ORIGINAL RESEARCH

Internalisation of neutrophils extracellular traps by macrophages aggravate rheumatoid arthritis via Rab5a

Haixin Ye,[1,2] Qian Yang,[1] Huaxia Guo,[2] Xing Wang,[1] Lifang Cheng,[1] Bingqi Han,[2] Mukeng Hong,[1,2] Fopei Ma,[2] Meng Li,[1] Xianghui Wu,[3] Feilong Chen,[2] Junqing Zhu ,[1] Shixian Chen ,[1] Songyuan Zheng,[1] Juan Li [1,2]

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HY, QY and HG contributed equally.

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For numbered affiliations see end of article.

Correspondence to
Dr Juan Li; lijuan@smu.edu.cn

Dr Songyuan Zheng; sean3017@163.com

Dr Shixian Chen; shixian@smu.edu.cn

ABSTRACT

Objectives Although elevated levels of neutrophil extracellular traps (NETs) have been reported in patients with rheumatoid arthritis (RA), the role of NETs in RA and the relationship between NETs and macrophages in the pathogenesis of RA requires further research. Here, we sought to determine the role of NETs in RA pathogenesis and reveal the potential mechanism.

Methods Neutrophil elastase (NE) and myeloperoxidase (MPO)-DNA were measured in human serum and synovium. NETs inhibitor GSK484 was used to examine whether NETs involved with RA progression. We stimulated macrophages with NETs and detected internalisation-related proteins to investigate whether NETs entry into macrophages and induced inflammatory cytokines secretion through internalisation. To reveal mechanisms mediating NETs-induced inflammation aggravation, we silenced GTPases involved in internalisation and inflammatory pathways in vivo and in vitro and detected downstream inflammatory pathways.

Results Serum and synovium from patients with RA showed a significant increase in NE and MPO, which positively correlated to disease activity. Inhibiting NETs formation alleviated the collagen-induced arthritis severity. In vitro, NETs are internalised by macrophages and located in early endosomes. Rab 5a was identified as the key mediator of the NETs internalisation and inflammatory cytokines secretion. Rab 5a knockout mice exhibited arthritis alleviation. Moreover, we found that NE contained in NETs activated the Rab5a-nuclear factor kappa B (NF-κB) signal pathway and promoted the inflammatory cytokines secretion in macrophages.

Conclusions This study demonstrated that NETs-induced macrophages inflammation to aggravate RA in Rab 5a dependent manner. Mechanically, Rab5a mediated internalisation of NETs by macrophages and NE contained in NETs promoted macrophages inflammatory cytokines secretion through NF-κB-light-chain-enhancer of activated B cells signal pathway. Therapeutic targeting Rab 5a or NE might extend novel strategies to minimise inflammation in RA.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Although elevated levels of neutrophil extracellular traps (NETs) have been reported in patients with rheumatoid arthritis (RA), the role of NETs in RA and the relationship between NETs and macrophages in the pathogenesis of RA requires further research.

WHAT THIS STUDY ADDS

⇒ NETs are enriched in patients with RA and associated with disease severity.
⇒ NETs induce macrophages inflammatory cytokines secretion dependent on Rab5a-mediated internalisation pathway.
⇒ Neutrophil elastase (NE) contained in NETs activates the Rab5a-nuclear factor kappa B (NF-κB) signal pathway and promotes the inflammatory cytokines secretion in macrophages.
⇒ An enhanced NE-Rab5a-NF-κB axis is shown in the synovium of patients with RA.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Rab5a mediating internalisation of NETs by macrophages and the proteins contained in NETs could be an appealing therapeutic strategy for RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic multifactorial autoimmune disorder characterised by autoantibodies, systemic inflammation, persistent synovitis and the destruction of bone and cartilage in multiple joints.[1] However, the potential mechanisms are as yet undefined. Recently, accumulating studies suggest that cell death plays an important role in the pathogenesis of various autoimmune diseases.[2 3] Especially, as the most abundant cell type in RA synovial fluid, neutrophils have a specific type of cell death, neutrophils extracellular traps (NETs), which release structural fibres containing chromatin and granular proteins. Prior studies have demonstrated that NETs levels are elevated in patients with RA, and NETs could promote anticitrullinated protein antibodies (ACPAs) and osteoclast formation.[4 5] However, the role of NETs in RA and the underlying mechanisms need further investigation.

Macrophages, which have been found to eliminate and degrade damaged, aged and dead cells for further recycling, are involved in NETs clearance.[6] The prior study suggests that macrophages internalised NETs-DNA and ultimately degraded in lysosomes. Internalisation disruption could cause excess NETs accumulation and lead to prolonged inflammation and self-antigen persistence.[7] Meanwhile, as crucial regulators of endosome production and trafficking, small GTP-binding proteins and GTPase (Rab proteins) also are involved in inflammatory signal pathway transduction through activating inflammatory pathways, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Toll-like receptors (TLRs) sensing signalling pathway, etc.[8 9] Abnormal Rab function activates downstream inflammatory pathways and has transformed the outcomes of patients with inflammatory diseases, such as infections.[9] Therefore, we speculated that excessive NETs could entry into macrophages and trigger subsequent inflammation through GTPase modulation.

Here, we found that NETs were enriched in patients with RA and inhibiting NETs could alleviate inflammation in collagen-induced arthritis (CIA) mice. We further demonstrated that NETs could spread into synovial macrophages and induce inflammatory cytokines secretion via small GTPase Rab5a modulation, which activates the NF-κB signal pathway.

METHODS

Human clinical cohorts and sample collection

Individuals diagnosed with RA according to the 2010 American College of Rheumatology/EULAR classification criteria for RA were included in the study. Disease Activity Score 28 (DAS28) was used to classify patients with RA. Inclusion and exclusion criteria were previously described in studies.[10 11] Inclusion criteria for patients with RA were age >18 years with a disease duration of at least 6 weeks. Exclusion criteria included a diagnosis of known autoimmune diseases other than RA, a history of severe chronic infection, any current infection, diagnosis of cancer, pregnancy and lactation. Healthy control (HC) individuals were included if they met the following criteria: age >18 years, with average values on recent screens for liver and kidney function. Exclusion criteria were the presence of autoimmune diseases.

Peripheral blood from patients with RA (n=25) and HCs (n=18) was obtained by venipuncture and collected in EDTA-containing tubes. Synovium tissues were obtained from the individuals according to criteria previously described.[12] Briefly, synovium tissues of patients with RA (n=5) were collected from patients with RA performing arthroplasty or synovectomy. Control samples were taken from trauma patients with normal synovium (n=5). The characteristics of the participants for blood and synovium collecting are listed in online supplemental table S1.

Mice

Male DBA/1J WT and C57BL/6 WT mice (8–10 weeks) were purchased from Charles River Bioscience Company (Beijing, China). Rab5a$^{-/-}$ mice (on a C57BL/6 background) were purchased from Cyagen Biosciences (Suzhou, China). All mice were housed in the specific pathogen-free environment on a 12:12 hours light/dark cycle. CIA experiments were carried out in the above mice and detailed experiments information was described in the following related methods.

