IgD enhances the release of neutrophil extracellular traps (NETs) via FcδR in rheumatoid arthritis patients

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Abstract

Background and Purpose Neutrophils and the release of neutrophil extracellular traps (NETs) play important roles in the pathogenesis of RA. However, IgD, which was abnormally higher in RA, has not been studied for its pathological role in neutrophil activation and NETs formation. Experimental Approach Peripheral blood of RA patients and healthy controls were collected and adjuvant-induced arthritis (AA) rat models were established. Body weights, the severity of arthritis of AA rats were monitored regularly. After being stimulated with IgD, expression of FcδR on neutrophils and NETs formation were analyzed with multiple approaches such as flow cytometry, scanning electron microscopy, western blot, and qPCR. IgD-Fc-Ig were used to block interactions between IgD-FcδR. Additionally, the effect of IgD-induced neutrophils or NETs on FLS was assayed. Key Results As a specific marker of NETs, the level of citrullinated histone H3 was positively correlated with sIgD and FcδR in RA patients. IgD enhances the release of NETs by activating neutrophils. IgD-Fc-Ig could significantly reduce NETs formation and FcδR expression on neutrophils in vitro. In vivo, IgD-Fc-Ig treatment significantly regulates the neutrophil activity and NETs formation. IgD-Fc-Ig could restrain IgD-induced neutrophil activation and NETs formation, thus inhibiting FLS proliferation. Conclusions and Implications Neutrophils were activated by IgD, which suggests that neutrophils play a role in inducing FLS proliferation in RA patients who have abnormally higher IgD levels, there by increasing the severity of the disease. Blocking FcδR inhibited neutrophil activation and may represent an additional attractive novel therapeutic strategy for the treatment of RA.

Ιγ∆ ενηανςες τηε ρελεασε οφ νευτροπηιλ εξτραςελλυλαρ τραπς (NETς) ια ΦςδP ιν ρηευματοιδ αρτηριτις πατιεντς

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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Competing interests

The authors declare that they have no competing interests.

Abstract

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Experimental Approach

Peripheral blood of RA patients and healthy controls were collected and adjuvant-induced arthritis (AA) rat models were established. Body weights, the severity of arthritis of AA rats were monitored regularly. After being stimulated with IgD, expression of FcδR on neutrophils and NETs formation were analyzed with multiple approaches such as flow cytometry, scanning electron microscopy, western blot, and qPCR. IgD-Fc-Ig were used to block interactions between IgD-FcδR. Additionally, the effect of IgD-induced neutrophils or NETs on FLS was assayed.

Key Results

As a specific marker of NETs, the level of citrullinated histone H3 was positively correlated with sIgD and FcδR in RA patients. IgD enhances the release of NETs by activating neutrophils. IgD-Fc-Ig could significantly reduce NETs formation and FcδR expression on neutrophils in vitro. In vivo, IgD-Fc-Ig treatment significantly regulates the neutrophil activity and NETs formation. IgD-Fc-Ig could restrain IgD-induced neutrophil activation and NETs formation, thus inhibiting FLS proliferation.

Conclusions and Implications

Neutrophils were activated by IgD, which suggests that neutrophils play a role in inducing FLS proliferation in RA patients who have abnormally higher IgD levels, thereby increasing the severity of the disease. Blocking FcδR inhibited neutrophil activation and may represent an additional attractive novel therapeutic strategy for the treatment of RA.

Keywords : Neutrophil; NETs; FcδR; IgD-Fc-Ig; adjuvant-induced arthritis

Abbreviations

AA, adjuvant-induced arthritis; ACPA, anti-citrullinated protein antibody; anti-CCP; anti-cyclic citrullinated peptide; CCK-8, Cell counting kit-8; CCP, cyclic citrullinated peptide; CitH3, Citrullinated histone H3; CFA, complete Freund's adjuvant; CRP, C-reactive protein; DG, IgD-Fc-Ig; ESR, erythrocyte sedimentation rate; FLS, fibroblast-like synoviocytes; FcδR, the IgD receptor; HC, healthy control; HE, hematoxylin-eosin; Ig, immunoglobulin; MHCII, major histocompatibility complex class II; MPO, myeloperoxidase; mIgD, transmembrane IgD; NE, neutrophil elastase; NETs, neutrophil extracellular traps; PAD4, Peptidylarginase

deiminase4; PB, peripheral blood; PMA, phorbol 12-myristate 13-acetate; RA, rheumatoid arthritis; RF, rheumatoid factors; sIgD, secreted IgD.

Introduction

Rheumatoid arthritis (RA) is characterized by synovial inflammation and hyperplasia, autoantibody production rheumatoid factor, and anti-citrullinated protein antibody (ACPA) (McInnes & Schett, 2017). Accumulating evidence has implicated the involvement of local immunoglobulin (Ig), such as IgG and IgA in the pathogenesis of RA(Mikuls et al., 2020; Sanz, 2016). Compared to other Igs, IgD remains an enigmatic antibody class. IgD includes transmembrane and secretory molecule, secreted IgD (sIgD) is released into the blood as well as respiratory, salivary, lacrimal, and mammary secretions (Chen & Cerutti, 2010; Chen et al., 2009; van der Linden et al., 2009). Recently, our studies investigated that in more than half of RA patients, the levels of sIgD were higher than the cut-off value of 80 μg/ml and sIgD levels were positively correlated with anti-cyclic citrullinated peptide (anti-CCP) and rheumatoid factors (RF) (Y. Wu et al., 2016; Zhang et al., 2020).

