. Previous studies have reported that serum SAA levels were elevated in RA patients compared with other inflammatory rheumatic diseases (IRD) patients [6]. High expression of SAA and its receptors had been shown in RA synovial tissues such as scavenger receptor class B member 1 (SR-B1), formyl peptide receptor-like 1 (FPRL1), and toll-like receptor 2 (TLR2) [7] [8] [9]. Given that, the intriguing feature of SAA is that it can bind to and activate a variety of structurally diverse receptors and further induce RA synovial hyperplasia and angiogenesis by activation of several downstream signaling pathways. For instance, SAA could enhance the proliferation and survival of endothelial cells and synovial fibroblasts through FPRL1, via the activation of ERK and Akt signaling pathways [7]. Similarly, we previously reported that SAA and SR-B1 interaction could induce angiogenesis via the p38 MAPK signaling pathway in RA patients [8].

Neutrophils are key innate immune effector cells that are rapidly recruited to the sites of infection and inflammation to provide early defense against invading

microorganisms [10]. Besides, neutrophil extracellular traps (NETs) are formed and released by neutrophils when they are properly stimulated. However, abnormal or excessive NETs formation can lead to the occurrence of autoimmune diseases [11] [12]. To date there are shred of evidences regarding the role of neutrophil extracellular traps (NETs) in the pathogenesis of RA [11] [13] [14]. It was proved that NETs promote joint and cartilage damage in RA, through the activation of fibroblast-like synoviocytes (FLS) that release cytokines [15]. Another study reported that NETs promote endothelial cell dysfunction in systemic lupus erythematosus [16]. Furthermore, in patients with pulmonary hypertension, NETs demonstrated its potential to promote pulmonary angiogenesis [17]. Although a potential pathological significance of NETs formation in RA was proven, its role in the angiogenesis process has yet to be elucidated. NETs formation can be induced by reactive oxygen species (ROS) such as hydrogen peroxide [18], antibodies [19], antigen-antibody complexes [20] [21], PMA, serum amyloid A [22], and microbial components like lipopolysaccharide (LPS) [23] [24].

Moreover, TLR4 was shown to play an important role in controlling the disease severity, and the progressive joint degeneration, and then might be a potent target in RA [25] [26] [27]. Recent studies showed that the fusion protein (F protein) of respiratory syncytial virus combined with TLR4 can induce the formation of NETs [28] [29], and high-mobility group box 1 (HMGB-1) can also be combined with TLR4 to induce the formation of NETs [30]. LPS binding to TLR4 on the neutrophil surface induced dose-dependent Nox-dependent NETs formation and its inhibition using TAK-242 (a TLR4 inhibitor) abolished this effect [31]. Interestingly, it has been reported that TLR4 is also an endogenous receptor of SAA [32] [33] [34]. Nevertheless, the detailed signal transduction pathway mediated by SAA/TLR4 and NETs in the neovascularization process remains incompletely defined in RA. Therefore, our current study launched a series of investigations attempting to reveal the possible molecular mechanisms by which SAA/TLR4/NETs may promote angiogenesis in RA.

2. Methods

Patients

The data collection was performed from March 2020 to December 2021 at General Hospital of Tianjin Medical University. The types of diseases and the respective number of patients from whom serum samples were obtained were as follows: rheumatoid arthritis (RA) (n=105); osteoarthritis (OA) (n = 64). Sixty age and gender-matched healthy control subjects were recruited. Besides, synovial membranes (SM) were obtained from RA (n = 8) and OA (n = 6) patients during synovectomy at General Hospital of Tianjin Medical University during the same period. All patients with RA fulfilled the American College of Rheumatology (ACR)/European League against Rheumatism (EULAR) criteria for RA [2], and all patients with OA fulfilled the ACR 1995 criteria for OA [35]. Pa-

tients who suffered from other chronic diseases or any acute infections within the past three months were excluded from this study. This study has been reviewed and approved by the Ethics Committee of Tianjin Medical University (ethical approval number IRB2019-KY-147), and all subjects have signed an informed consent form. All the human material or human data were performed in accordance with the Declaration of Helsinki.

Disease activity related indicators

The disease activity was measured with DAS28-ESR.

DAS28-ESR, a validated scoring system for predicting disease activity, was employed in order to calculate disease activity using the following formula: DAS28-ESR = 0.56xsqrt (number of tender joints) + 0.28xsqrt (number of swollen joints) + 0.70xLn (ESR) + 0.014xvisual analogue scale (VAS).

Erythrocyte Sedimentation Rate (ESR) was detected by westergren method. Male value less than 15 mm/h, and female value less than 20 mm/h were considered as normal reference values.

Serum CRP levels were detected by immunoturbidimetry, and CRP level less than 8 mg/l was considered a normal reference value.

ELISA

ELISA was first used to detect the content of SAA in serum and synovial fluid of patients. Latterly, it has been used to measure the levels of myeloperoxidase (MPO) and neutrophil elastase (NE) in the serum of RA patients. SAA, MPO, and NE ELISA kits were purchased from Shanghai Xinle Biotechnology Company. Each test was conducted according to the manufacturer's recommendations and has been repeated at least three times.

Immunohistochemistry

All the synovial membrane sections (5 μm thick) were dewaxed, hydrated, and incubated for 1 hour at room temperature with 5% bovine serum albumin (BSA, Amresco). The slides were then incubated overnight at 4˚C with the rabbit anti-human TLR4 antibody (Abcam, UK) at 1:100 dilution. After washing with PBS 3 times, the slides were incubated with HRP anti-rabbit IgG secondary antibody (ZSGB-BIO) for 1 hour. The chromogenic reaction was developed with diaminobenzidine (DAB, Beijing Conway Century Company) for 5 - 10 min. The sections were counterstained with hematoxylin, dehydrated, and mounted. Sections were observed using light microscopy (Nikon E400 in Japan) with original magnification ×10.

Relative quantitative detection of DNA in serum by fluorescence

All specimens were of early morning fasting venous blood. After centrifugation, the serum was separated into the centrifuge tube and placed in refrigerator at −80˚C for freezing. The specimens were sequentially added to a black 96-well plate for chemiluminescence, with 100 μl diluted solution per hole. We added DAPI dye with 50 μl per hole avoiding light. After mixing, the plate was tested with biotek multifunction enzyme marker. (Two results with more than 20% variation rate were discarded).

Immunofluorescence detection of the presence of NETs in the synovial tissue of RA patients

All the synovial specimens from RA and OA patients (5 μm thick) were dewaxed, hydrated, and dried for more than 2 hours in a constant temperature oven at 60˚C, then the proteins were taken out and dyed after solidification. We added 1:300 diluted rabbit anti-human neutrophil elastase antibody (diluted with 5% BSA solution), incubated overnight at 4˚C then 1:200 diluted Rhodamine (TRITC; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) labeled goat anti-rabbit IgG antibody for 1 h at room temperature in the dark. After DAPI dye solution removal, we observed immediately the results under a fluorescent microscope.

DNA fluorescence staining to compare NETs formation in RA and HC sera

We diluted the extracted neutrophils from RA patients and healthy control to 3×10^5 cells/l in a 24-well plate divided into 3 groups (HC, LPS, and SAA groups), and incubated at 37˚C in a 5% CO_2 cell incubator for 30 min, then 4 h, successively. After the fixation process and staining, a Nikon upright fluorescence microscope (Japan, NIKON Co., Ltd) was used to observe each cell slide at 200 times magnification. Finally, we randomly select 10 fields of view, count the number of NETs and the total number of cells in the field of view, and calculate the percentage of NETs.

DNA fluorescence staining to investigate the mechanism of NETs formation induced by SAA stimulation

We inoculated the extracted healthy human peripheral blood neutrophil suspension adjusted to a concentration of 3×10^5 cells/l into a 24-well plate. The 24-well plate was divided into six groups: negative control group, SAA group, (SAA + TAK-242) group, LPS group, (LPS + anti-TAK-242) group, and TAK-242 group, then we incubated them at 37˚C in a 5% CO_2 cell incubator for 30 min, then 4 h, successively. We covered each slide with the primary rabbit anti-human neutrophil elastase antibody (Abcam, UK), incubated overnight at 4˚C. Then, we added TRITC-labeled goat anti-rabbit secondary antibody and incubated in the dark at room temperature for 1 h. After DAPI removal, a Nikon upright fluorescence microscope was used to observe each cell slide at 200 times magnification.

Detection and analysis of SAA-induced NETs formation in the supernatant (DNA testing)

First, we dissolved 1mg calf thymus DNA in 20 ml enzyme-free water, shook slowly in a refrigerator at 4˚C overnight. We further prepared DNA standard solutions using five 1.5 ml centrifuge tubes and labeled them as 1, 2, 3, 4, and 5, with an adjusted DNA concentration of 160 ng/ml using a standard diluent, in the tube No. 1. After, 200 μl of standard dilution was progressively added to an equal volume of each previous tube DNA standard solution to obtain half concentration. Using a black 96-well plate, we successively added 100 μl of standard diluent to the blank wells, 100 μl of DNA standard solutions of various concen-

trations to the standard wells, and 100 μl of supernatants from each group to the experimental wells: negative control group, SAA group, (SAA + TAK-242 group, LPS group, (LPS + TAK-242) group and TAK-242 group. Then, 100 μl of Pico-Green (American Invitrogen Company) working dye solution was added to each well and shook for 10 min at room temperature away from light. Finally, a multifunctional microplate reader (BioTek Synergy2, American Biotek Company) was used to measure the fluorescence intensity, and the DNA content in the supernatant was calculated.

DNA fluorescence staining to explore whether NETs formation induced by SAA was dependent or independent on NADPH oxidase pathway

Adjust the concentration of the extracted healthy human peripheral blood neutrophil suspension to 3×10^5 cells/l, and pipette 500 μl of the cell suspension into 24-wells, and divide it into four groups: SAA group, LPS group, (SAA + apocynin) group, (SAA + VAS2870). Each group has three auxiliary holes. The final concentration of SAA was 25 mg/l, and the final concentration of LPS was 1 mg/l. Apocynin and VAS2870 are NADPH oxidase inhibitors and their concentrations were 100 μM and 5 μM, respectively. Place the 24-well plate again in a 37˚C 5% CO_2 cell incubator for 4 h. After the fixation, cover each slide with primary rabbit anti-human neutrophil elastase antibody, and incubate overnight in a refrigerator at 4˚C. Then, add TRITC-labeled secondary goat anti-rabbit antibody. After DAPI removal, the results were observed with a Nikon upright fluorescent microscope.