Induction of CIA

CIA was induced in DBA/1J mice by immunisation on days 1 and 21 based on previously described methods.[11 13] Briefly, for the first immunisation (day 1), mice were injected intradermally at the tail-base with 150 mg Bovine Collagen Type II (Chondrex, Redmond, Washington, USA) emulsified in complete Freund's adjuvant (Chondrex) in equal volumes. The second immunisation, conducted on day 21, was performed with 75 mg of CII emulsified in Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, Missouri, USA). The severity of arthritis was assessed after the second immunisation and scored according to the following criterion: 0, standard; 1, slight swelling or/and erythema of digits; 2, moderate swelling and erythema extending from the ankle to the mid-foot (tarsals); 3, pronounced swelling extending from the ankle to the metatarsal joints and 4, complete swelling and erythema encompassing the ankle, foot and digits, resulting in deformity and/or ankyloses.[12] The total clinical score (0–16) was calculated every 2 days. CIA induced in C57BL/6 was performed in a single intradermal injection of 200 mg native Bovine Collagen Type II collagen, and arthritis scores were evaluated in the same way. To investigate the role of NETs in CIA, the DBA/1J mice were randomly divided into two groups, with five mice in each group: naive group, CIA model group. To investigate the role of inhibition of formation of NETs in CIA, the DBA/1J mice were randomly divided into three groups, with five mice in each group: naive group, vehicle-treated CIA model group and GSK484-treated CIA model group. In GSK484-treated CIA model group, DBA/1J mice were treated with GSK484 (2 mg/kg intraperitoneally every 3 days, inhibitor of PAD4, an enzyme required for NETs formation, HY-100514, MedChemExpress, USA) at day 18, 3 days before second immunisation.[14–17] To investigate the role of Rab5a in CIA, the C57BL/6 WT and the C57BL/6 Rab5a$^{-/-}$ mice were randomly divided into four groups, with five mice in each group: WT naive group, WT CIA model group, Rab5a$^{-/-}$ naive group (data did not show) and Rab5a$^{-/-}$ CIA model group. To investigate the role of NE in CIA, the DBA/1J mice were randomly divided into two groups, with five mice in each group: vehicle-treated CIA model group and sivelestat-treated CIA model group. In sivelestat-treated CIA model group, DBA/1J mice were treated with sivelestat (10 mg/kg intraperitoneally every 3 days, inhibitor of NE, LY544349,

MedChemExpress, USA) at day 18, 3 days before second immunisation.[18 19]

Cell isolation

Bone-marrow-derived macrophages (BMDMs) from CIA mice were generated as described previously.[20–22] After 7 days culture, BMDMs were ready for further experimentation. Whole blood from patients with RA and HCs were used to isolate neutrophils by density gradient centrifugation, using Human Neutrophils Enrichment Kit (Solarbio science & technology, Beijing, China) according to the manufacturer's specification. For isolation of mouse bone marrow-derived neutrophils, tibias and femurs were obtained after euthanasia and then bone marrow cells were collected to isolate neutrophils by using Mouse Neutrophils Enrichment Kit (Solarbio science & technology), according to the manufacturer's specifications.

NETs generation and quantification

Mouse NETs generation using a previously described method.[4 23] Briefly, mice neutrophils were seeded in a six-well tissue culture with or without 500 nM phorbol-12-myristate-13-acetate (PMA, Solarbio science & technology) for 6 hours.[24 25] The supernatants were harvested and centrifuged at 1000 g at 4°C for 10 min. The cell-free supernatants containing NETs were collected and transferred to a fresh tube and stored at −80°C until used. BCA Kit (Beyotime, Jiangsu, China) was used to quantify the collected NETs. Human neutrophils were seeded in a confocal-well tissue culture plate incubated for 6 hours to observe the spontaneously generated NETs.

Assessment of NETs internalisation by macrophages

BMDMs were seeded in confocal-well at a density of 1×10^6/well, followed by addition of 50 µg of mice NETs and further incubation for 1, 2 or 6 hours. To investigate the NETs location, BMDMs were pretreated with 1 µg/mL Dynasore (HY-15304, MedChemExpress, USA),[26 27] or 40 µg/mL of Bafilomycin-A1 (HY-100558, MedChemExpress, USA) for 30 min in complete media.[28] To investigate the role of Rab5a or other GTPases, BMDMs were pretreated with siRNA transfection, detailed in the methods of siRNA Knockdown. To investigate role of NETs containing proteins, NETs were pretreated with a proteinase K (40 µg /mL, ST532, Beyotime, Jiangsu, China) and heat-inactivated (65°C for 10 min).[5 29] To investigate the role of Neutrophil elastase (NE), BMDMs were treated with 50 nM recombinant NE (4517-SE-010, R&D Systems, Minnesota, USA) for 6 hours.[30] Supernatants were then harvested to detect the levels of IL-6 and TNF-α. BMDMs were washed twice with phosphate-buffered saline (PBS), then either lysed or fixed for additional experiments.

Immunofluorescence staining

Human synovialis and mouse paws were fixed with 4% paraformaldehyde, followed by penetration. Frozen tissues were sliced into 14 µm sections. For staining, the sections were washed by the PBS three times and penetrated with methanol at −20°C for 8 min. Non-specific binding was then blocked with 5% goat serum at room temperature (RT) for 1 hour. Cells for immunofluorescence were fixed with 4% paraformaldehyde for 25 min at RT, washed with PBS and permeabilised with or without 0.2% Triton X-100 in PBS for 20 min. Cells were then blocked in PBS with 5% goat serum for 1 hour at RT. Subsequently, the samples were then treated overnight at 4°C with diluted primary antibodies or isotope-matched control IgG. After extensive washing, the frozen sections were incubated with the respective fluorescent secondary antibodies. Finally, DAPI was then used for counterstaining the nuclei. Images were obtained by laser scanning confocal microscopy (LSM780, Zeiss), and mean fluorescence intensity was analysed by Image J software. Detailed information of antibodies used in immunofluorescence staining is listed in online supplemental table S3.

ELISA

The levels of human or mouse inflammatory cytokines (IL-6, TNF-α) and NETs (myeloperoxidase (MPO)-DNA and NE) were detected by ELISA kits (Boshen Biotechnology, Jiangsu, China) according to the manufacturer's instructions.

Histological analysis and immunohistochemistry

Mouse tissues were fixed in 4% paraformaldehyde (Thermo Scientific) for 24 hours at 4°C, washed with PBS, embedded in paraffin and sectioned at 4 µm thickness for H&E staining or immunohistochemistry.