The new effector function of neutrophils was known as the product of neutrophil extracellular traps (NETs) have was found in 2004 (Brinkmann et al., 2004). NETs can damage host tissue and play a vital role in many diseases including infectious and non-infectious diseases, autoimmune diseases (Jorch & Kubes, 2017). NETs are decorated with citrullinated histone H3 (CitH3), myeloperoxidase (MPO), neutrophil elastase (NE), peptidyl arginase deiminase 4 (PAD4), and other intracellular molecules, with filamentous web-like structures (Honda & Kubes, 2018). NETs formation can be triggered by diverse stimuli (Alemán, Mora, Cortes-Vieyra, Uribe-Querol, & Rosales, 2016; Khan et al., 2017). In addition, anti-neutrophil cytoplasmic antibodies are considered the important autoantibodies to stimulate neutrophil activation, and then NETs formation (Söderberg & Segelmark, 2016). Thus, further investigation to explore the crucial factors in regulating NETs is important for RA.

Among the different Ig isotypes, IgA has been reported to enhance NETs formation via Fcα receptor I on neutrophils (Aleyd et al., 2014). As the plasma concentrations of IgD were abnormally higher in part of RA patients. But till now, the correlation between the abnormally increased IgD and the formation of NETs has not been reported yet.

Our previous study suggests that IgD can bind to a specific Fc receptor (FcδR or IgDR), also demonstrated that FcδR expression on fibroblast-like synoviocytes (FLS) in RA patients was higher than in HC (Y. J. Wu et al., 2017). Our group reported that high levels of IgD may accompany high expression FcδR in RA patients. Thus, investigating IgD-activated neutrophils may offer a potential therapeutic strategy that can be used to treat overexpressed NETs in RA. To block interactions between IgD-FcδR, IgD-Fc-Ig fusion protein was synthesized (patent license NO.ZL201510600762X, ZL201810692531X), it has been shown that IgD-Fc-Ig has the potential to be a therapeutic drug in RA via T cell regulation (Han et al., 2020; Hu et al., 2021; Zhang et al., 2020).

In this study, we sought to compare the expressions of FcδR on neutrophils in RA patients and HC. We further investigated how IgD can regulate NETs formation and IgD-Fc-Ig whether decrease excessive NETs in adjuvant-induced arthritis (AA) rat model.

Materials and methods

Samples collection and animals

The study protocol was carried out following the Declaration of Helsinki and approved by the Ethics Committee of Anhui Medical University (PZ-2021-022). Blood samples from 44 RA patients and 13 age-matched healthy donors were collected from the First Affiliated Hospital, Anhui Medical University. Written informed consent was obtained from each donor. Six-week-old male SD rats (160 ± 20 g) (Certificate No. 110324211103177231) were obtained from Beijing Si Bei Fu Laboratory Animal Technology Co., Ltd., and maintained in a specific pathogen-free animal laboratory at Anhui Medical University.

Clinical, serological assessments and human plasma IgD and CitH3 detection

Medical history, baseline characteristics, and demographic data were recorded and physical examination was performed for each patient at the beginning of the study. The levels of sIgD and CitH3 were determined in serum samples using the ELISA method according to the manufacturer's instructions. The clinical indexes of 44 RA patients were collected, including gender, age, RF (rheumatoid factor), CRP (C-reactive protein), ESR (erythrocyte sedimentation rate), and anti-CCP (anti-cyclic citrullinated peptide).

Drugs and reagents

The IgD-Fc-Ig fusion protein (Han et al., 2020) and IgG_1-Fc protein were supplied by the Institute of Clinical Pharmacology, Anhui Medical University (Hefei, China), and the purity of the IgD-Fc-Ig is more than 90%. Complete Freund's adjuvant (CFA)and Phorbol 12-myristate 13-acetate (PMA) were purchased from SigmaAldrich (St. Louis, MO, USA). rhTNFR:Fc fusion protein was purchased from Guojian Pharmaceutical Company (Shanghai, China). Anti-human/rat MPO (ab208670), anti-human/rat anti-histone H3 (citrulline R2+R8+R17) antibody (ab5103) were obtained from Abcam (USA). Brilliant Violet 421-anti-human CD15(323040), PE-anti-human-CD177 (315806), and PE-anti-human HLA-DR (307605) were purchased from Biolegend (USA). APC-anti-human-CD16 (561304) was purchased from BD Pharmingen (USA). FITC-anti-human myeloperoxidase(92008) was obtained from Invitrogen (USA). Human IgD protein (FITC-IgD) was labeled with FITC fluorescent labeling kit (DOJINDO LABORATORIES). Anti-human/rat NE(AF0010) were purchased from Affinity Biotechnology (China). Alexa Fluor 647-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Cat. No. FMS-Msaf48801, Fcmacs, China) second antibodies were purchased for this study.

Isolation and treatment of human neutrophils

Neutrophils were isolated from peripheral blood that was obtained from HC and RA patients using Polymorphprep^{TM}(Axis-Shield, Dundee, UK)according to the manufacturer's instructions. Briefly, density gradient centrifugation, then to remove any erythrocytes contamination of the neutrophils, resuspend the cell pellet in 3 ml of Solution ammonium chloride lysis buffer (0.83%(w/v)NH_4Cl, 10 mM Hepes-NaOH, PH 7.4) and incubate at 37degC for 5 min at room temperature (RT). After lysis, Neutrophils were washed with PBS. Cells were resuspended in RMPI 1640 supplemented with 10% fetal bovine serum (HyClone, Carlsbad, CA, USA). The cell preparations contained 90-95% neutrophils with viability higher than 95%, as established by exclusion with trypan blue.

For neutrophil stimulation, neutrophils were plated in 24-well plates at a concentration of $1.5x10^6$ cells/ml in 1ml RPMI1640medium for each well. Neutrophils were cultured for 6 h after incubated with various concentrations of IgD (0.1, 0.3, 1, 3, 10μg/ml) or IgD (3μg/ml) combined with IgD-Fc-Ig(1,3,9μg/ml). Following treatment for 6 h, neutrophils were collected for further analysis, and the supernatant was collected for NETs isolation.