Isolation of neutrophils

The isolation of neutrophils was performed using a density gradient centrifugation system. First, 3ml Histopaque-1119 (Sigma-Aldrich, Germany) was added into a 1.5 ml centrifuge tube, followed by an equal addition of 3 ml Histopaque-1077 on the top of Histopaque-1119. Then, 6 mL of anticoagulated whole blood was added above the liquid level of Histopaque-1077, and centrifuge at 700 × g for 30 minutes at room temperature. After centrifugation, we removed the plasma layer and transferred the neutrophil layer and a small amount of liquid under it to another new 1.5 ml centrifuge tube in a phenol red-free 1640 medium (Corning Company) and centrifuge at 450 × g for 5 min at room temperature. The remaining red blood cells were lysed (Beijing Soleibao Technology Co., Ltd) at room temperature for 2 - 3 min, and we centrifuged again at 450 × g for 5 min at room temperature. After discarding the supernatant, the neutrophils were washed and centrifuged at 450 × g for 5 min at room temperature, twice. Take 10 μl of cell suspension and count the number of cells in 4 large squares, and calculate the cell concentration, using a bovine Bowman cell counting plate. HE and DAPI staining were used to observe the percentage of neutrophils and the nuclei morphology, respectively.

Induction and extraction of NETs

We added 1 ml of cell suspension with neutrophils density at 1×10^6 cells/ml to each of the 6-well plate, placed in a 37˚C 5% CO_2 cell incubator (American Thermo Company) for 40 min. Then, we added SAA (25 mg/l; Sigma-Aldrich,

Germany) to 3 wells, and added solvent control to the remaining three wells. The culture plates were taking out after 4 h of stimulation into the incubator. DAPI working dye solution was added, and NETs formation was observed under a fluorescence microscope at 200 times magnification. After randomly select 10 fields of view, we calculated the percentage of NETs formed, according to the count number of NETs over the total number of cells in the field of view. After washing the bottom of the plate with cold PBS solution and the addition of 1 mL of pre-warmed phenol red 1640 medium, the bottom of the plate was repeatedly pipetted to promote the dissolution of NETs liquid. We transferred the liquid to a 1.5 ml centrifuge tube, centrifuged at 350 × g at 4˚C for 10 min to remove intact cells and cell debris, and transferred the supernatant to another centrifuge tube, which was the extracted NETs.

Cell culture

Human umbilical vein endothelial cells (HUVECs) culture model was used for *in vitro* experiments. Primary human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cords via collagenase digestion, and subsequently maintained in Dulbecco's modified Eagle's medium (DMEM, American Gibco Company) supplemented with 10% FBS (Tianjin Zhaoran Biological Company) at 37˚C in an atmosphere of 5% CO_2. All HUVECs were used no more than five passages.

MTT assay

HUVECs (100 μl) were seeded in 96-well plates at a density of 3×10^5 cells/well in complete DMEM medium until the cells reached 50% - 60% confluency. The cells were stimulated with extracted NETs (0.28 mg/L) or a mixture of extracted NETs and DNaseI (10 U/mL) in a DMEM medium containing 1% FBS for 72 h. Then, a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (20 μl, 5 mg/ml) was added to each of the wells and incubated for 4 h at 37˚C. Then the dimethylsulphoxide (DMSO) (150 μl) was added to solubilize the formazan crystals. Absorbance at 490 nm was measured by an automated microplate reader.

Wound healing assay

HUVECs were seeded on to 6-well plates and grown to confluence. A sterile pipette was used to make a single scratch through the middle of each well and the injury line was marked. First, HUVECs were stimulated with extracted NETs (0.28 mg/l) in the presence or not of DNaseI (10 U/ml) in DMEM medium, supplemented with 1% fetal bovine serum. The healing area of the scratch area is calculated by Image J software, and the healing rate = (initial scratch area-current scratch area)/initial scratch area × 100%.

Tube formation assay

Tube formation was measured using a three-dimensional in-vitro model as previously described [8], with some minor modifications. A type I collagen from rat tail tendon (50 μl, Shengyou Biological Technology Co., Ltd, Jiangsu, China), and 190 μl of HUVECs suspension (2.0×10^5 cells/ml) were added to 96-well culture plates and allowed to polymerize for 20 min at room temperature. Then,

the DMEM medium was added to each well, and the cells were stimulated with extracted NETs (0.28 mg/l) in the presence or absence of DNaseI (10 U/ml). Then, the endothelial cells tube formation was visualized using an inverted microscope (Olympus, Japan) and photographed at 24, 48, and 72 h. At least three biological replicates were done for the evaluation of cell angiogenesis ability.

Statistical analysis

All data were analyzed using SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA), and GraphPad Prism 5 software was used for graphing. All data are measured by mean ± SD. Comparisons between the two groups were performed using t-test, comparisons between multiple groups were performed using single-factor analysis of variance, and multiple comparisons between groups were performed using Dunnett's T test. The test level was two-sided $\alpha = 0.05$.

3. Results

Increased expression of the TLR4 receptor in the synovial membranes, and elevated serum levels of SAA, in RA patients.

To identify the expression of TLR4 in RA synovium, immunohistochemistry was performed on tissue sections. The results showed that TLR4 expression was remarkably detected in all RA tissue samples compared to a minimal expression in all OA tissue sections. The positive staining of TLR4 receptor staining (dark brown) was mainly distributed in the synovial lining and underlining layers, as well as the inflammatory cells (synovial fibroblasts, macrophages, and endothelial cells), and was also found around blood vessels (**Figure 1(a)** and **Figure 1(c)**). Meanwhile, in the synovial tissue of OA patients, TLR4 receptor staining was scattered only in the synovial lining and fibroblasts, with lower expression and lighter coloration (**Figure 1(b)** and **Figure 1(d)**). Immunostaining with an isotype-matched control IgG was utterly negative in all samples examined. Besides, an ELISA assay was performed to detect serum levels of SAA. Data were analyzed using one-way ANOVA. The results revealed that there was a statistically significant difference among the serum levels of SAA in RA and OA groups (6.63 ± 0.21 mg/l vs. 1.40 ± 0.09 mg/l, $p < 0.001$) (**Figure 1(e)**).

The levels of MPO and NE in the serum of RA patients increase, and the level of DNA decreases

Serum MPO levels were measured by ELISA. Compared to healthy controls, the serum level in RA patients (4.845 ± 0.6428 mg/l) was significantly higher than those in healthy controls (1.050 ± 0.1202 mg/l, $p < 0.0001$), about 4 times as high (**Figure 2(a)**). The serum levels of NE in RA patients was 1.992 ± 0.07389 mg/l, and healthy volunteers was 1.707 ± 0.06374 mg/l, $p < 0.0001$; and the difference between the two groups was also statistically significant (**Figure 2(b)**). In the same batch of serum, the relative fluorescence quantitative results of DNA showed that the absolute free DNA content in serum of RA group (RA 17.51 ± 1.319 RFU) was significantly lower than that in healthy controls group (22.68 ± 0.9035 RFU, $p < 0.01$) as indicated in **Figure 2(c)**.

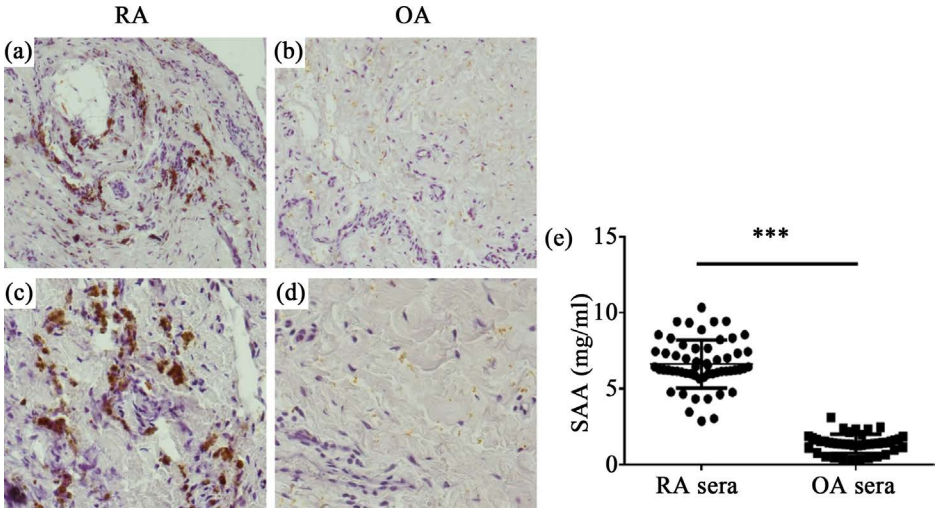

Figure 1. Increased expression of TLR4 in the SM, and of SAA in RA serum. SM sections from RA (n = 8) and OA patients (n = 6) were stained with anti-TLR4 antibodies. A brown stain indicated cells positive for TLR4. In RA patients TLR4 expression was observed in synovial vascular endothelial cells, perivascular areas, and inflammatory cells (a) and (c). Meanwhile, lighter staining of perivascular areas and fibroblast like synovial cells was observed in OA tissue (b) and (d). (e) ELISA analysis of serum SAA in RA and OA patients (n = 57 and 43, respectively). ***p < 0.001 versus RA SAA concentration. Magnification: (a) and (b), ×100; (c) and (d), ×200.