Western blot

Mouse paws and the harvested BMDMs were sonicated in ice-cold RIPA lysis buffer cell lysis buffer (Beyotime, Jiangsu, China) containing a phosphatase inhibitor cocktail (beyotime) and a protease inhibitor cocktail (Beyotime, Jiangsu, China), and the lysates were subjected to centrifugation at 12 000 g for 15 min. BCA kit (Beyotime, Jiangsu, China) was used to measure the protein concentration of the supernatants. After 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis(SDS-PAGE), protein samples were transferred onto PVDF membranes (EMD Millipore, Billerica, Massachusetts, USA) and processed for immunoblot analyses with primary antibody overnight. Then, Blots were treated with HRP-coupled secondary at RT for 60 min. Detection was performed by Bio-Rad ChemiDoc XRS+. Grey scale values of the image were analysed by ImageJ. Detail information of antibody used in Western blot is listed in online supplemental table S3.

siRNA knockdown

BMDMs were collected and fasted by serum-free medium before transfection. Then BMDMs were transfected with nonspecific control siRNA or mouse siRNA nucleotide fragment (GenePharma) using the LipofectaminTM RNAiMAX (Invitrogen, California, USA) for 36 hours and then BMDMs were subjected to internalisation

experiments or western blotting to assess the knockdown efficiency. Target primer sequences for RNAi knockdown are listed in online supplemental table S2.

Statistical analysis

All the statistical analyses were performed using GraphPad Prism V.9 software, and error bars indicate as means±SEM. The number of independent experiments, the number of events and information about the statistical details and methods are indicated in the relevant figure legends. Normal distribution of data was analysed using Shapiro-Wilk test for data with a normal distribution and homogeneity of variance, Student's t-test was used to identify differences between the two groups. Three or more groups using one-way ANOVA. For data with non-normal distribution, Mann-Whitney test was applied. Correlation analyses were performed based on the Spearman's r statistic, and data visualisation was performed by using R V.3.5.0.

RESULTS

Abundant NETs in patients with RA are positively correlated with inflammation

To examine the relationship between NETs and RA development, we established CIA mouse model and detected MPO, NE and peptidyl-arginine deiminase 4 (PAD4)-specific markers of neutrophils and NETosis (online supplemental figure S1A–C).[31] Notably, in CIA mice, higher serum levels of MPO–DNA and NE were consistent with the joint levels of NETs (online supplemental figure S1D–H), as evaluated by western blot analyses of MPO, NE and PAD4 (online supplemental figure S1E, F) and immunofluorescence staining for MPO and NE (online supplemental figure S1G, H). To investigate the clinical importance of NETs, serum samples were collected from patients with RA (n=25) and HCs (n=18) (online supplemental table S1). Notably, neutrophils isolated from patients with RA showed spontaneous NETs formation (figure 1A). In addition, we observed that patients with RA exhibited higher serum MPO-DNA, NE and inflammatory cytokines (IL-6, TNF- α) than HCs (figure 1B–E). Next, we analysed the correlation between NETs and the parameters of RA disease activity (DAS28-CRP, DAS28-ESR, RF, CRP, ESR, ACPA, IL-6 and TNF- α) (figure 1F). We found that NE had a strong positive correlation with inflammatory cytokines (figure 1G,H). To further confirm the correlation between NETs and RA, we collected synovial samples from patients with RA (n=5) and control patients (n=5). Similar to the above findings, the synovial tissues of patients with RA exhibited more abundant NETs infiltration (figure 1I,J). Together, these results, consistent with previous reports,[5 32] imply that excessive amounts of NETs could form in the blood and synovialis of patients with RA, positively correlate with disease activity and facilitate the subsequent development of RA.

Inhibiting the formation of NETs alleviate arthritis in CIA mice

To investigate whether NETs formation is involved in RA development, we injected CIA mice with GSK484, a peptidylarginine-deiminase-4 inhibitor to inhibit NETs formation (figure 2A).[14–17] Compared with the CIA group, GSK484 administration reduced the expression of MPO, NE and PAD4 in the synovialis (figure 2B,C). As visualised by confocal microscopy, less formation of NETs was observed in the synovialis of mice treated with GSK484 (figure 2D,E). Notably, in comparison with the CIA mice, GSK484 administration reduced the severity of arthritis (figure 2F,G). Meanwhile, GSK484 treatment also decreased serum IL-6 and TNF-α levels (figure 2H). We used immunohistochemical staining to detect the infiltration of macrophages into the synovialis of mice in each group and found that GSK484 significantly reduced macrophages infiltration (online supplemental figure S2A, B). We also observed excessive NETs accompanied with macrophages infiltration in patients with RA (online supplemental figure S2C, D). Collectively, these findings indicate that inhibition of NETs alleviated arthritis and inhibited inflammatory responses.

Internalisation of NETs by macrophages promote inflammatory cytokine secretion

To study the function of NETs in promoting macrophages inflammation, PMA-induced NETs were incubated with BMDMs for 1, 2 and 6 hours (figure 3A,B).[4 23] Using confocal microscopy, NE was used as a hallmark of NETs staining based on its specific expression in neutrophils,[33] and we found that the proportion of macrophages internalising NETs increased over time (figure 3C, online supplemental figure S3A). Moreover, the levels of IL-6 and TNF-α secreted by macrophages were significantly associated with the proportion of internalised NETs (figure 3D). To visualise the cytosolic localisation of NETs during macrophages internalisation, BMDMs were stained with antibodies against NE and early endosomal (EEA1) or lysosomal markers (LAMP1) (figure 3E).[34] After 6 hours of internalisation, NE in the cytosol was observed at a higher frequency in early endosomes than in lysosomes (figure 3E). To further confirm these observations, we pretreated macrophages with dynasore (an early endosome inhibitor) or bafilomycin (a late endosome inhibitor) to determine the function of NETs in the cytosol.[26–28] Interestingly, we found that dynasore administration, but not bafilomycin administration, significantly reduced the levels of IL-6 and TNF-α, suggesting that NETs triggering macrophages inflammation might be associated with early endosomes (figure 3F). Notably, we found a distinct NE staining associated with early endosomes in the RA synovialis in comparison with controls (figure 3G). Similar results were observed in the synovialis of CIA mice (online supplemental figure S3A,B). These findings suggest that NETs trigger macrophages inflammation via internalisation.