Flow cytometric analysis

After cells were collected, surface and intracellular markers of neutrophils were evaluated by flow cytometry. After fixation, neutrophils were stained with fluorescently labeled mAbs against surface molecules (Brilliant Violet 421-anti-CD15, APC-anti-CD16, and FITC-FcδR). MPO and anti-histone H3 (citrulline R2 + R8 + R17)(CitH3) are highly specific markers of NETs and are used to evaluate NETs. In short, cells were fixed and permeabilized with a fixation/permeabilization kit (Invitrogen) and incubated with combinations of the following antibodies; FITC-conjugated anti-MPO antibody and rabbit anti-CitH3 antibody as a primary antibody for 30 min at 4, then use Alexa Fluor 647 goat IgG anti-rabbit as a secondary antibody to detect CitH3. Neutrophils untreated with primary antibodies were used as a negative control. Each incubation was followed by a wash with 2% BSA in PBS and centrifugation at 3000 rpm at 4°C for 5 minutes. Samples were then resuspended in 2% BSA in PBS. All data were collected using flow cytometry (Beckman Coulter)and analyzed with CytExpert (2.1.0.92).

Imaging flow cytometry acquisition and analysis

It has described the rapid quantification of NETs *in vitro* and whole blood samples by imaging flow cytometry(Lelliott et al., 2019). Neutrophils (3×10^5) were treated as mentioned above. Subsequently, cells were resuspended in 80 ml HBSS-2% BSA. The cells were analyzed in an imaging flow cytometer (ImageStreamX MkII) using the 60 objectives. Cells were filtered through a cell strainer directly before the acquisition, a minimum of 30,000events per sample was acquired. Data were analyzed by the IDEAS software package with gating first on single cells; second, on focused cells; and third, with criteria on MPO and CitH3 positive cells. The same analysis template file was used for each sample for gating.

NETs isolation

After 6-hour treatment of neutrophils, supernatants were collected and centrifuged at 450g for 5 min and then by centrifugation (1,8000g, 10 min) at 4°C. NETs were prepared by suspending the cold PBS.NETs supernatants were transferred to a fresh tube, obtained DNA concentrations in each sample were measured with spectrophotometry. An adequate concentration in the sample should range between 140-180 ng/μl and be stored at-80°C until used (Najmeh, Cools-Lartigue, Giannias, Spicer, & Ferri, 2015).

Western blot analysis

Neutrophils from HC were collected after treatment. Then, cells are lysed in lysis buffer on ice for 30 minutes. Primary antibodies of MPO (rabbit,1:500, ab208670), NE (rabbit,1:300, AF0010), CitH3 (rabbit,1:500,ab5103), and β-actin (mouse, 1:1000, Cat. No. 66009-I-Ig, proteitech, USA) were then incubated at 4overnight, and goat anti-rabbit (1:30000, Cat. No. 7074P2, Cell Signaling, Germany)/mouse conjugated secondary antibodies (1:30000, Cat. No. SA00001-1, Proteintech, USA) were incubated for 2 h at 37 °C. The membrane was scanned using GS-700 Imaging Densitometer. The image was analyzed with Image J software(1.51j8).

Confocal immunofluorescence microscopy analysis

Neutrophils were seeded at a density of 3×10^5cells/ml on glass coverslips in a 24-well plate. The cells were incubated with or without IgD (3μg/ml) and IgD-Fc-Ig (10μg/ml) for 6 h. Then, cells were fixed with 4% paraformaldehyde for 15 min and were permeabilized with 0.1% Triton X-100 in PBS for 10 min. After blocking with 5% BSA/PBS for 1 h, cells were incubated with anti-MPO, anti-NE, or anti-CitH3 antibodies at 4 overnight and subsequently with Alexa Fluor 647-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG was incubated at a 1:200 ratio in a 4 incubator for 1 h. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, cat. No. C1005) for 5 min. Images were acquired on a Leica SP8 TCS STED ×3 confocal microscope system.

Scanning electron microscopy analysis

Add 100μl cells to the 24-well plate and keep it for 10 minutes. Then cells were fixed with 2.5 % glutaraldehyde for 30min,then the cells were washed with PBS. Thereafter, the samples were dehydrated in an ascending gradient of 35%, 50%, 75%, 95%,100% ethanol with 10 min. Transfer the sample from 100% ethanol to 1:2 (HMDS: 100% ethanol) solution, and let stand for 20 minutes, then transfer to 2:1 (HMDS: 100% ethanol) solution and let stand for 20 minutes. Finally, transfer the sample to 100% HMDS solution and overnight in a ventilated place.Images were scanned under a scanning electron microscope (ZEISS GeminiSEM 300) (Fischer, Hansen, Nair, Hoyt, & Dorward, 2012).

Induction and treatment of AA rats

We established a rat adjuvant-induced arthritis (AA) model via intradermal injection of CFA. Each arthritis rat was induced by a single intradermal injection of 0.15ml of CFA into the right hind paw for the AA model rat (Luo et al., 2020). Rats were maintained in a specific pathogen-free animal laboratory at Anhui Medical University. The rats were divided into six treatment groups: normal, AA model, IgD-Fc-Ig (3,9 mg/kg), IgG1-Fc (9mg/kg), Etanercept (3 mg/kg). After the onset of arthritis at day 14, the rats were treated with IgD-Fc-Ig, IgG1-Fc, or etanercept through subcutaneous injection (every three days, for 24 days). The rats in the normal control group were treated with an equal volume by subcutaneous injection.