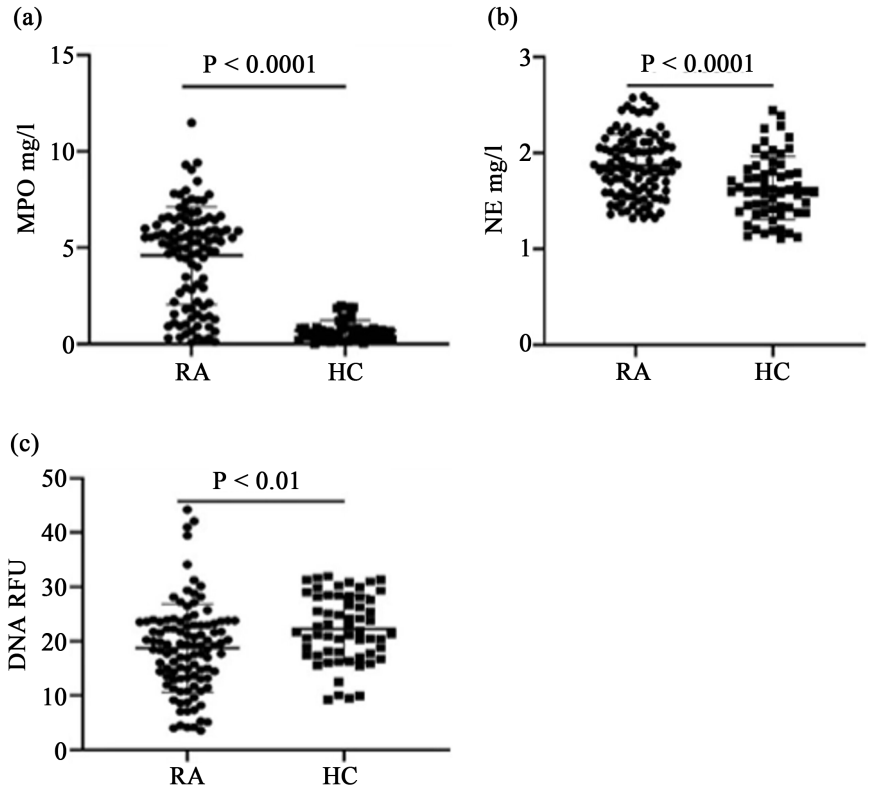

Figure 2. Serum levels of MPO, NE and DNA. (a) Serum levels of MPO in RA patients were significantly higher than those in HC ($p < 0.0001$). (b) Serum levels of NE in RA patients were significantly higher than those in HC ($p < 0.0001$). (c) Serum DNA levels in RA were significantly decreased compared to those of HC ($p < 0.01$). RA, rheumatoid arthritis; HC, healthy control; MPO, myeloperoxidase; NE, neutrophil elastase; DNA, deoxyribonucleic acid; RFU, relative fluorescence unit.

SAA levels were associated with MPO and NE, which correlated with RA disease activity

We examined the relationship between SAA, MPO, and NE. The results showed that SAA was positively correlated with MPO ($R^2 = 0.548$, $p < 0.0001$) and NE ($R^2 = 0.131$, $p < 0.0001$), as shown in **Figure 3(a)** and **Figure 3(b)**.

Moreover, the results showed that serum levels of MPO and NE in RA patients were significantly higher than those in healthy controls. However, did their levels correlate with disease activity? So, we examined the relationship between MPO, NE and RA disease activity. As well, the relationship between MPO, NE and autoantibodies (anti-CCP, RF-IgA; RF-IgG) was examined. MPO was positively correlated with DAS28 ($R^2 = 0.296$, $p < 0.0001$) and ESR ($R^2 = 0.235$, $p < 0.0001$), but weakly correlated with CRP ($R^2 = 0.071$, $p = 0.006$) as indicated in **Figures 3(c)-(e)**, respectively. However, there was no significant correlation between MPO and anti-CCP ($R^2 = 0.005$, $p = 0.477$), RF-IgG ($R^2 = 0.005$, $p = 0.459$), and RF-IgA ($R^2 = 0.007$, $p = 0.393$), in RA patients (**Figures 3(f)-(h)**).

Besides, NE was positively correlated with DAS28 ($R^2 = 0.093$, $p = 0.0015$) as shown in **Figure 3(k)**. However, there was no significant correlation between NE and ESR ($R^2 = 0.03$, $p = 0.06$) (**Figure 3(j)**), CRP ($R^2 = 0.01$, $p = 0.18$) (**Figure 3(i)**), anti-CCP ($R^2 = 0.006$, $p = 0.397$), RF-IgG ($R^2 = 0.002$, $p = 0.589$), and RF-IgA ($R^2 = 0.0046$, $p = 0.487$) (**Figures 3(l)-(n)**).

The presence of NETs was detected in the synovial tissues of RA patients

Tissue immunofluorescence staining used rhodamine (TRITC) to label NE in red, and DAPI to stain DNA in blue. Under normal circumstances, histones and DNA together form chromatin and are located within the nucleus. Since neutrophil elastase (NE) is an important participant and main component in the formation of NETs [36], immunofluorescence was used to observe the overlap of NE staining and DNA staining under a fluorescence microscope. When NETs are formed, MPO and NE translocate into the nuclei, in which they synergize their action and promote the chromatin decondensation [37]. Eventually, the nuclear membrane and cell membrane are ruptured, and NE and DNA are released outside the cell. That is, the co-localization of NE and DNA indicates the existence of NETs (**Figure 4**). The results showed that there was obvious NETs formation in RA synovial tissue, while no NETs release was observed in OA synovial tissue.

Neutrophils of RA patients are primed to undergo NETs formation and SAA stimulation enhanced this process

The DNA fluorescence staining was performed to assess the NETs formation after SAA stimulation. It was found that the nucleus morphology of some neutrophils was destroyed with the disappearance of boundaries of the nuclear membrane that became blurred, and the nucleus spread out to form a fiber network structure (**Figure 5(a)**). The results showed that the spontaneous NETs formation was higher in the RA patients group compared to the HC group ($7.394\% \pm 0.5270\%$ vs. $4.85\% \pm 0.3659\%$, $p < 0.05$). As well, in SAA stimulated

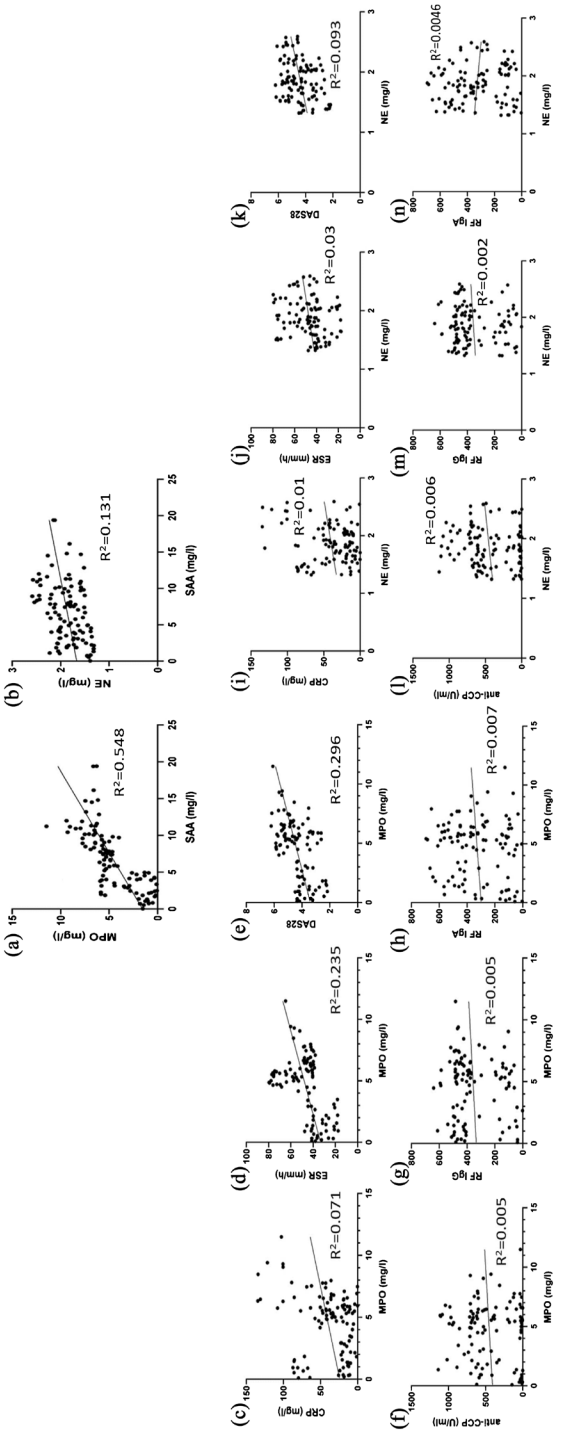

Figure 3. Scatter plots demonstrating the correlations of SAA levels with MPO, NE and the association of MPO and NE with disease activity in rheumatoid arthritis.: (a) SAA levels with MPO ($R^2 = 0.548$; $p < 0.0001$); (b) SAA with NE ($R^2 = 0.131$; $p < 0.0001$); (c) Serum MPO levels with CRP ($R^2 = 0.071$; $p = 0.0059$); (d) MPO with ESR ($R^2 = 0.235$; $p < 0.001$); (e) MPO with DAS28 score ($R^2 = 0.295$; $p < 0.0001$); (f) MPO with anti-CCP ($R^2 = 0.005$; $p = 0.47$); (g) MPO with RF IgG ($R^2 = 0.005$; $p = 0.45$). (h) MPO with RF IgA ($R^2 = 0.007$; $p = 0.39$); (i) Serum NE levels with CRP ($R^2 = 0.01$; $p = 0.18$); (j) NE with ESR ($R^2 = 0.03$; $p = 0.06$); (K) NE with DAS28 score ($R^2 = 0.093$; $p = 0.0015$); (l) NE with anti-CCP ($R^2 = 0.006$; $p = 0.39$); (m) NE with RF IgG ($R^2 = 0.002$; $p = 0.58$). (n) NE with RF IgA ($R^2 = 0.0046$; $p = 0.48$). SAA, serum amyloid A; MPO, myeloperoxidase; NE, neutrophil elastase; DAS28, disease activity score for 28 joints; ESR, erythrocyte sedimentation rates; CRP, C reactive protein; anti-CCP, anti-cyclic citrullinated peptide; RF, rheumatoid factor; IgG, immunoglobulin G; IgA, immunoglobulin A; R^2, correlation coefficient.