NETs internalised by macrophages induce inflammation in Rab5a-dependent manner

Previous studies suggest that GTPases modulate the process of endosomes internalising cargo and mediate

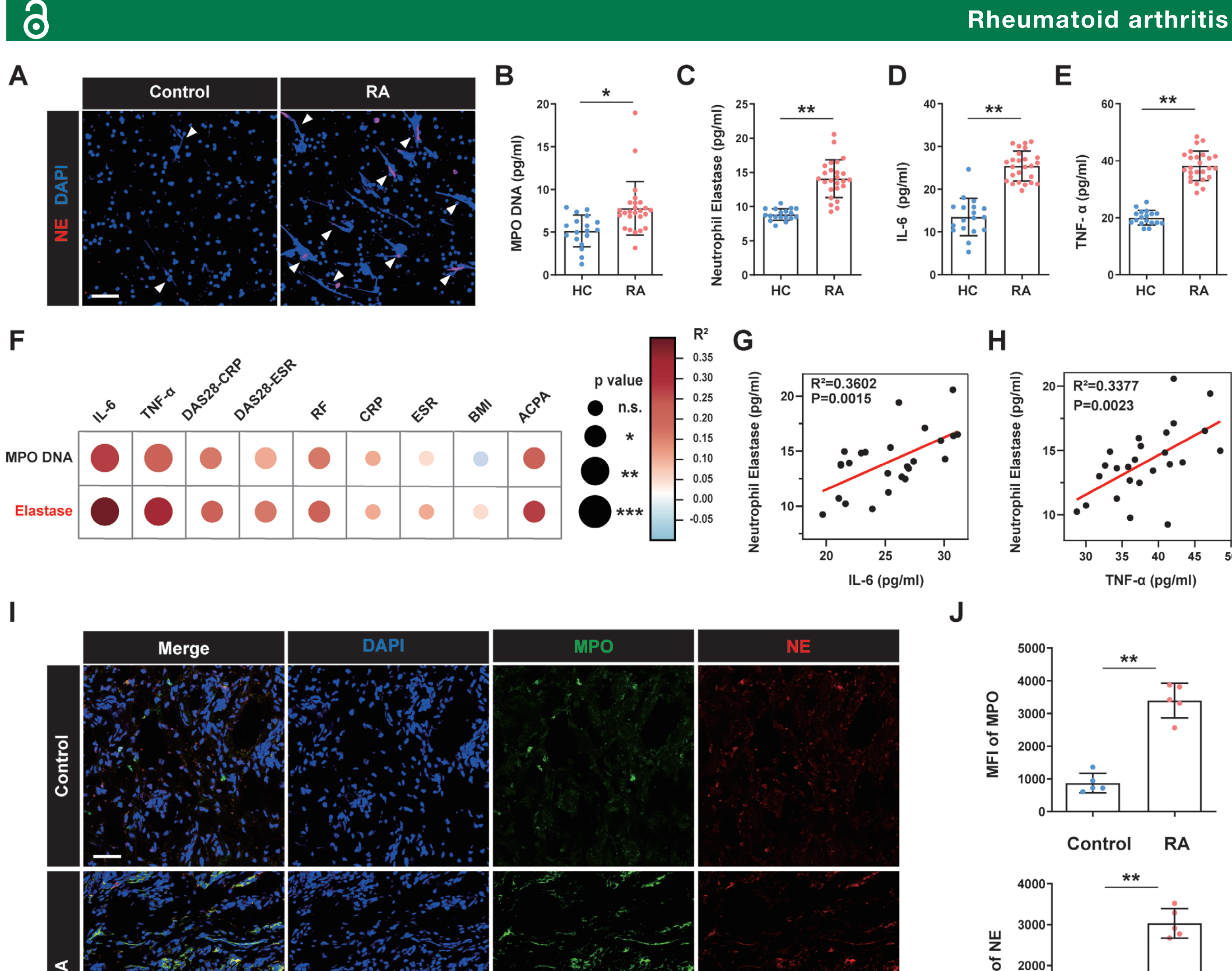

Figure 1 Abundant NETs in patients with RA are positively correlated with inflammation. (A) Representative immunofluorescence images staining of NETs in neutrophils isolated from patients with RA and healthy controls (HCs) peripheral blood, white arrows indicate NETs, costained with NE and DAPI. Scale bars, 100 µm. (B–E) Blood samples of patients with RA (n=25) and HCs (n=18) were collected, and serum levels of MPO-DNA (B), NE (C), IL-6 (D) and TNF- α (E) were measured. (F) Visual representation of significant correlations of serum levels of NETs (MPO-DNA, NE) measured in patients with RA (n=25) with clinical parameters (DAS28-CRP, DAS28-ESR, RF, CRP, ESR, ACPA, IL-6 and TNF-α). Colour gradient represents R^2 values. (G, H) Spearman's correlation between serum neutrophils elastase level with serum IL-6 level and TNF-α level in patients with RA. (I, J) Representative immunofluorescence images staining of NETs in synovialis from patients with RA (n=5) and control patients (n=5), costained with MPO, NE and DAPI. Scale bars, 100 µm. Student's t-test was used to calculate p values between RA and HCs in (B–E, J) between RA and controls. *p<0.05, **p<0.01. ACPA, anticitrullinated protein antibodies; BMI, body mass index; DAS28-CRP, Disease Activity Score 28-C reactive protein; DAS28-ESR, Disease Activity Score 28-Erythrocyte sedimentation rate;RF,rheumatoid factor; MPO, myeloperoxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap; RA, rheumatoid arthritis.

potential downstream inflammatory signal pathways.[8 9] To determine which GTPase dominantly mediates the internalisation of NETs, we pretreated macrophages with siRNA against the formation of early endosomes (Rab4a, Rab5a, Rab13, Rab21 and Rab22a, key GTPases involved in this process) and maturation of endosomes (Rab7a, the critical GTPase for early-to-late endosome transition) (online supplemental figure S4A).[35] We found that the levels of IL-6 and TNF-α secreted by macrophages were substantially reduced following Rab5a knockdown rather than other Rab genes (figure 4A,B). Subsequently, we confirmed that knockdown of Rab5a could reduce the recruitment of early endosomes in macrophages induced by NETs (figure 4C).[35] More importantly, the internalisation of NETs into macrophages was reduced after Rab5a knockdown (figure 4D, online supplemental figure S4B). To confirm these findings in vivo, we measured these critical factors in the joints of CIA mice. We found that