Arthritis assessment

Two independent observers with no knowledge of the treatment protocol evaluated the severity of AA. The severity of arthritis was evaluated every three days about arthritis index, paw volume,the number of joint swelling, global scoring,and body weight.The evaluation criteria of arthritis index are as follows: 0: no redness and swelling; 1: slight swelling of the small toe joint; 2: swelling of the toe joint and plantar; 3: swelling of the paw below the ankle; 4: swelling of all-paw including the ankle. The evaluation criteria of the swelling joint count are as follows: one foot of each rat counted one ankle (or wrist) and five fingers (toe) joints. The evaluation criteria of global scoring are as follows: Ears: 0: no nodules or redness, 1: nodules or redness in one ear, 2: nodules or redness in both ears; Nose: 0: no connective tissue swelling, 1: obvious connective tissue swelling; Tail: 0: no nodules, 1: nodules; forefoot: 0: no swelling, 1: swelling of one forefoot, 2: swelling of two forefeet; Hindfoot: 0: no swelling, 1: swelling of one hind paw, 2: swelling of two hind paws. The rat paw volume was measured before CFA injection and after CFA injection using a plethysmometer (Panlab, Spain).

Rat plasma sIgD, CitH3, and anti-CCP2 Detection

Rat whole blood was collected on day 35 and then centrifuged at 2,500 rpm for 10 min to isolate the plasma. Rat plasma levels of sIgD, citrullinated histone (CitH3), and anti-CCP2 were quantified by ELISA (Shanghai Enzyme-linked Biotechnology Co. Ltd). The absorbance at 450 nm of each sample was detected with an Infinite M1000 PRO microplate reader.

Histopathological analysis

The hind paws and spleen of rats in each group were fixed in 4% formaldehyde. For histological assessment, the fixed joint, and knee tissues were decalcified and embedded in paraffin, and 5 μm slides were prepared and stained with hematoxylin-eosin (HE). Inflammatory cell infiltration, cartilage erosion, vascular pannus formation, and synovial hyperplasia were semi-quantitatively assessed for each joint. The spleen tissue sections were evaluated for parameters such as peripheral arterial lymphatic sheath density, germinal center hyperplasia, marginal zone hyperplasia, and red pulp. Cartilage damage was evaluated by staining with safranin-O.

Measurement of apoptosis

Cell apoptosis was analyzed after Annexin V-FITC/PI double staining, collection of neutrophils from rat peripheral blood, cells were washed by PBS twice, centrifuged, and resuspended in 500 μl binding buffer 5μl Annexin V-FITC was added to the cell suspension and mixed thoroughly, kept in the dark 15 min, then 10μl PI was added to the cell suspension 5 min, followed by detected by flow cytometry. Cell apoptosis was analyzed with CytExpert (2.1.0.92).

Measurement of ROS production

Neutrophils were incubated with 10μM dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) as a peroxide-sensitive fluorescent probe for 30 min at 37 °C and washed three times with serum-free RPMI 1640. Intracellular ROS accumulation was measured via flow cytometry (Beckman Coulter, Beckman Coulter Biotechnology, CA, USA).

Immunofluorescence was performed to detect the levels of neutrophils

Rat ankle tissues were fixed with 4% paraformaldehyde overnight and subsequently embedded in paraffin. Serial sections of 5 μm thickness were cut and deparaffinized in xylene and rehydrated by decreasing alcohol-water series (100%-90%-75% ethanol-water). Then, the antigen was retrieved with 2% citrate antigen retrieval solution at 100 for 10 minutes and blocked with 5% BSA-PBS buffer for 1 hour at room temperature. Then, the sections were incubated separately with MPO antibodies at 4 overnight. Nuclei were counterstained with DAPI (2 μg/ml) and washed three times with PBS. Fluorescence images were captured using the 3D HISTECH (Pannoramic MIDI).

Real-time quantitative PCR (qPCR)

To quantify the mRNA expression levels of MPO, PAD4, and NE. The samples were collected from the treated human neutrophils and rats' spleen. Total RNA from each group was extracted in 1 mL Trizol® reagent (Invitrogen, CA, USA), and centrifuged at 12,000 rpm at 4 for 10 min. The extracted total RNA was reverse-transcribed into cDNA.Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control gene for mRNA expression. PAD4, MPO, and NE genes were synthesized using specific primer sequences (Sangon Biotech, China). Transcription levels of target genes were analyzed by qPCR using an ABI 7500 (Applied Biosystems) and SYBR Green Master Mix (Vazyme). The novel primer sequences of human and rat PAD4, MPO, and NE are as follows:

Human:

MPO- Forward 5' -CCAAATTGCAGTGGATGAGATC-3'

Reverse 5' -AGATGTTGTTCTTAGACACGGT-3'

NE-Forward 5' -CTCCCGCTGCTCCCTCTGTC-3'

Reverse 5' -TCACCGCTCAGAACCTCAGTCTC-3'

PAD4-Forward 5' -CCCTGCCCTCGTGTTGCTTTC-3'

Reverse 5' -AACTCCACTCCTCACTCCACATCC-3'

GPADH- Forward 5' -GACTTCAACAGCGACACCCACTC-3'

Reverse 5' -CCCAGCCACATACCAGGAAATGAG-3'

Rat:

PAD4-Forward 5' -CAGCGGTTATTCCAGCAGTGAGAG-3'

Reverse 5' -CCCACAAAGAGCCAATCGGAGAAG-3'

MPO-Forward 5' -CACACCCTCATCCAACCCTTCATG-3'

Reverse 5' -GCCACCTTCCAGCACAACTCTC-3'

NE-Forward 5' -CCGAGGAGGCTGTGGATCTGG-3'

Reverse 5' -GGTCATCATGGCTGCGGATAATGG-3'

GAPDH-Forward 5' -ACGGCAAGTTCAACGGCACAG-3'

Reverse 5' -CGACATACTCAGCACCAGCATCAC-3'