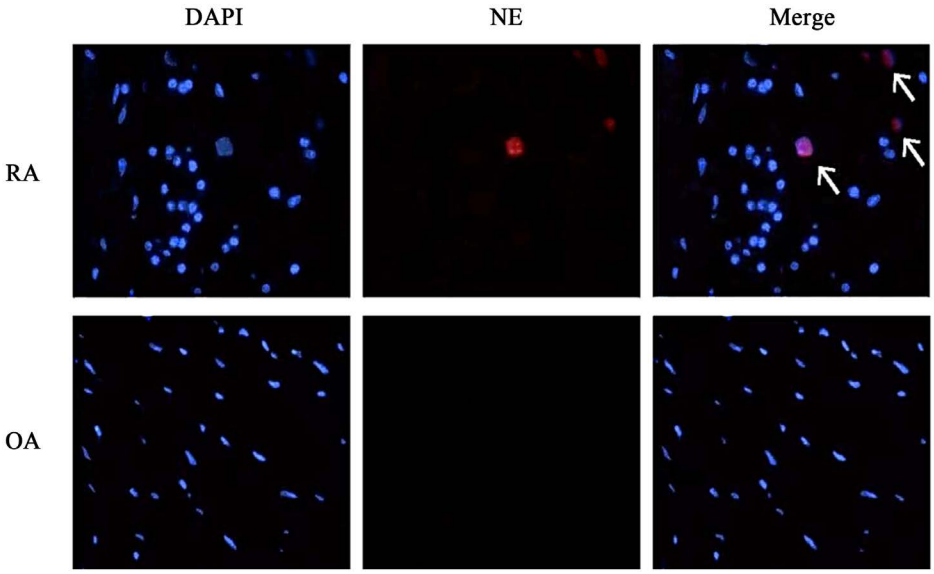

Figure 4. Presence of NETs in RA synovial tissues. Tissue immunofluorescence staining uses rhodamine (TRITC) to label NE in red, and DAPI to stain DNA in blue. NETs were observed in RA synovial tissue with the co-localization of DNA and NE, while no NETs were unobserved in OA synovial tissue. Original magnification ×200.

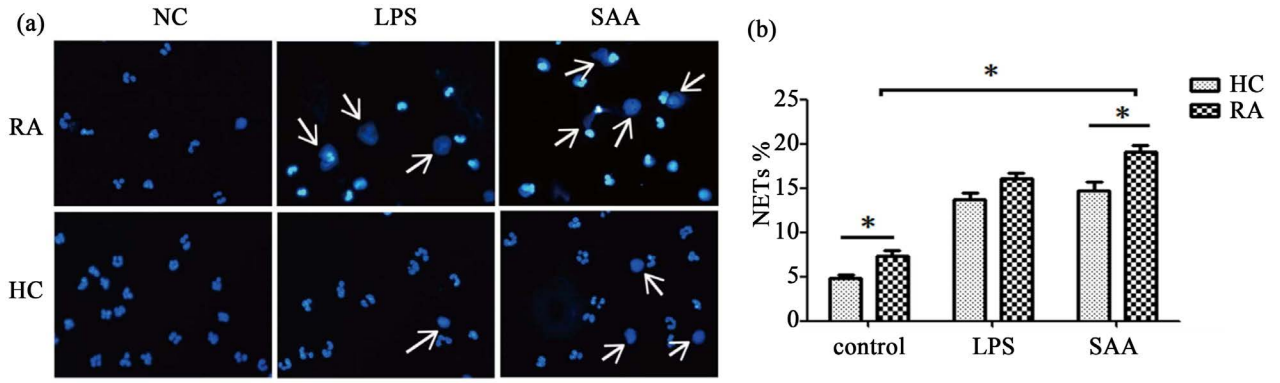

Figure 5. Spontaneous NETs formation and enhanced NETs expression under stimulation in RA patients. (a) Neutrophils in RA patients were primed to form NETs with an enhanced expression after LPS or SAA stimulation. DAPI was used to stain neutrophils' nuclei in blue and the arrows indicated the web-like structures of the forming NETs. (b) Statistical measurements of NETs in each group. In a basal condition, NETs expression in RA group was statistically higher than that of HC group; Under LPS or SAA stimulation, the formed NETs were higher than that in HC. However, compared with LPS group, the NETs expression induced in SAA was significantly higher. The data were expressed as the percentage of NETs; *$p < 0.05$.

neutrophils group, the amounts of NETs formed in the RA patients group were significantly elevated than those of the HC group (19.10% ± 0.7922% vs. 14.67% ± 1.078%, $p < 0.05$) (Figure 5(b)). Those results suggest that, in RA patients, neutrophils were primed to form NETs, and SAA stimulation potentiated this effect.

SAA can induce the formation of NETs and increased total DNA concentration through its interaction with TLR4

The isolated neutrophils accounted for more than 95% of all cells (data not shown). Since neutrophil elastase (NE) is an important participant and main component in the formation of NETs [36], immunofluorescence was used to observe the overlap of NE staining and DNA staining under a fluorescence mi-

croscope. The results showed that some neutrophils lost their original lobular nucleus or rod-shaped nucleus, and the DNA loosely diffused to form a fibrous network (blue), and there were NE staining positive parts (red), namely NETs (partial Purple); while the blue nucleus in the picture, surrounded by the red NE staining positive part, were considered normal neutrophils (Figure 6(a)). Compared

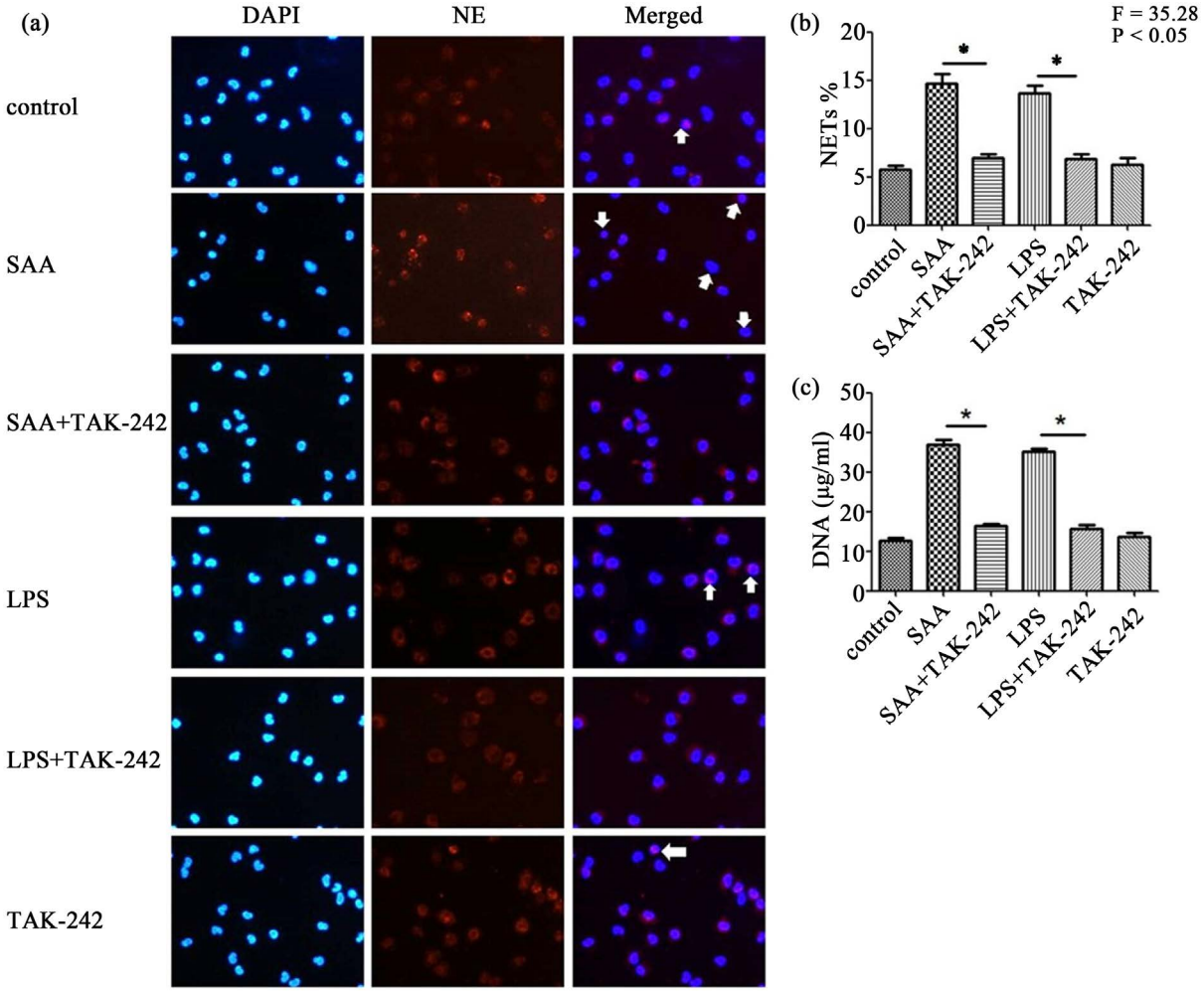

Figure 6. SAA induced NETs formation and increased DNA concentration was mediated by TLR4 activation. (a) immunofluorescence of NETs formation into the different groups. The overlap of NE staining (red) and DNA staining (blue) was used to represent the formed NETs. As indicated by the arrows, some neutrophils lost their original lobular nucleus or rod-shaped nucleus, and the DNA loosely diffused to form a fibrous network (blue), and there were NE staining positive parts (red), namely NETs (partial Purple), which were seen in the SAA and LPS groups; while the blue nuclei in the picture, surrounded by the red NE staining positive part, were normal neutrophils, more pronounced in (SAA+TAK-242) and (LPS+TAK-242). (b) quantitative measure of NETs formed into the different groups. Compared with the control group (5.734% ± 0.428%), the SAA stimulation group (14.58% ± 1.058%) significantly increased the formation of NETs ($p < 0.05$). Whereas, the pretreatment of neutrophils with TAK-242, and despite the same stimulation by SAA, the formation of NETs was significantly reduced (6.915% ± 0.418% *vs.* 14.58% ± 1.058%, $p < 0.05$). Similarly, the (LPS+TAK-242) group (6.905% ± 0.4646%) compared with the LPS group (13.71% ± 0.7951%), the formation of NETs was significantly reduced ($p < 0.05$). (c) DNA quantification in the neutrophils supernatants. There was a significantly lower DNA concentration in the culture supernatant of SAA group pretreated with TAK-242 (16.34 ± 0.611 μg/ml) compared with the SAA group (36.89 ± 1.288 μg/ml) ($p < 0.05$); As well, similar results were found in the LPS group (35.23 ± 0.7689 μg/ml) and (LPS+anti-TAK-242) group (15.77 ± 0.8893 μg/ml) ($p < 0.05$). There was no statistically significant change in the TAK-242 group and the control group ($p > 0.05$).

with the control group (5.734% ± 0.428%), the NETs formed in the SAA stimulation group (14.5% ± 1.058%) were significantly increased ($p < 0.05$). But after pretreatment with TAK-242, and despite the same stimulation by SAA, the formation of NETs was significantly reduced (6.915% ± 0.418% vs. 14.58% ± 1.058%, $p < 0.05$) (**Figure 6(b)**).