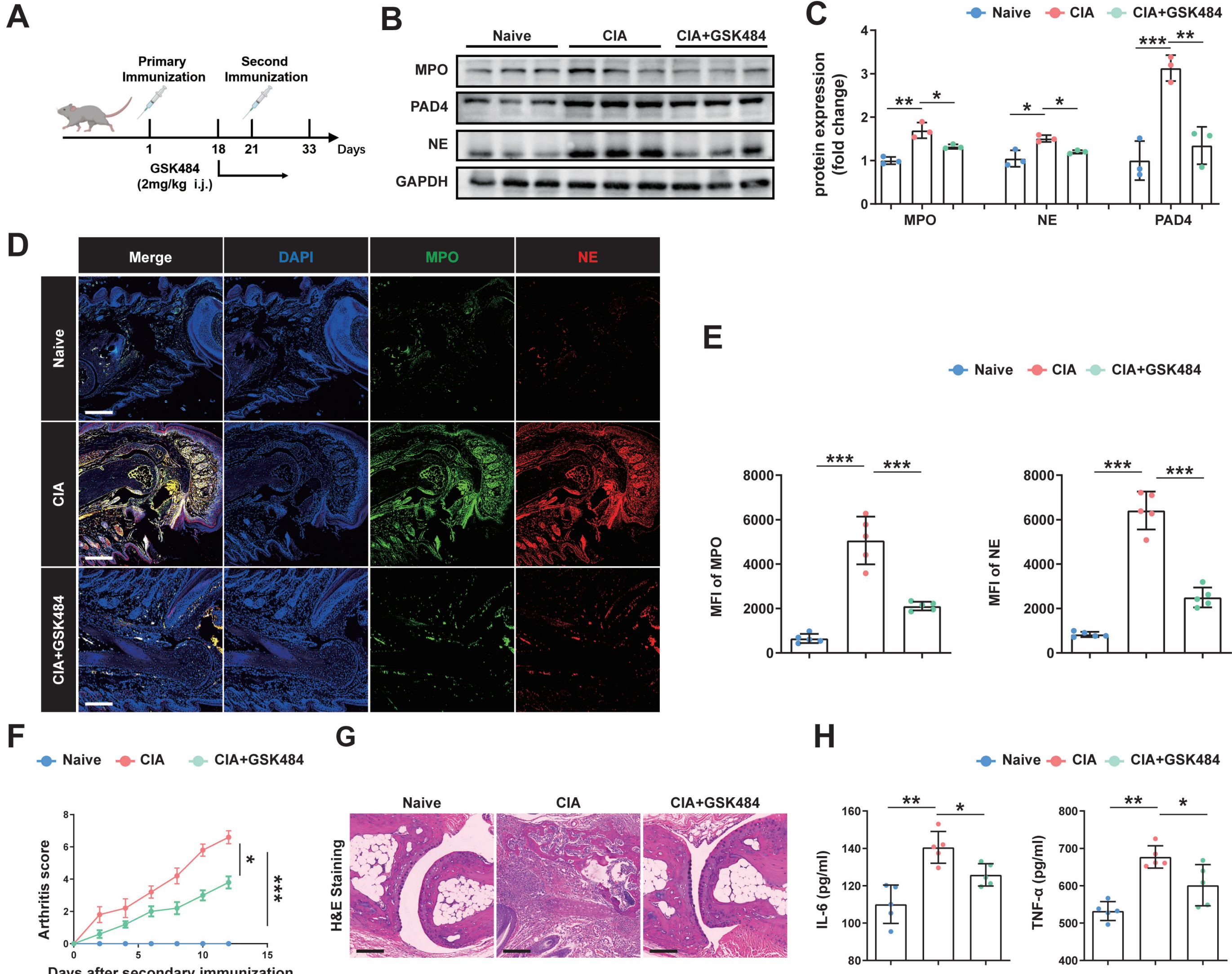

Figure 2 Inhibiting the formation of NETs alleviate arthritis in CIA mice. (A) Schematic figure of CIA mice receiving GSK484 administrations. Mice in CIA+GSK484 group were treated every 3 days by intraperitoneal injections of the PAD4 inhibitor GSK484. (B, C) Western blot was performed on NETs expression (MPO, PAD4, NE) in mice paws. (D, E) Representative Immunofluorescence images staining of NETs in paws from CIA mice (n=5/group), costained with MPO, NE and DAPI. Scale bars, 100 µm. (F) The arthritis score is shown. (G) H&E staining of paw joints from mice. Scale bars, 100 µm. (H) Mice serum inflammatory cytokines levels (IL-6, left; TNF-α, right; n=5). Two-way ANOVA was used to calculate p values in (B). One-way ANOVA was used to calculate p values in (C, E, H). *p<0.05, **p<0.01, ***p<0.001. ANOVA, analysis of variance; CIA, collagen-induced arthritis; MPO, myeloperoxidase; NETs, neutrophil extracellular traps.

inhibiting NETs with GSK484 treatment could downregulate the expression of EEA1 and Rab5a (figure 4E,F). Notably, compared with control subject, increased NE infiltration and Rab5a expression were observed in joints of CIA mice and the synovialis of patients with RA (figure 4G,H). Collectively, these data describe Rab5a as crucial regulator of macrophages for NETs internalisation and inflammatory effects.

Rab5a deficiency alleviates arthritis in CIA mice

Prior data indicated that both the degree of macrophages internalisation of NETs and the activity of RA are closely related to inflammatory cytokine levels. Previous studies suggest that NETs could activate NF-κB pathway to induce transcription of inflammatory cytokines.[36] Therefore, we further investigated whether Rab5a modulated NETs-induced macrophages inflammation through NF-κB pathway activation. First, we observed NETs strongly induced IκBα degradation and increased p65 and p50 phosphorylation (figure 5A,B), while Rab5a depletion downregulated the NF-κB pathway (figure 5C,D). To further demonstrate the role of Rab5a in RA, CIA was induced in WT and Rab5a KO mice. When Rab5a was absent, the arthritis score was significantly reduced compared with that in CIA mice (figure 5E). Likewise, Rab5a KO mice exhibited less immune cell infiltration (figure 5F), and also decreased serum IL-6 and TNF-α levels (figure 5G). Furthermore, the NE internalised by macrophages was decreased in Rab5a KO mice

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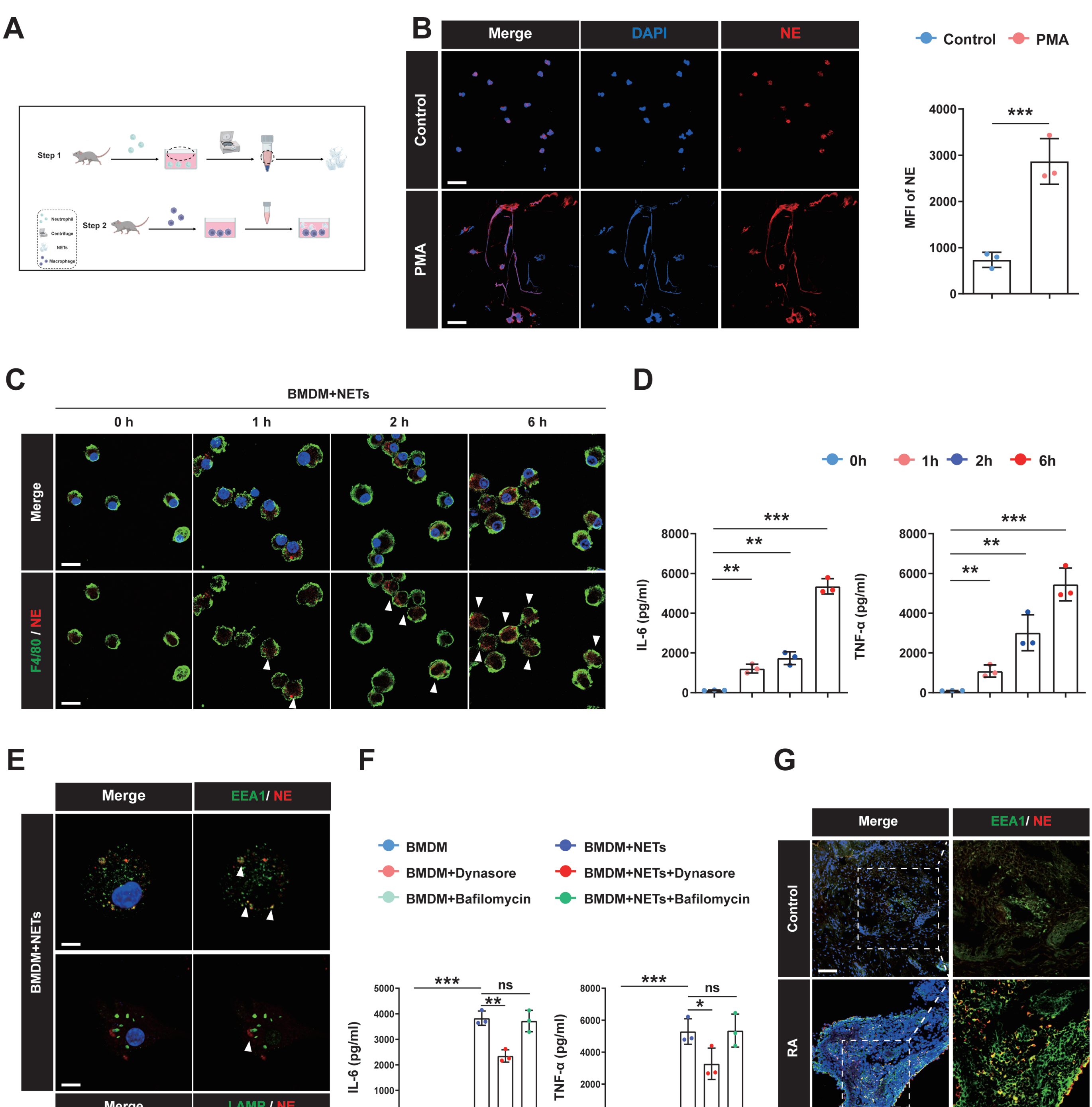