Isolation and treatment of FLS

FLS from RA patients (RA FLS) and AA model rats (AA FLS) were isolated (Song et al., 2020). For CCK-8 analysis, RA FLS were cultured in the presence or absence of generated human NETstreated with IgD (3μg/ml), IgD (3μg/ml)+IgD-Fc-Ig(1,3,10ug/ml), PMA(20nmol), or DnaseI(100u/ml)in 96-well plates for 48 hours at 37°C in an atmosphere of 5% CO_2. In the AA rat study, neutrophils were isolated from rat peripheral blood in six different groups (normal, AA model, IgD-Fc-Ig (3,9 mg/kg), IgG_1-Fc (9mg/kg), and Etanercept (3 mg/kg) groups) were co-cultured with AA FLSfor 18h. For flow cytometric assay, RA FLS were added to 6-well plates at $1x10^6$ cells/well, stimulated with NETs from different treatment groups, and cultured at 37 degC with 5% CO_2. After 48 h, RA FLS were collected and centrifuged at 3000/rpm for 5 min at 4 degC. Cells were stained with labeled anti-human HLA-DR for 30 min at 4 degCand analyzed by flow cytometry as mentioned before.

Cell counting kit-8 (CCK-8) analysis

To assess the proliferative responses of cultured cells, a CCK-8 (Cat. No. CK04, DOJINDO, Japan) assay was used to explore the viability of FLS. 10 μl of CCK-8 solution was added per well at the end of treatment. The cells were incubated for 4 h at 37 °C in an atmosphere containing 5% CO_2, and the absorbance at 450 nm was measured on a microplate reader (Infinite M1000 PRO, TECAN, Switzerland).

Statistical analysis

Data were analyzed using GraphPad Prism 7 software. For samples with non-Gaussian distribution, we used Student's 2-tailed Mann-Whitney U test. Multiple comparisons were analyzed by one-way ANOVA with Dunnett'sT3 test. Correlations were calculated using Spearman's correlation. Results are presented as the means ± SEM. P values of less than 0.05 were considered significant.

Results

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We identified the clinical and demographic characteristics of 44 RA patients and 13 healthy controls (**Table1**). Then assessed neutrophil FcδR using immunofluorescence microscopy and flow cytometry in neutrophils isolated from RA or HC. A significant increase in neutrophil FcδR in RA patients' neutrophils compared to HC was observed (**Fig.1A-C**). Next, we determined whether plasma CitH3 levels in RA patients correlate with sIgD and FcδR. As compared with plasma samples from 13 HC, RA patients showed higher levels of CitH3 (5.8289±1.692 ng/ml *vs.* 2.268±0.211 ng/ml) and sIgD (87.475±34.385 μg/ml *vs.* 43.860±7.708 μg/ml) in their plasma compared to HC (**Figure.1D and E**). CitH3 is generally regarded as relatively specific for NETs formation. The data showed that sIgD and CitH3 demonstrated a significant correlative relationship (**Figure.1F**), the association between FcδR and CitH3 was significant as well (**Figure.1G**).

Effects of IgD on neutrophilsfrom Healthy Controls.

Following detection of FcδR and the presence of FcδR in RA patients, we sought to determine whether neutrophils were capable of inducing NETs release and the increase of FcδR by IgD. This prompted us to examine the effect of IgD on neutrophils. To confirm the effect of IgD on neutrophil activation in HC, neutrophils were treated with IgD(0.1 ,0.3 ,1, 3 and 10 μg/ml)within 6h. We observed changes of FcδR in neutrophils (**Figure.2A, B)** similar to those found in RA patients with active neutrophils. Then, we examined NETs formation by neutrophils in the presence or absence of IgD. NETting neutrophils, defined as MPO^+CitH3^+ events were increased in IgD (3 and 10 μg/ml) treatment groups (**Figure.2C, D)** , indicating that signs of NETs were increased than control. By western blotting, little CitH3 was observed in the control group, while MPO, NE, and CitH3 were observed to increase in the IgD (3μg/ml) group(**Figure.2E-H)** .

Effects of IgD-Fc-Ig on IgD activated neutrophils.

The observations described above prompted us to explore the role of IgD-Fc-Ig in neutrophils activated by IgD (3μg/ml). Human neutrophils treated with IgD (3μg/ml) have been shown to induce NETs forming and FcδR expression from peripheral blood in HC. To confirm this finding, neutrophils were incubated with different concentrations of IgD-Fc-Ig(1,3, 10 μg/ml) combined with IgD (3μg/ml) for 6 h. Then, the effect of IgD-Fc-Ig on NETs formation and FcδR expression in neutrophils was observed by flow cytometry. Compared with the IgD(3μg/ml) group, IgD-Fc-Ig(10μg/ml) strongly inhibited NETs formation and FcδR expression (**Figure.3A-D**). The mRNA of MPO, PAD4, NE after IgD-Fc-Ig-treatment for 6 h was measured via qPCR**(Figure.3E-G)** . The result showed significantly decreased MPO, NE, and PAD4 mRNA levels compared to the IgD(3μg/ml) group.

Next, we examined NETs formation by neutrophils in the presence or absence of IgD-Fc-Ig observed by imaging flow cytometry, confocal microscopy, and scanning electron microscopy. First, We intended to visually observe NETs formation by imaging flow cytometry. Consistent with the findings by flow cytometry, MPO and CitH3 fluorescent signals were increased after IgD (3μg/ml) treatment, and IgD-Fc-Ig (10 μg/ml) could significantly downregulate the signals (**Figure.4A, B**). Next, we visualized NETs location using confocal microscopy, neutrophils from the IgD (3 μg/ml) group significantly augmented NETs which were presented

by MPO, NE, and CitH3, and IgD-Fc-Ig (10 μg/ml) could decrease the release of NETs (**Figure.4C-E**). And some neutrophils appeared non-activated, with a distinctive lobulated nucleus while others showed signs of nuclear decondensation, which is a characteristic of NETs. Scanning electron microscopy analysis showed that the surface of neutrophils covered with plasma membrane elaborated into irregular ridges or small ruffles in the normal group. In IgD (3 μg/ml) group, it has shown a range of morphological variations. Neutrophils were covered mainly by a smooth plasma membrane in which there were crater-like pits. Those neutrophils which have plasma membrane contours consistent with the existence of bulges, and pits on their surface had cytoplasm which seemed markedly disordered. And IgD-Fc-Ig (10μg/ml) can significantly reduce NETs generation(**Figure.4F**).