Then, the PicoGreen DNA quantification was used to determine the DNA concentration in the supernatants of neutrophils. The results showed that the SAA stimulation group (16.34 ± 0.611 μg/ml) after adding TAK-242, and compared with the SAA group (36.89 ± 1.288 μg/ml) had significantly lower DNA concentration in the culture supernatant ($p < 0.05$). Similar results were found in the LPS group (35.23 ± 0.7689 μg/ml) and (LPS+TAK-242) group (15.77 ± 0.8893 μg/ml) ($p < 0.05$). However, there was no statistically significant change in the TAK-242 group and the control group ($p > 0.05$) (**Figure 6(c)**).

SAA/TLR4 induced the formation of NETs through the dependent NADPH oxidase (NOX) pathway

DNA fluorescence staining was used to determine whether SAA/TLR4 induced NETs formation was independent or dependent on NADPH pathway. The results showed that there was a significant decreased NETs formation when neutrophils were stimulated with SAA in presence of NADPH oxidase inhibitors (SAA + apocynin or SAA + VAS2870; $p < 0.0001$) compared to SAA alone (**Figure 7(a)** and **Figure 7(b)**). Similarly, there was a significant decreased serum DNA levels when neutrophils were stimulated with SAA in presence of NADPH oxidase inhibitors (SAA + apocynin or SAA + VAS2870; $p < 0.0001$) compared to SAA alone (**Figure 7(c)**).

NETs induced the proliferation, migration, and tube formation of HUVECs

The MTT assay was used to explore the effect of NETs on the proliferation of endothelial cells. First, the neutrophils were isolated from the healthy volunteers, and the extracted NETs formation used in the experiment were induced *in vitro*. The results showed that compared with the control group, NETs could significantly promote the proliferation of HUVECs (1.241 ± 0.068 vs. 0.405 ± 0.445, $p < 0.05$). After pretreatment of NETs with DNaseI, their proliferation ability on HUVECs was abrogated (0.919 ± 0.056 vs. 1.241 ± 0.068, $p < 0.05$), but there was still an ability to promote endothelial cell proliferation (0.919 ± 0.056 vs. 0.405 ± 0.445, $p < 0.05$) (**Figure 8(a)**).

Afterward, the scratch assay was applied to detect the influence of NETs on the migration of HUVECs. The cell migration in each group was observed under an inverted microscope after 12 h of stimulation, and the scratch healing rate of each group was quantitatively analyzed. The results showed that compared with the control group (basal), the cells in the NETs (0.28 mg/l) stimulation group migrated significantly, and the cell scratch was almost completely repaired (78.43% ± 1.784% vs. 16.57% ± 1.066%, $p < 0.05$). Whereas, after pretreatment of NETs with DNaseI for 10 min, followed by co-incubation with HUVECs, the cells' migration was significantly reduced (44.57% ± 1.798% vs. 78.43% ± 1.784%, p

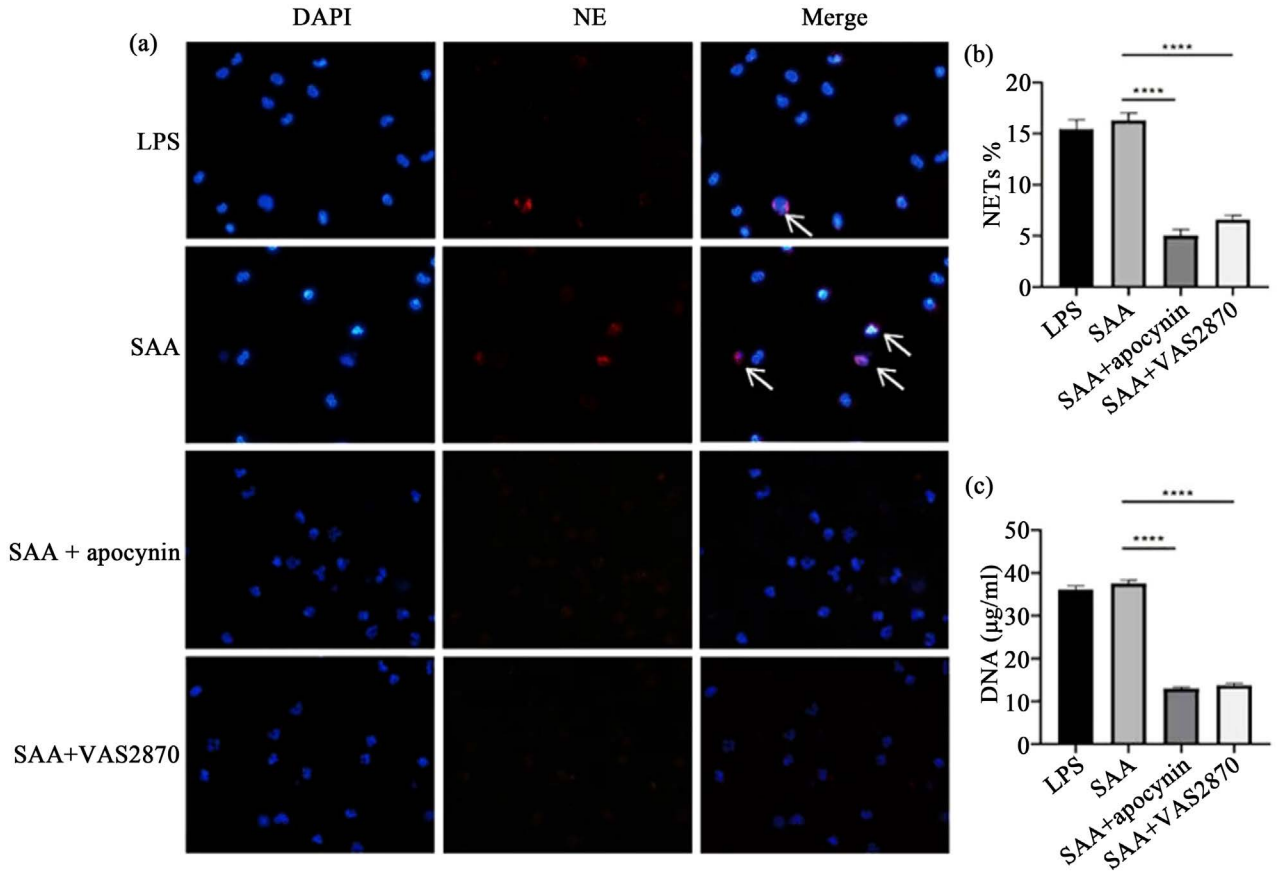

Figure 7. Pathway analysis of NETs formation. (a) Morphologic observation of neutrophils exposed to stimulants in presence or not of NADPH inhibitors (apocynin or VAS2870). (b) Statistical analysis of NETs formation in the different groups. (c) Statistical analysis of DNA serum levels in the different groups. DAPI, 4', 6-diamidino-2-phenylindole; NE, neutrophil elastase; LPS, lipopolysaccharide; SAA, serum amyloid A. **** ($p < 0.0001$).

< 0.05), but there was still a residual ability of endothelial cells to migrate (44.57% ± 1.798% vs. 16.57% ± 1.066%, $p < 0.05$) (**Figure 8(b)** and **Figure 8(c)**). What's more, the tube formation assay was used to analyze the effect of NETs on the morphological angiogenesis process of HUVECs. The vascular tubule formation *in vitro* was observed after 72 h of stimulation using an inverted microscope. The results showed that in the NETs (0.28 mg/l) stimulation group, there were obvious interconnections between cells to form tubules, and the tubules interconnected to form a complex network structure, suggesting that NETs can promote vascular tubules formation (2.857 ± 0.3401 vs. 1.143 ± 0.4041, $p < 0.05$). However, following NETs pretreatment (DNaseI), the tube formation was nearly unobserved (1.571 ± 0.3689 vs. 2.857 ± 0.3401, $p < 0.05$) (**Figure 8(d)** and **Figure 8(e)**). Together, these results indicated that DNA components in NETs play an important role in the promotion of endothelial cells' proliferation, migration, and vascular tube formation.