Figure 3 Internalisation of NETs by macrophages promote inflammatory cytokine secretion. (A) Diagram of assessment of NETs internalisation by macrophages, detail in related methods. (B) Immunofluorescence images staining of PMA-induced NETs in mouse neutrophils (n=3 replicates), costained with NE and DAPI. Scale bars, 50 µm. (C) Immunofluorescence images staining of NETs internalisation by BMDMs for 0, 1, 2, 6 hours (n=3 replicates), white arrow indicated NETs entry into BMDMs, costained with F4/80 (membrane marker of macrophages) and NE and DAPI. Scale bars, 20 µm. (D) Levels of the IL-6 (Left) and TNF-α (Right) in the culture medium were measured (n=3 replicates). (E) Immunofluorescence images staining of BMDMs treated with NETs for 6 hours, costained with NE, EEA1 or LAMP, white arrow indicated the overlap area (n=3 replicates). Scale bars,10 µm, (F) Levels of the IL-6 (Left) and TNF-α (Right) in the culture medium which macrophages were pretreated with dynasore or bafilomycin in the presence or absence of NETs (n=3 replicates). (G) Representative immunofluorescence images staining of NETs and early endosome in synovialis from patients with RA (n=5) and control patients (n=5), costained with EEA1, NE and DAPI. Scale bars, 100 µm. One-way ANOVA was used to calculate p values in (D, F). **p<0.01, ***p<0.001. ANOVA, analysis of variance; BMDMs, bone-marrow-derived macrophages; NE, neutrophil elastase; NETs, neutrophil extracellular traps; PMA, phorbol-12-myristate-13-acetate.