Effects of IgD-Fc-Ig on inflammatory index assessment of rat

Secondary joint inflammation was apparent on approximately day 14 following the first immunization. AA rat developed typical manifestations of severe arthritis and showed front and hind paw swelling and redness **(Figure.5A, B)** . Administration of IgD-Fc-Ig (3 and 9mg/kg) significantly alleviated these abnormalities in varying degrees **(Figure.5C and Supplementary figure 1)** . Results from the H&E staining and Safranin O-fast green cartilage staining of the ankle joint sections showed that synoviocyte proliferation and inflammatory infiltration, pannus formation, damaged articular cartilage, as well as cartilage erosion, were significantly attenuated following administration of IgD-Fc-Ig (3 and 9mg/kg)**(Figure.6A-F)** . The effects of IgD-Fc-Ig(3 and 9mg/kg) on AA rats were similar to the effects of the positive control drug etanercept. Similarly, the histopathology of the spleen, which was characterized by the density of periarterial lymphatic sheath, the emergence of germinal centers, marginal zone hyperplasia, and red pulp proliferation was significantly alleviated by IgD-Fc-Ig (9mg/kg) treatment (**Figure.6H-L**). Etanercept treatment exhibited similar effects to that observed with IgD-Fc-Ig treatment. Thus, IgD-Fc-Ig treatment significantly alleviated arthritis in AA rats.

Effects of IgD-Fc-Ig on the levels of IgD, CitH3, and CCP2 in AA rat

Rat serum levels of sIgD, CitH3, and CCP2 were detected by ELISA. As we expected, IgD levels in AA rats were significantly higher than those in normal rats. Compared with the normal group rats, the levels of serum CitH3 and CCP2 were also significantly elevated in AA rats. With the administration of IgD-Fc-Ig (9mg/kg), sIgD, CitH3, and CCP2 levels were significantly reduced in AA rats (**Figure5D-F**).

Effects of IgD-Fc-Ig on the neutrophils function of AA rats

In comparison with the normal group, the model group showed a significant increase in ROS levels from neutrophils. Administration of IgD-Fc-Ig (3 and 9mg/kg) significantly reduced the neutrophil ROS production (**Figure.7A, B**). The levels of apoptosis neutrophils in AA rats were significantly decreased, IgD-Fc-Ig (9mg/kg)administration could promote neutrophil apoptosis (**Figure.7C, D**). Etanercept (3 mg/kg) could downregulate the expression of ROS and increase the apoptosis rate of neutrophils in AA rats. We further performed an immunohistochemistry assay to assess MPO expression in the ankle joints of rats. The results showed that the expression of MPO was low in the normal rats. The high expression of MPO was observed as rings associated with pannus formation. IgD-Fc-Ig (9mg/kg) treated rats exhibited decreased expression of MPO in the ankle joints (**Figure.6G**). There were significant effects of IgD-Fc-Ig on MPO, NE, PAD4 mRNA expression, IgD-Fc-Ig (9mg/kg) significantly decrease the expression of MPO, NE, CitH3 mRNA relative to neutrophils in the AA model group (**Figure.7F-H**). Etanercept (3 mg/kg) exhibited similar effects to that observed in IgD-Fc-Ig treatment groups.

Effects of IgD andIgD-Fc-Ig on neutrophil-FLS interaction.

To observe the effect of IgD-induced NETs RA FLS function. We observed the major histocompatibility complex (MHC) class II expression and proliferation of FLSs after cocultured with NETs isolated from IgD and IgD-Fc-Ig treatment groups. The expression of surface MHCII was upregulated in RA FLS in IgD (3μg/ml) stimulated NETs group compared to FLS not exposed to NETs, and IgD-Fc-Ig(9μg/kg) NETs group could downregulate the expression of MHCII **(Figure.8B, C)** .NETs stimulated with IgD (3μg/ml)

increased the proliferation of FLS, while IgD-Fc-Ig(10μg/ml) treatment NETs decreased FLS proliferation after cocultured for 48h**(Figure.8D)** . To confirm our finding, *in vivo* study we observed the proliferation changes of AA FLS cocultured with neutrophils isolated from different groups (normal, AA model, IgD-Fc-Ig (3,9 mg/kg), IgG1-Fc (9mg/kg), and etanercept (3 mg/kg)). Compared with the control group, the proliferation of AA FLS increased significantly after coculturing with neutrophils from the AA model rats group, neutrophils from IgD-Fc-Ig (9mg/kg) and etanercept groups could significantly decrease the abnormal proliferation of FLS **(Figure.8E)** .