4. Discussion

Angiogenesis is one of the most important pathological manifestations of the

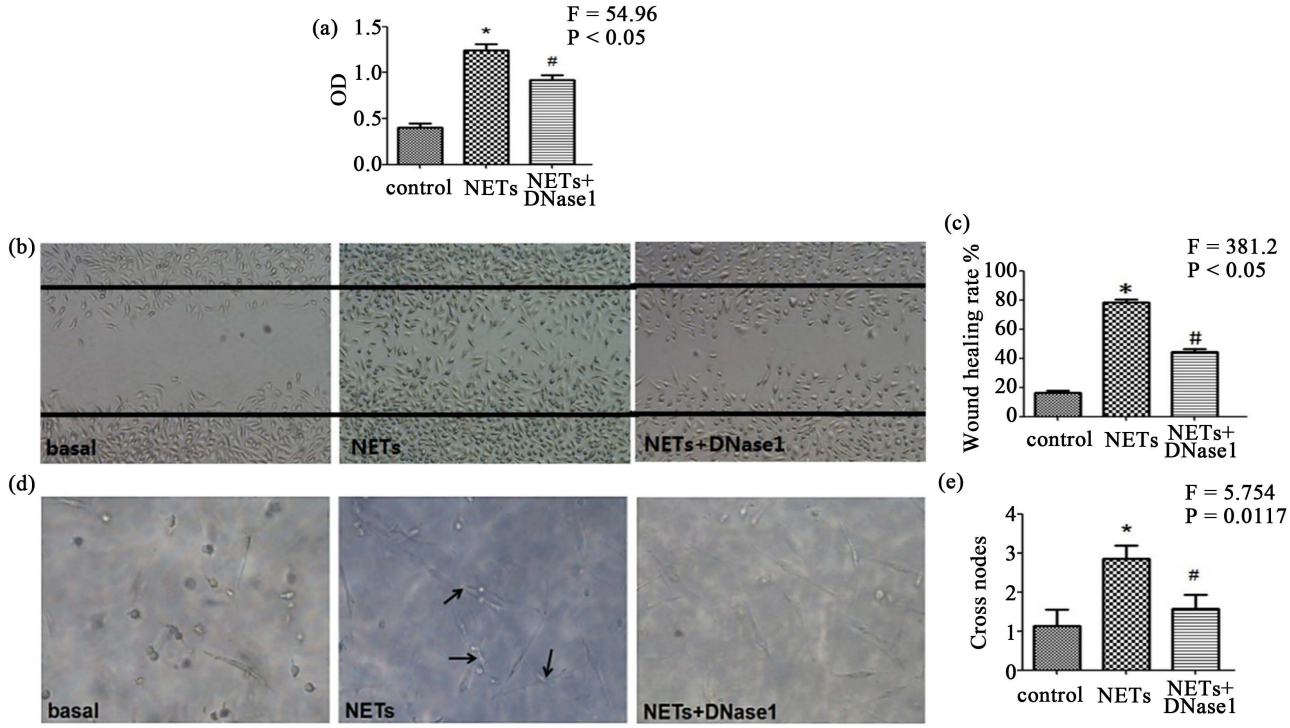

Figure 8. NETs induced the proliferation, migration and the vascular tube formation of HUVECs. (a) NETs induced endothelial cells proliferation *in vitro*. Significant increases in proliferation of HUVECs stimulated with NETs (0.28 mg/l) were showed after 72 hours of incubation, while a significant decreases were observed after the addition of a mixture of extracted NETs and DNaseI (10 U/ml) for the same time of incubation. The data were expressed as the optical density (OD) measurement at 490 nm; *compared with control group, $p < 0.05$ versus #compared with NETs group, $p < 0.05$. (b) NETs induced endothelial cells migration *in vitro*. The representative photomicrograph showed HUVECs repopulating the wound in response to NETs (0.28 mg/l). However, the pre-treatment with DNaseI (10 U/ml) displayed a significantly decreased migration of HUVECs. Original magnification: ×4. (c) Statistical results of the figure B; the data were expressed as the healing rate = (initial scratch area-current scratch area)/initial scratch area × 100%; *compared with control group, $p < 0.05$ versus #compared with NETs group, $p < 0.05$. (d) NETs induced HUVECs to form vascular tubes network. The representative photomicrograph showed that NETS (0.28 mg/l) induced HUVECs to form small complex interconnected tube vessels network over 72 hours of incubation, while they lack to interweave after their pre-treatment with DNaseI (10 U/ml). The arrows indicate the structure of blood vessels formed *in vitro* as the number of cross-nodes. Original magnification: ×10. (e) Statistical results of the figure D; the data were expressed as the number of cross nodes; *compared with control group, $p < 0.05$ versus #compared with NETs group, $p < 0.05$.

joint disorder in RA patients. In the current work, we confirmed the high expression of TLR4 in the synovial membranes, and elevated serum levels of SAA, in RA patients. Subsequently, our results indicated that SAA was associated to NETs components which correlated with disease activity. Moreover, neutrophils in RA were primed to undergo NETs formation. Latterly, we demonstrated NETs formation in ST of RA patients. Then, we indicated that SAA/TLR4 induced NETs formation and SAA-induced NETs formation was dependent on NADPH pathway. Finally, we demonstrated that extracted NETs could induce angiogenesis.

We first showed that TLR4 was highly expressed in the synovial tissues of RA patients compared to OA patients. A previous study also reported that TLR4 was widely expressed in early and longstanding RA [38]. Moreover, as we previously demonstrated [39], this current research also showed that serum levels of SAA in

RA patients were significantly increased compared to those of the OA patients. As for the specific role of SAA and TLR4 in RA, there was still a lack of systemic reports, and collectively, those observations may also suggest that excessive production of SAA and TLR4 could play an important role in RA pathogenesis.

Afterwards, we reported an increased MPO and NE levels in the sera of RA patients, and a relatively decreased DNA levels compared to HC group. Set apart, the levels of MPO and NE in RA were seen to be correlated to the disease activity. In addition, SAA serum levels were seen to correlate with MPO and NE serum levels. Neutrophils represent the first line of immune response that rapidly immigrates to the site of inflammation and/or infection [40]. Also, NE and MPO are two of the primary enzymes stored in azurophilic granules and are found in abundance in NETs [41]. Corroborating our data, Sur Chowdhury C *et al.* demonstrated elevated concentrations of NE and MPO in RA patients [42], and those results could be explained by the increased formation of NETs. However, we found out a decreased serum DNA levels that were similar to the results of Marina D *et al.* [43]. Paradoxically, other studies showed a substantial increase in cell-free DNA (cfDNA) concentration in plasma or serum of RA patients [44] [45]. These disparencies data on serum cfDNA levels in RA may at least in part be explained by the use of different techniques (for example, qPCR, ELISA or UV-spectrophotometry) and the different DNA targets chosen to perform the analyses. In addition, differences in the state of disease progression and/or activity at the time of sampling may have affected the results, as indicated by the differences observed for early RA (eRA) and established RA (esRA) [43]. It is also tempting to speculate on the role of drugs used for treatment, as it was previously indicated reduced levels of cfDNA in esRA patients, but not in eRA patients [43].

On the other hand, our results indicated a positive correlation of SAA with MPO and NE, which were all correlated with RA disease activity. C. Perez-Sanchez *et al.* reported that NETs and NETs-derived products as potential biomarker to assess disease activity [14]. In their study, they indicated that NETs formation was higher in RA patients along with increased myeloperoxidase and neutrophil elastase protein levels. In a similar way, Bach, M *et al.* reported that the serum levels of NETs in RA patients were correlated with the disease activity [46]. Although there are not systemic reports on the association between SAA, MPO, and NE, taken together, all the above findings strengthen our results.

Furthermore, we showed that NETs was highly expressed in the synovial tissues of RA patients compared to OA patients. Besides, consistent with our results, netting neutrophils in joints were also previously found in patients with RA [14] [15], demonstrating an active and widespread NETosis in this context. In RA, netting neutrophils were showed to easily infiltrate the synovial tissues [15] [47].

What's more, we demonstrated that in RA, neutrophils were primed to undergo NETs formation, and the NETs formation was enhanced under SAA stimulation. Our results are consistent with the results of Sur Chowdhury *et al.* re-

search in which neutrophils from RA cases exhibited increased spontaneous NETs formation *in vitro* [42]. As well, Wang, W *et al.* suggested in their study that the disease status of RA is associated with increased NETs formation [13], and also reported that isolated neutrophils from RA patients showed higher spontaneous NETs formation than neutrophils isolated from HC [13]. Thus, this priming may be due to the inflammation state in RA patients that keep the neutrophils in a pre-activated state, which makes them more likely to form NETs. For instance, antibodies were shown to participate in the effects of RA serum on normal neutrophils following the depletion of IgG from serum of ACPA-positive and -negative RA patients and healthy controls. As results, compared with non-depleted sera, IgG depletion of both ACPA-positive and -negative sera markedly reduced NETs induction to levels of normal serum [42]. Moreover, a study showed that the level of NETs induction by IL-17 in RA neutrophils was comparable to induction observed by positive control (PMA). However, in control neutrophils, IL-17A itself could not significantly enhance NETs formation, whereas, upon priming with TNF-α, recombinant IL-17A led to significant increased NETs formation [15]. Those observations are in accordance with our results and strengthen our assumption that SAA enhances NETs formation in RA patients.

Moreover, our results revealed that SAA can induce the formation of NETs by combining with TLR4. Then we demonstrated that SAA-induced NETs formation was dependent on NADPH pathway. TLR4 has been involved in a variety of inflammatory processes leading to the sustaining of chronic inflammatory state observed in immune and non-immune diseases [48] [49] [50] [51] [52]. TLR4 is expressed by cells within the RA joint [27] [38], and various endogenous TLR4 ligands are present within the inflamed joints of patients with RA. Some authors reported that TLR4 activation significantly up-regulated the expression of pro-inflammatory cytokines such as IL-1β, IL-6, and IL-8, which may exert direct angiogenic activity or may act indirectly via VEGF-dependent pathways [53]. Furthermore, a study demonstrated the ability of IL-1β, and IL-8 in eliciting oxidative burst and NETs formation, which were inhibited by NADPH oxidase and myeloperoxidase inhibitors [54]. Similar to our results, previous authors determined an induced dose-dependent Nox-dependent NET formation through LPS binding to TLR4 on the neutrophil surface and that effect was abolished using TAK-242 [31]. Collectively, these data strengthen our assertion that SAA could induce NETs formation through its binding to TLR4, however we still don't know whether the NETs formation induced by SAA was in a lytic or a non-lytic way. Subsequently, further studies are needed to delineate this mechanism.

Latterly, we demonstrated that NETs enhanced endothelial cells' migration, proliferation and induced new vascular tube formation. Our observations were in agreement with a recent study reporting that NETs release could mediate neutrophils adhesion onto ECs and exert proangiogenic activities on EC capil-

lary-like tube formation [55]. For instance, it has recently been shown that NETs mediate the proteolysis of the intercellular junction protein (vascular endothelial cadherin) and nuclear translocation of junctional β-catenin [56]. Together, these data indicated a potential role of NETs, participating in proinflammatory and proangiogenic activities in RA. Nevertheless, further studies are needed to better delineate how NETs contribute to these biological activities.