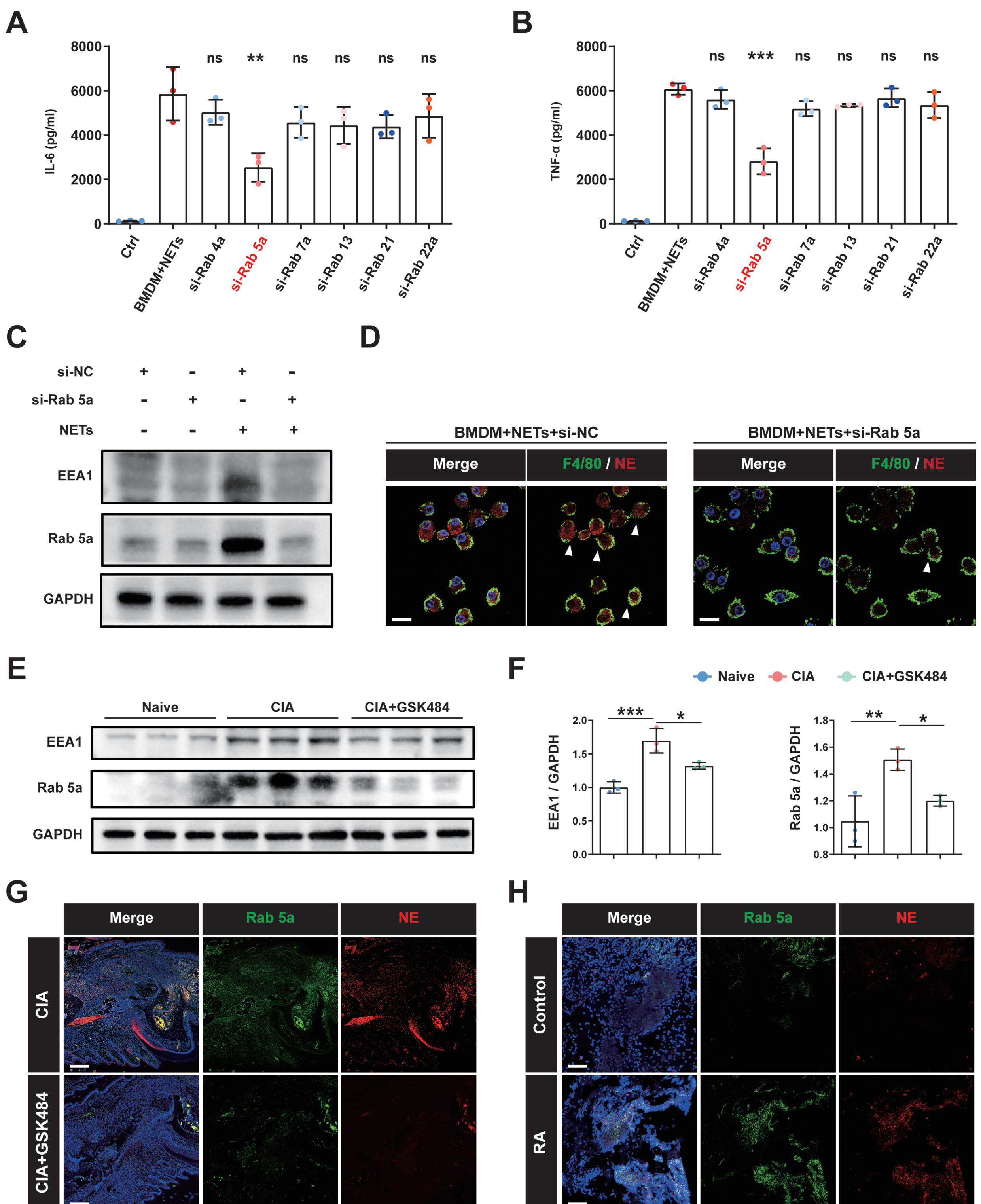

Figure 4 NETs internalised by macrophages induce inflammation in Rab5a-dependent manner. (A, B) Levels of inflammatory cytokines (IL-6, left; TNF-α, right) in Rab-knockdown BMDMs with stimulation of NETs. (C) Western blot was performed in Rab5a knockdown BMDMs (n=3 replicated). (D) Immunofluorescence images staining of NETs internalisation by BMDMs with si-NC or si-Rab5a for 6 hours (n=3 replicates), white arrow indicated NETs entry into BMDMs, costained with F4/80 and NE and DAPI. Scale bars, 20 µm. (E, F) Western blot was performed in mice paws. (G) Representative immunofluorescence images staining in paws from CIA mice (n=5/group), costained with Rab5a, NE and DAPI. Scale bars, 100 µm. (H) Representative immunofluorescence images staining in synovialis from patients with RA (n=5) and control patients (n=5), costained with Rab5a, NE and DAPI. Scale bars, 100 µm. Two-way ANOVA was used to calculate p values in (A, B). One-way ANOVA was used to calculate p values in (F) *p<0.05, **p<0.01, ***p<0.001. ANOVA, analysis of variance; BMDMs, bone-marrow-derived macrophages; CIA, collagen-induced arthritis; NE, neutrophil elastase; NETs, neutrophil extracellular traps; RA, rheumatoid arthritis.

Figure 5 Rab5a deficiency alleviates arthritis in CIA mice. (A, B) Western blot was performed in BMDMs with or without stimulation of NETs (n=3 replicated). (C, D) Western blot was performed in BMDMs with si-NC or si-Rab5a in the presence or absence of NETs (n=3 replicated). (E) The arthritis score is shown. (F) H&E staining of paw joints from mice. Scale bars, 100 µm. (G) Mice serum inflammatory cytokines levels (IL-6, left; TNF-α, right; n=5). (H, I) Representative immunofluorescence images staining in paws from CIA mice (n=5/group), costained with F4/80, NE and DAPI. Scale bars, 100 µm. Student's t-test was used to calculate p values in (B, I). Two-way ANOVA was used to calculate p values in (E). One-way ANOVA was used to calculate p values in (D, G). *p<0.05, **p<0.01, ***p<0.001. ANOVA, analysis of variance; BMDMs, bone-marrow-derived macrophages; CIA, collagen-induced arthritis; NE, neutrophil elastase; NETs, neutrophil extracellular traps.

(figure 5H,I). Overall, these results indicated that NETs induce macrophages inflammation through Rab5a-NF-κB pathway to aggravated arthritis.

NE activates Rab5a-NF-κB signalling

As the specific hallmark of neutrophils, NE is enriched in patients with RA and positively correlated with serum inflammatory cytokines (figure 1F–H), which also exhibits proinflammatory effects in some diseases.[33 37] To verify the role of NE, we eliminated proteins component with protease,[5 29] and found that protein depletion reduced NETs internalisation and inhibited cytokines secretion (figure 6A,B). Of note, recombinant NE treatment could trigger macrophages inflammation, which could be diminished by Rab5a depletion (figure 6C, online supplemental figure S5A). More importantly, NE can also activate the NF-κB pathway in macrophages and promote the phosphorylation and nuclear translocation of P65, which was also regulated by Rab5a (figure 6D–G).

To investigate whether NETs activating the Rab5a-NF-κB pathway in CIA mice were similarly NE

Figure 6 Neutrophils elastase activates Rab5a-NF-κB signalling. (A) Immunofluorescence images staining of NETs, pretreated with or without protease, internalisation by BMDMs for 6 hours (n=3 replicates), white arrow indicated NETs entry into BMDMs, costained with F4/80 and NE and DAPI. Scale bars, 20 µm. (B) Levels of the IL-6 (left) and TNF-α (right) in the BMDMs culture medium were measured (n=3 replicates). (C) Levels of the IL-6 (Left) and TNF-α (Right) in the BMDMs culture medium with si-NC or si-Rab5a in the presence or absence of NE were measured (n=3 replicates). (D, E) Western blot was performed for NF-κB signalling in BMDMs with si-NC or si-Rab5a in the presence or absence of NE (n=3 replicated). (F, G) Immunofluorescence staining for p-P65 translocated into the nucleus, costained with p-P65, NE and DAPI. Scale bars, 20 µm. Student's t-test was used to calculate p values in (B). One-way ANOVA was used to calculate p values in (C, E, G). *p<0.05, **p<0.01, ***p<0.001. ANOVA, analysis of variance; BMDMs, bone-marrow-derived macrophages; NE, neutrophil elastase; NETs, neutrophil extracellular traps.