Discussion

RA is a systemic inflammatory autoimmune disorder in which genetic and environmental risk factors contribute to disease development. Immunoglobulins are essential components of the adaptive immune system to fight against pathogens. For the past six decades, IgD has remained an enigmatic antibody that belongs to an ancient form of immune surveillance and can profoundly influence immune responses mediated by binding to effector molecules such as the Fc receptor via its carboxyl-constant regions. Several FcRs, namely FcR for IgG, IgE, IgA, and IgM, have been characterized at both the protein and nucleic acid levels. In contrast, FcδR has defied genetic identification. In the past decade, our research group has been committed to the research of IgD and FcδR in RA (Basu et al., 2016; Hu et al., 2021; Y. Wu et al., 2016). Our previous experiments proved the existence of FcδR on T cells, B cells, and FLSs through quantitative immunofluorescence techniques (Dai et al., 2019; Y. J. Wu et al., 2017). It has been demonstrated that IgD serves as a ligand for FcδR expressed on $CD4^+$ T cells (Coico et al., 1985). As we know, neutrophils express a large number of cell surface receptors for the recognition of microbial invasion, which are capable of innate recognition of microbial structures or activation of the adaptive immune response (Futosi, Fodor, & Mócsai, 2013). For instance, when IgA immune complexes cross-linked IgA Fc receptor (FcαRI) on neutrophils results in neutrophil recruitment and the release of NETs, which induces joint damage in RA patients (Aleyd, Al, Tuk, van der Laken, & van Egmond, 2016). Our group has reported abnormal levels of IgD in plasma and FcδR on $CD4^+$T cells from RA patients and arthritis rodent animal models. While it is currently not known the expression of FcδR and roles of IgD on neutrophils. In this study, the expression of FcδR on human neutrophils was observed. As a result, the expression of FcδR was higher on RA-neutrophils than on HC-neutrophils.

We have reported that in RA patients the levels of sIgD were increased (Y. Wu et al., 2016) and positively correlated with RF and anti-CCP (Zhang et al., 2020). Here we showed that besides plasma IgD, the levels of plasma CitH3 in RA patients were significantly higher than that of HC. The association between plasma IgD, FcδR expression on neutrophils, and plasma CitH3 in RA patient blood samples were observed. MPO, NE, and CitH3 are proposed as target biomarkers reflecting NETs formation (Mauracher et al., 2018). MPO catalyzes the oxidation of halides by hydrogen peroxide. NE is a neutrophil-specific serine protease that degrades virulence factors and kills bacteria (Papayannopoulos, Metzler, Hakkim, & Zychlinsky, 2010). We observed that the levels in plasma CitH3 in RA patients were significantly higher than in HC. It has also been found that IgD significantly triggered NETs release and these structures were composed of DNA decorated with MPO, NE, and CitH3, which revealed the typical NETs-like structures triggered by IgD. Scanning electron microscopy analysis was used to observe the morphology of IgD-induced NETs. Our data clearly show that IgD significantly increases the expression of FcδR and NETs formation in neutrophils, indicating that sIgD and FcδR as potential specific immunological hallmarks in RA diagnosis and therapeutic targets.

The aberrant formation of NETs from neutrophils has been demonstrated in the pathogenesis of RA (Berthelot, Le Goff, Neel, Maugars, & Hamidou, 2017). First, NETs are responsible for autoantigen formation in RA patients. Second, during NETs formation, potent enzymes which can promote tissue injury are released into the extracellular space. Third, impaired clearance of NETs in autoimmune diseases prolongs the presence of active NETs and their components and, in this way, accelerates immune responses (Fousert, Toes, & Desai, 2020). Thus, NETs may be an important therapeutic target in treating RA. Although there have been many medications taken for RA therapy in recent years (Smolen et al., 2020), they are not sufficiently safe or effective. Therefore, an increasing number of methods for the targeted treatment of RA

with FcRs are emerging (Y. Wu, Pan, Hu, Zhang, & Wei, 2021). To neutralize overexpressed IgD in RA, we synthesized a fusion protein known as IgD-Fc-Ig successfully by connecting human IgD-Fc and IgG1-Fc domains. We herein used *in vitro* IgD treatment to simulate the aberrant formation of NETs in RA. As we had hypothesized, IgD-Fc-Ig could selectively inhibit the IgD-induced formation of NETs in HC *in vitro* , and the imbalance of NETs was restored. After treatment with IgD-Fc-Ig, the specific blocking of the activated neutrophils induced by IgD suggests that IgD-Fc-Ig may repress neutrophil activation and NETs generation through selectively targeting FcδR.

We further assess the therapeutic effects and pharmacology mechanisms of IgD-Fc-Ig on neutrophils in the AA rat model. Consistent with our previous reports in CIA animal models (Han et al., 2020; Tai et al., 2021; Zhang et al., 2020), the disease progression of AA rats was significantly alleviated after IgD-Fc-Ig treatment, restored the arthritis index, global scoring, paw volume, and swollen joint counts of AA rats. AA rats presented higher levers of IgD, CitH3, and CCP2 in serum, administration of IgD-Fc-Ig significantly reduced abnormal levels of IgD, CitH3, and CCP2, suppressed the proliferation of synoviocytes, cartilage erosion, inflammatory cell infiltration, and pannus formation of AA rats' joints, and improved the pathological deterioration of joints.

The NETs signal-transduction cascade included the generation of ROS, nuclear de-lobulation involving the MPO and NE, and chromatin modification via the citrullination of histones by PAD4. These signaling elements are significantly enhanced to promote NETs in RA compared with HC (Sur Chowdhury et al., 2014). NETs can be initiated by ROS, it can also promote the morphological changes that occur during NETs (Remijsen et al., 2011). AA rats presented a higher level of ROS in plasma, IgD-Fc-Ig (9mg/kg) administration could significantly inhibit ROS overproduction. It has been shown that mitochondrial ROS combine with increased intracellular calcium levels to activate PAD enzymes (e.g., PAD4) leading to hypercitrullination of histones and NETs release (Douda, Khan, Grasemann, & Palaniyar, 2015). And consistent with our*in vitro* study results, the mRNA expression of PAD4 was significantly reduced in AA rats after IgD-Fc-Ig (9mg/kg) administration.

Apoptosis is crucial for neutrophil turnover and the resolution of inflammation (Greenlee-Wacker, 2016). The levels of apoptosis were significantly delayed in AA rats compared to normal rats. However, following IgD-Fc-Ig treatment, we discovered that IgD-Fc-Ig (9mg/kg) significantly promoted apoptosis of activated neutrophils. In the ankle joints of rats, high expression of MPO was observed as rings associated with pannus formation andIgD-Fc-Ig treated rats exhibited decreased expression of MPO. Our *in vivo* data indicated that IgD-Fc-Ig could reduce neutrophil infiltration in the inflamed tissue.