5. Conclusion

Our current study aimed to explore the molecular mechanism underlying angiogenesis in rheumatoid arthritis. SAA, TLR4 and NETs expressions were significantly increased in RA synovial tissues. Besides, SAA which associated with DAS-28, also correlated with MPO and NE, both being NETs components. In parallel, MPO and NE also correlated with DAS-28. Further, TLR4 which is also expressed on neutrophils, bound with SAA to induce NETs formation dependent on NADPH pathway. In return, the extracted SAA-induced NETs promoted angiogenesis on endothelial cells. This pivotal role of SAA/TLR4 inducing angiogenesis through formed NETs may lead to a synergism of action, and therefore, this study offers new perspectives for a better understanding of RA pathogenicity and its management.

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Ethics Committee Approval

This study has been reviewed and approved by the Ethics Committee of Tianjin Medical University (ethical approval number IRB2019-KY-147), and all subjects have signed an informed consent form. All the human material or human data were performed in accordance with the Declaration of Helsinki.

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[2017XK030605 to F.Z].

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Smolen, J.S., Aletaha, D. and McInnes, I.B. (2016) Rheumatoid Arthritis. *Lancet*, **388**, 2023-2038. https://doi.org/10.1016/S0140-6736(16)30173-8

[2] Aletaha, D., Neogi, T., Silman, A.J., Funovits, J., Felson, D.T., Bingham III, C.O., *et*

al. (2010) 2010 Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology/European League against Rheumatism Collaborative Initiative. *Arthritis & Rheumatology*, **62**, 2569-2581. https://doi.org/10.1002/art.27584

[3] Szekanecz, Z., Besenyei, T., Szentpetery, A. and Koch, A.E. (2010) Angiogenesis and Vasculogenesis in Rheumatoid Arthritis. *Current Opinion in Rheumatology*, **22**, 299-306. https://doi.org/10.1097/BOR.0b013e328337c95a

[4] Lv, M., Xia, Y.F., Li, B., Liu, H., Pan, J.Y., Li, B.B., *et al.* (2016) Serum Amyloid A Stimulates Vascular Endothelial Growth Factor Receptor 2 Expression and Angiogenesis. *Journal of Physiology and Biochemistry*, **72**, 71-81. https://doi.org/10.1007/s13105-015-0462-4

[5] Sack Jr., G.H. (2018) Serum Amyloid A—A Review. *Molecular Medicine*, **24**, Article No. 46. https://doi.org/10.1186/s10020-018-0047-0

[6] Soric Hosman, I., Kos, I. and Lamot, L. (2020) Serum Amyloid A in Inflammatory Rheumatic Diseases: A Compendious Review of a Renowned Biomarker. *Frontiers in Immunology*, **11**, Article 631299. https://doi.org/10.3389/fimmu.2020.631299

[7] Lee, M.S., Yoo, S.A., Cho, C.S., Suh, P.G., Kim, W.U. and Ryu, S.H. (2006) Serum Amyloid A Binding to Formyl Peptide Receptor-Like 1 Induces Synovial Hyperplasia and Angiogenesis. *The Journal of Immunology*, **177**, 5585-5594. https://doi.org/10.4049/jimmunol.177.8.5585

[8] Hong, C., Shen, C., Ding, H., Huang, S., Mu, Y., Su, H., *et al.* (2015) An Involvement of SR-B1 Mediated p38 MAPK Signaling Pathway in Serum Amyloid A-Induced Angiogenesis in Rheumatoid Arthritis. *Molecular Immunology*, **66**, 340-345. https://doi.org/10.1016/j.molimm.2015.03.254

[9] Radstake, T.R., Roelofs, M.F., Jenniskens, Y.M., Oppers-Walgreen, B., van Riel, P.L., Barrera, P., *et al.* (2004) Expression of toll-like receptors 2 and 4 in Rheumatoid Synovial Tissue and Regulation by Proinflammatory Cytokines Interleukin-12 and Interleukin-18 via Interferon-γ. *Arthritis & Rheumatology*, **50**, 3856-3865. https://doi.org/10.1002/art.20678

[10] Prince, L.R., Whyte, M.K., Sabroe, I. and Parker, L.C. (2011) The Role of TLRs in Neutrophil Activation. *Current Opinion in Pharmacology*, **11**, 397-403. https://doi.org/10.1016/j.coph.2011.06.007

[11] Lee, K.H., Kronbichler, A., Park, D.D., Park, Y., Moon, H., Kim, H., *et al.* (2017) Neutrophil Extracellular Traps (NETs) in Autoimmune Diseases: A Comprehensive Review. *Autoimmunity Reviews*, **16**, 1160-1173. https://doi.org/10.1016/j.autrev.2017.09.012

[12] Song, W., Ye, J., Pan, N., Tan, C. and Herrmann, M. (2020) Neutrophil Extracellular Traps Tied to Rheumatoid Arthritis: Points to Ponder. *Frontiers in Immunology*, **11**, Article 578129. https://doi.org/10.3389/fimmu.2020.578129

[13] Wang, W., Peng, W. and Ning, X. (2017) Increased Levels of Neutrophil Extracellular Trap Remnants in the Serum of Patients with Rheumatoid Arthritis. *International Journal of Rheumatic Diseases*, **21**, 415-421. https://doi.org/10.1111/1756-185X.13226

[14] Perez-Sanchez, C., Ruiz-Limon, P., Aguirre, M.A., Jimenez-Gomez, Y., Arias-de la Rosa, I., Abalos-Aguilera, M.C., *et al.* (2017) Diagnostic Potential of NETosis-Derived Products for Disease Activity, Atherosclerosis and Therapeutic Effectiveness in Rheumatoid Arthritis Patients. *Journal of Autoimmunity*, **82**, 31-40. https://doi.org/10.1016/j.jaut.2017.04.007

[15] Khandpur, R., Carmona-Rivera, C., Vivekanandan-Giri, A., Gizinski, A., Yalavarthi, S., Knight, J.S., *et al.* (2013) NETs Are a Source of Citrullinated Autoantigens and

Stimulate Inflammatory Responses in Rheumatoid Arthritis. *Science Translational Medicine*, **5**, 178ra40. https://doi.org/10.1126/scitranslmed.3005580

[16] Carmona-Rivera, C., Zhao, W., Yalavarthi, S. and Kaplan, M.J. (2015) Neutrophil Extracellular Traps Induce Endothelial Dysfunction in Systemic Lupus Erythematosus through the Activation of Matrix Metalloproteinase-2. *Annals of the Rheumatic Diseases*, **74**, 1417-1424. https://doi.org/10.1136/annrheumdis-2013-204837

[17] Aldabbous, L., Abdul-Salam, V., McKinnon, T., Duluc, L., Pepke-Zaba, J., Southwood, M., *et al.* (2016) Neutrophil Extracellular Traps Promote Angiogenesis: Evidence from Vascular Pathology in Pulmonary Hypertension. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **36**, 2078-2087. https://doi.org/10.1161/ATVBAHA.116.307634

[18] Fuchs, T.A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., *et al.* (2007) Novel Cell Death Program Leads to Neutrophil Extracellular Traps. *Journal of Cell Biology*, **176**, 231-241. https://doi.org/10.1083/jcb.200606027

[19] Kessenbrock, K., Krumbholz, M., Schonermarck, U., Back, W., Gross, W.L., Werb, Z., *et al.* (2009) Netting Neutrophils in Autoimmune Small-Vessel Vasculitis. *Nature Medicine*, **15**, 623-625. https://doi.org/10.1038/nm.1959

[20] Garcia-Romo, G.S., Caielli, S., Vega, B., Connolly, J., Allantaz, F., Xu, Z., *et al.* (2011) Netting Neutrophils Are Major Inducers of Type I IFN Production in Pediatric Systemic Lupus Erythematosus. *Science Translational Medicine*, **3**, 73ra20. https://doi.org/10.1126/scitranslmed.3001201

[21] Lande, R., Ganguly, D., Facchinetti, V., Frasca, L., Conrad, C., Gregorio, J., *et al.* (2011) Neutrophils Activate Plasmacytoid Dendritic Cells by Releasing Self-DNA-Peptide Complexes in systemic Lupus Erythematosus. *Science Translational Medicine*, **3**, 73ra19. https://doi.org/10.1126/scitranslmed.3001180

[22] Hasler, P., Giaglis, S. and Hahn, S. (2016) Neutrophil Extracellular Traps in Health and Disease. *Swiss Medical Weekly*, **146**, w14352. https://doi.org/10.4414/smw.2016.14352

[23] Neeli, I., Dwivedi, N., Khan, S. and Radic, M. (2009) Regulation of Extracellular Chromatin Release from Neutrophils. *Journal of Innate Immunity*, **1**, 194-201. https://doi.org/10.1159/000206974

[24] Lim, M.B., Kuiper, J.W., Katchky, A., Goldberg, H. and Glogauer, M. (2011) Rac2 Is Required for the Formation of Neutrophil Extracellular Traps. *Journal of Leukocyte Biology*, **90**, 771-776. https://doi.org/10.1189/jlb.1010549

[25] Abdollahi-Roodsaz, S., Joosten, L.A., Roelofs, M.F., Radstake, T.R., Matera, G., Popa, C., *et al.* (2007) Inhibition of Toll-Like Receptor 4 Breaks the Inflammatory Loop in Autoimmune Destructive Arthritis. *Arthritis & Rheumatology*, **56**, 2957-2967. https://doi.org/10.1002/art.22848

[26] Murad, S. (2014) Toll-Like Receptor 4 in Inflammation and Angiogenesis: A Double-Edged Sword. *Frontiers in Immunology*, **5**, Article 313. https://doi.org/10.3389/fimmu.2014.00313

[27] Li, X., Xu, T., Wang, Y., Huang, C. and Li, J. (2014) Toll-Like Receptor-4 Signaling: A New Potential Therapeutic Pathway for Rheumatoid Arthritis. *Rheumatology International*, **34**, 1613-1614. https://doi.org/10.1007/s00296-013-2890-1

[28] Funchal, G.A., Jaeger, N., Czepielewski, R.S., Machado, M.S., Muraro, S.P., Stein, R.T., *et al.* (2015) Respiratory Syncytial Virus Fusion Protein Promotes TLR-4-Dependent Neutrophil Extracellular Trap Formation by Human Neutrophils. *PLOS ONE*, **10**, e0124082. https://doi.org/10.1371/journal.pone.0124082