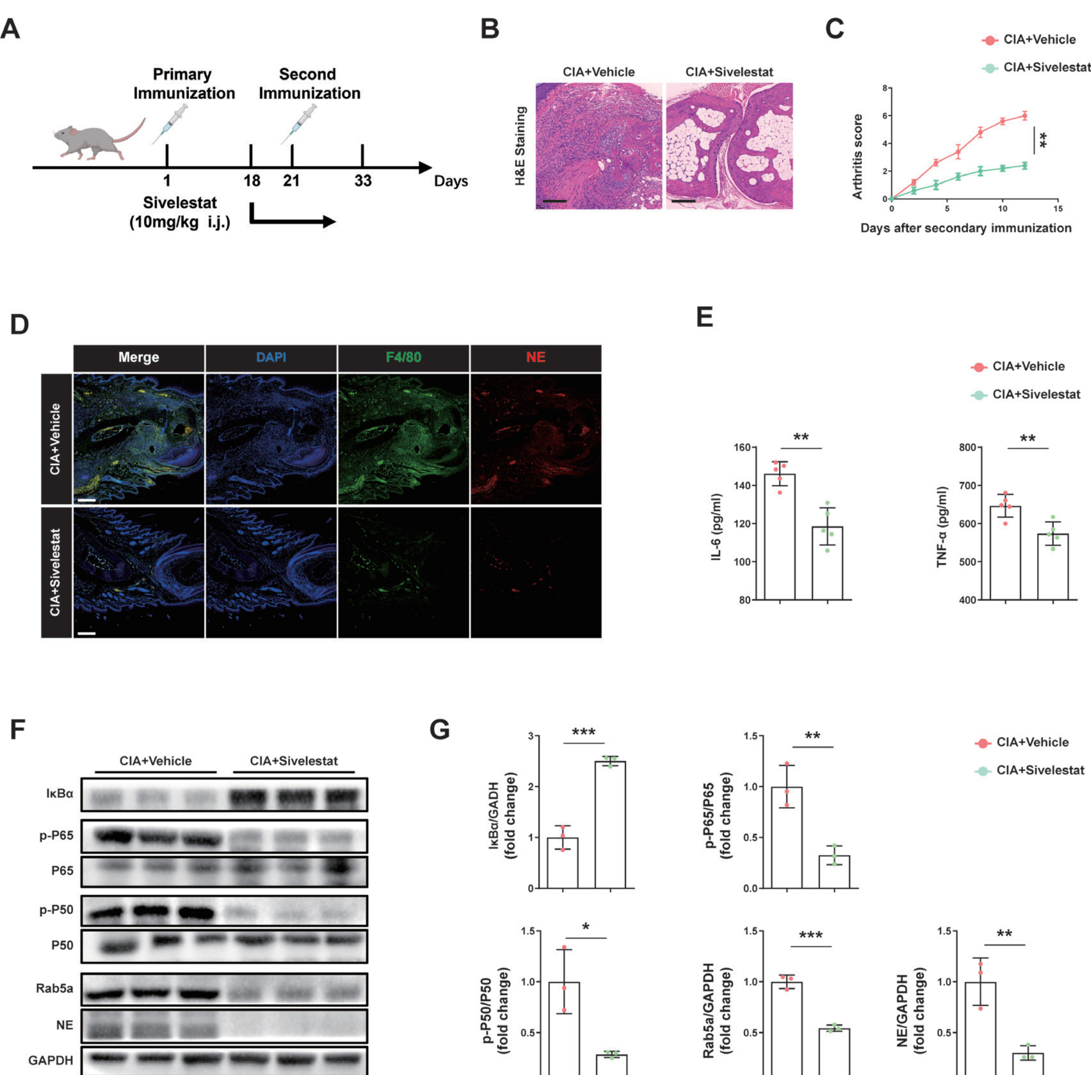

Figure 7 Sivelestat (NE inhibitor) alleviates arthritis in CIA mice. (A) Schematic figure of CIA mice receiving Sivelestat administrations. Mice in CIA+sivelestat group were treated every 3 days by intraperitoneal injections of the NE inhibitor Sivelestat (10 mg/kg). (B) H&E staining of paw joints from mice. Scale bars, 100 µm. (C) The arthritis score was shown. (D) Representative immunofluorescence images staining of internalisation of NETs by macrophages in paws from CIA mice, costained with NE, F4/80 and DAPI. Scale bars, 100 µm. (E) Mice serum inflammatory cytokines levels (IL-6, left; TNF-α, right). (F, G) Western blot was performed on Rab5a-NFκB pathway expression in mice paws. Two-way ANOVA was used to calculate p values in (C). Student's t-test was used to calculate p values in (E) and (G). *p<0.05, **p<0.01, ***p<0.001. ANOVA, analysis of variance; BMDMs, bone-marrow-derived macrophages; CIA, collagen-induced arthritis; NE, neutrophil elastase; NETs, neutrophil extracellular traps; NFκB, nuclear factor kappa B cells.

dependent, we injected the NE-specific inhibitor sivelestat in CIA mice (figure 7A). We found that sivelestat was effective in reducing arthritis in CIA mice (figure 7B,C). Meanwhile, fewer internalisation of NETs by macrophages were observed in the paws of CIA mice receiving sivelestat (figure 7D). More importantly, inhibition of NE can effectively reduce the levels of inflammatory cytokines (figure 7E) and the activation of the Rab5a-NF-κB pathway in CIA mice (figure 7F,G). All these results indicate that NE, as a key protein contained in NETs, activates the Rab5a-NF-κB pathway of the macrophages to aggravate arthritis.

DISCUSSION

RA is a chronic, systemic inflammatory disease, which is associated with citrullinated autoantigens. Recent studies suggested that cell death, especially NETs, plays a crucial role in RA progress through promoting bone erosion and generation of citrullinated autoantigens.[4 38] Here, we aimed to investigate the mechanism by which NETs promote RA development. Consistent with previous reports,[5 32] we found that excessive amounts of NETs in patients with RA were positively correlated with disease activity and levels of inflammatory cytokines. Inhibiting NETs can alleviate arthritis by reducing the secretion of

inflammatory cytokines by macrophages. Moreover, our data demonstrate that this was due to the activation of Rab5a-NF-κB signalling pathways following NETs internalisation by macrophages in RA.

Macrophages are key cell types for secreting proinflammatory cytokines, inducing neutrophil infiltration and bone destruction in RA. Here, we observed that NETs could be internalised by macrophages and promote cytokines secretion. The dead cells or debris internalised by macrophages was a process that required the GTPase dynamin. Given that GTPase dynamin binding and interactions with the Rab family are necessary for internalisation and proinflammation, we silenced GTPase proteins and identified Rab5a as the key regulator of NETs internalisation and mediating NETs-induced inflammation. Mechanistically, internalised NETs activated the NF-κB pathway and promoted P65 and P50 nuclear translocation. Overall, our findings revealed the mechanism mediating the macrophages inflammation in RA induced by excessive NETs.

Contents of NETs containing granule proteins and chromatin, which mediate the effects NETS on the pathogenesis. The prior study demonstrates that NETs-associated carbamoylation and histones trigger osteoclast formation.[4] Actually, depleting protein component with protease could diminish the inflammatory effect caused by NETs stimulation. Of note, we found that neutrophil-specific protein, NE, which has a significant correlation with RA disease activity, could activate Rab5a- NF-κB signalling pathway to induce the cytokines secretion of macrophages.

In this study, we identified NE as the representative NETs containing cargo to preliminary investigate the potential mechanisms by which NETs induce immune responses. However, as a multicomponent complex, the mechanism of immune response induced by NETs remains to be explored. For example, in our study, we found that Rab5a deletion could effectively reduce internalisation of NETs by macrophages but could not completely inhibit NFκB activation. This is possible because the NETs-DNA can be recognised with macrophage surface receptors like Toll-like receptors and thus cause NFκB activation.[39] Other studies also suggest that NETs-DNA could aggravate inflammation in various diseases via cGas-sting or ERK/JNK mitogen-activated protein kinase pathways.[37 38 40] Moreover, proteins in NETs can be post-translational modified to alter structure, which leading more potential immune responses.

CONCLUSIONS

In summary, we provided clinical data confirming the correlation between NETs and RA pathogenesis, particularly in terms of inflammation. Second, NETs were discovered to be internalised by macrophages through a Rab5a-dependent manner, engulfed in early endosomes, leading to activation of the NF-κB signalling pathway. Above all, we suggested that Rab5a mediating the internalisation of NETs by macrophages and the proteins contained in NETs could be an appealing therapeutic strategy for RA.

Author affiliations

[1]Department of Rheumatology and Immunology, Nanfang Hospital , Southern Medical University, Guangzhou, Guangdong, China
[2]Department of Traditional Chinese Internal Medicine, School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong, China
[3]Laboratory Animal Research Center, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, China

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ORCID iDs

Junqing Zhu http://orcid.org/0000-0003-4642-2981
Shixian Chen http://orcid.org/0000-0002-7200-433X
Juan Li http://orcid.org/0000-0002-2977-2966

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