[29] Sebina, I. and Phipps, S. (2020) The Contribution of Neutrophils to the Pathogenesis of RSV Bronchiolitis. *Viruses*, **12**, Article No. 808. https://doi.org/10.3390/v12080808

[30] Tadie, J.M., Bae, H.B., Jiang, S., Park, D.W., Bell, C.P., Yang, H., *et al.* (2013) HMGB1 Promotes Neutrophil Extracellular Trap Formation through Interactions with Toll-Like Receptor 4. *American Journal of Physiology—Lung Cellular and Molecular Physiology*, **304**, L342-L349. https://doi.org/10.1152/ajplung.00151.2012

[31] Khan, M.A., Farahvash, A., Douda, D.N., Licht, J.C., Grasemann, H., Sweezey, N., *et al.* (2017) JNK Activation Turns on LPS- and Gram-Negative Bacteria-Induced NADPH Oxidase-Dependent Suicidal NETosis. *Scientific Reports*, **7**, Article No. 3409. https://doi.org/10.1038/s41598-017-03257-z

[32] Sandri, S., Rodriguez, D., Gomes, E., Monteiro, H.P., Russo, M. and Campa, A. (2008) Is Serum Amyloid A an Endogenous TLR4 Agonist? *Journal of Leukocyte Biology*, **83**, 1174-1180. https://doi.org/10.1189/jlb.0407203

[33] Ebert, R., Benisch, P., Krug, M., Zeck, S., Meissner-Weigl, J., Steinert, A., *et al.* (2015) Acute Phase Serum Amyloid A Induces Proinflammatory Cytokines and Mineralization via Toll-Like Receptor 4 in Mesenchymal Stem Cells. *Stem Cell Research*, **15**, 231-239. https://doi.org/10.1016/j.scr.2015.06.008

[34] Hirai, K., Furusho, H., Kawashima, N., Xu, S., de Beer, M.C., Battaglino, R., *et al.* (2019) Serum Amyloid A Contributes to Chronic Apical Periodontitis via TLR2 and TLR4. *Journal of Dental Research*, **98**, 117-125. https://doi.org/10.1177/0022034518796456

[35] Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., *et al.* (1988) The American Rheumatism Association 1987 Revised Criteria for the Classification of Rheumatoid Arthritis. *Arthritis & Rheumatism*, **31**, 315-324. https://doi.org/10.1002/art.1780310302

[36] Yang, H., Biermann, M.H., Brauner, J.M., Liu, Y., Zhao, Y. and Herrmann, M. (2016) New Insights into Neutrophil Extracellular Traps: Mechanisms of Formation and Role in Inflammation. *Frontiers in Immunology*, **7**, Article 302. https://doi.org/10.3389/fimmu.2016.00302

[37] Papayannopoulos, V., Metzler, K.D., Hakkim, A. and Zychlinsky, A. (2010) Neutrophil Elastase and Myeloperoxidase Regulate the Formation of Neutrophil Extracellular Traps. *Journal of Cell Biology*, **191**, 677-691. https://doi.org/10.1083/jcb.201006052

[38] Ospelt, C., Brentano, F., Rengel, Y., Stanczyk, J., Kolling, C., Tak, P.P., *et al.* (2008) Overexpression of Toll-Like Receptors 3 and 4 in Synovial Tissue from patients with Early Rheumatoid Arthritis: Toll-Like Receptor Expression in Early and Longstanding Arthritis. *Arthritis & Rheumatism*, **58**, 3684-3692. https://doi.org/10.1002/art.24140

[39] Shen, C., Sun, X.G., Liu, N., Mu, Y., Hong, C.C., Wei, W., *et al.* (2015) Increased Serum Amyloid A and Its Association with Autoantibodies, Acute Phase Reactants and Disease Activity in Patients with Rheumatoid Arthritis. *Molecular Medicine Reports*, **11**, 1528-1534. https://doi.org/10.3892/mmr.2014.2804

[40] Malech, H.L., Deleo, F.R. and Quinn, M.T. (2014) The Role of Neutrophils in the Immune System: An Overview. In: Quinn, M. and DeLeo, F., Eds., *Neutrophil Methods and Protocols. Methods in Molecular Biology*, Vol. 1124, Humana Press, Totowa, 3-10. https://doi.org/10.1074/mcp.M500143-MCP200

[41] Lominadze, G., Powell, D.W., Luerman, G.C., Link, A.J., Ward, R.A. and McLeish, K.R. (2005) Proteomic Analysis of Human Neutrophil Granules. *Molecular & Cel-*

lular Proteomics, **4**, 1503-1521. https://doi.org/10.1074/mcp.M500143-MCP200

[42] Sur Chowdhury, C., Giaglis, S., Walker, U.A., Buser, A., Hahn, S. and Hasler, P. (2014) Enhanced Neutrophil Extracellular Trap Generation in Rheumatoid Arthritis: Analysis of Underlying Signal Transduction Pathways and Potential Diagnostic Utility. *Arthritis Research & Therapy*, **16**, Article No. R122. https://doi.org/10.1186/ar4579

[43] Dunaeva, M., Buddingh, B.C., Toes, R.E., Luime, J.J., Lubberts, E. and Pruijn, G.J. (2015) Decreased Serum Cell-Free DNA Levels in Rheumatoid Arthritis. *Autoimmunity Highlights*, **6**, 23-30. https://doi.org/10.1007/s13317-015-0066-6

[44] Zhong, X.Y., von Muhlenen, I., Li, Y., Kang, A., Gupta, A.K., Tyndall, A., *et al.* (2007) Increased Concentrations of Antibody-Bound Circulatory Cell-Free DNA in Rheumatoid Arthritis. *Clinical Chemistry*, **53**, 1609-1614. https://doi.org/10.1373/clinchem.2006.084509

[45] Bartoloni, E., Ludovini, V., Alunno, A., Pistola, L., Bistoni, O., Crino, L., *et al.* (2011) Increased Levels of Circulating DNA in Patients with Systemic Autoimmune Diseases: A Possible Marker of Disease Activity in Sjogren's Syndrome. *Lupus*, **20**, 928-935. https://doi.org/10.1177/0961203311399606

[46] Bach, M., Moon, J., Moore, R., Pan, T., Nelson, J.L. and Lood, C. (2020) A Neutrophil Activation Biomarker Panel in Prognosis and Monitoring of Patients with Rheumatoid Arthritis. *Arthritis & Rheumatology*, **72**, 47-56. https://doi.org/10.1002/art.41062

[47] Chen, W., Wang, Q., Ke, Y. and Lin, J. (2018) Neutrophil Function in an Inflammatory Milieu of Rheumatoid Arthritis. *Journal of Immunology Research*, **2018**, Article ID: 8549329. https://doi.org/10.1155/2018/8549329

[48] Benhamou, Y., Bellien, J., Armengol, G., Brakenhielm, E., Adriouch, S., Iacob, M., *et al.* (2014) Role of Toll-Like Receptors 2 and 4 in Mediating Endothelial Dysfunction and Arterial Remodeling in Primary Arterial Antiphospholipid Syndrome. *Arthritis & Rheumatology*, **66**, 3210-3220. https://doi.org/10.1002/art.38785

[49] Hernanz, R., Martinez-Revelles, S., Palacios, R., Martin, A., Cachofeiro, V., Aguado, A., *et al.* (2015) Toll-Like Receptor 4 Contributes to Vascular Remodelling and Endothelial Dysfunction in Angiotensin II-Induced Hypertension. *British Journal of Pharmacology*, **172**, 3159-3176. https://doi.org/10.1111/bph.13117

[50] Kuzmich, N.N., Sivak, K.V., Chubarev, V.N., Porozov, Y.B., Savateeva-Lyubimova, T.N. and Peri, F. (2017) TLR4 Signaling Pathway Modulators as Potential Therapeutics in Inflammation and Sepsis. *Vaccines*, **5**, Article No. 34. https://doi.org/10.3390/vaccines5040034

[51] Ma, B., Dohle, E., Li, M. and Kirkpatrick, C.J. (2017) TLR4 Stimulation by LPS Enhances Angiogenesis in a Co-Culture System Consisting of Primary Human Osteoblasts and Outgrowth Endothelial Cells. *Journal of Tissue Engineering and Regenerative Medicine*, **11**, 1779-1791. https://doi.org/10.1002/term.2075

[52] Rahimifard, M., Maqbool, F., Moeini-Nodeh, S., Niaz, K., Abdollahi, M., Braidy, N., *et al.* (2017) Targeting the TLR4 Signaling Pathway by Polyphenols: A Novel Therapeutic Strategy for Neuroinflammation. *Ageing Research Reviews*, **36**, 11-19. https://doi.org/10.1016/j.arr.2017.02.004

[53] Wang, W., Deng, M., Liu, X., Ai, W., Tang, Q. and Hu, J. (2011) TLR4 Activation Induces Nontolerant Inflammatory Response in Endothelial Cells. *Inflammation*, **34**, 509-518. https://doi.org/10.1007/s10753-010-9258-4

[54] Keshari, R.S., Jyoti, A., Dubey, M., Kothari, N., Kohli, M., Bogra, J., *et al.* (2012) Cytokines Induced Neutrophil Extracellular Traps Formation: Implication for the

Inflammatory Disease Condition. *PLOS ONE*, **7**, e48111. https://doi.org/10.1371/journal.pone.0048111

[55] Lavoie, S.S., Dumas, E., Vulesevic, B., Neagoe, P.E., White, M. and Sirois, M.G. (2018) Synthesis of Human Neutrophil Extracellular Traps Contributes to Angiopoietin-Mediated *in Vitro* Proinflammatory and Proangiogenic Activities. *The Journal of Immunology*, **200**, 3801-2813. https://doi.org/10.4049/jimmunol.1701203

[56] Pieterse, E., Rother, N., Garsen, M., Hofstra, J.M., Satchell, S.C., Hoffmann, M., *et al.* (2017) Neutrophil Extracellular Traps Drive Endothelial-to-Mesenchymal Transition. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **37**, 1371-1379. https://doi.org/10.1161/ATVBAHA.117.309002