Recently, it has been described that neutrophil-FLS interactions in the RA synovium might play crucial roles in the promotion of joint damage and the development of systemic dysregulated innate and adaptive immunity against citrullinated intracellular autoantigens (Carmona-Rivera et al., 2017). We studied the effect of neutrophils and NETs on FLS from RA patients and AA rats. We observed that the production of NETs treated with IgD could induce not only the proliferation but also surface expression of MHCII on RA FLS. IgD-Fc-Ig could repress the promotion effect of IgD stimulated NETs. At the same time, after co-culture of rat neutrophils and FLS, the proliferation of FLS from the IgD-Fc-Ig group and etanercept group rats were significantly reduced compared with AA rats. FLS can acquire nonprofessional antigen-presenting cell capabilities in the RA synovial environment by internalizing NETs containing citrullinated autoantigens, up-regulating MHCII, and presenting the modified autoantigens to $CD4^+$ T cells (Carmona-Rivera et al., 2017). Our data indicated that neutrophil-FLS interactions may take part in the development of the AA model. There were parallels between human *in vitro* results and rats *in vivo* assays, suggesting that the therapeutic mechanism of IgD-Fc-Ig may be related to the regulation of neutrophil-mediated immune responses.

Taken together, our study showed a correlation between aberrant NETs formation and IgD-FcδR in RA pathogenesis. We demonstrated that cross-linking of FcδR by IgD may act via a positive feedback loop mechanism contributing to neutrophil activation and leading to NETs generation and processing of RA (**Figure 9**). IgD-Fc-Ig selectively blocks IgD binding to FcδR, suppresses the abnormal NETs generation and

activation of neutrophils induced by IgD, recovers the imbalance of NETs. However, further studies focusing on the molecular mechanisms of IgD-Fc-Ig on FcδR and downstream signaling pathways in neutrophils are needed. This may help to explain how neutrophils act in patients with RA and animal models and the exact pharmacological therapeutic effect of IgD-Fc-Ig.

Conclusions

In summary, activation of neutrophils was reduced when FcδR was blocked. Our results show that neutrophils are activated by IgD via FcδR. Because neutrophils are found abundant in RA patients, and IgD can activate neutrophils. After releasing NETs and upregulating the CitH3 and anti-CCP levels, which are involved in the collaboration of FLS, in this way worsens disease in RA patients. IgD-induced inflammation by neutrophils might be prevented by blocking FcδR using IgD-Fc-Ig therapy, which may help to alleviate disease in RA patients.

Author contributions

WWP performed the experiments and wrote the manuscript. XXH, QLX, and JX participated in the experiments. ZYC and JJH collected the clinical samples and information. TL analyzed clinical results. WW conceived of the study and revised the manuscript. YJW designed the study, participated in the experiments, and revised the manuscript. All authors read and approved the final manuscript.

Reference

Alemán, O. R., Mora, N., Cortes-Vieyra, R., Uribe-Querol, E., & Rosales, C. (2016). Differential Use of Human Neutrophil Fcγ Receptors for Inducing Neutrophil Extracellular Trap Formation. *J Immunol Res, 2016* , 2908034. doi:10.1155/2016/2908034

Aleyd, E., Al, M., Tuk, C. W., van der Laken, C. J., & van Egmond, M. (2016). IgA Complexes in Plasma and Synovial Fluid of Patients with Rheumatoid Arthritis Induce Neutrophil Extracellular Traps via FcαRI.*J Immunol, 197* (12), 4552-4559. doi:10.4049/jimmunol.1502353

Aleyd, E., van Hout, M. W., Ganzevles, S. H., Hoeben, K. A., Everts, V., Bakema, J. E., & van Egmond, M. (2014). IgA enhances NETosis and release of neutrophil extracellular traps by polymorphonuclear cells via Fcα receptor I. *J Immunol, 192* (5), 2374-2383. doi:10.4049/jimmunol.1300261

Basu, M., Lenka, S. S., Paichha, M., Swain, B., Patel, B., Banerjee, R., . . . Samanta, M. (2016). Immunoglobulin (Ig) D in Labeo rohita is widely expressed and differentially modulated in viral, bacterial and parasitic antigenic challenges. *Vet Immunol Immunopathol, 179* , 77-84. doi:10.1016/j.vetimm.2016.08.008

Berthelot, J. M., Le Goff, B., Neel, A., Maugars, Y., & Hamidou, M. (2017). NETosis: At the crossroads of rheumatoid arthritis, lupus, and vasculitis. *Joint Bone Spine, 84* (3), 255-262. doi:10.1016/j.jbspin.2016.05.013

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., . . . Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science, 303* (5663), 1532-1535. doi:10.1126/science.1092385

Carmona-Rivera, C., Carlucci, P. M., Moore, E., Lingampalli, N., Uchtenhagen, H., James, E., . . . Kaplan, M. J. (2017). Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. *Sci Immunol, 2* (10). doi:10.1126/sciimmunol.aag3358

Chen, K., & Cerutti, A. (2010). New insights into the enigma of immunoglobulin D. *Immunol Rev, 237* (1), 160-179. doi:10.1111/j.1600-065X.2010.00929.x

Chen, K., Xu, W., Wilson, M., He, B., Miller, N. W., Bengtén, E., . . . Cerutti, A. (2009). Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. *Nat Immunol, 10* (8), 889-898. doi:10.1038/ni.1748

Coico, R. F., Xue, B., Wallace, D., Pernis, B., Siskind, G. W., & Thorbecke, G. J. (1985). T cells with receptors for IgD. *Nature, 316* (6030), 744-746. doi:10.1038/316744a0

Dai, X., Wu, Y. J., Jia, X. Y., Chang, Y., Wu, H. X., Wang, C., & Wei, W. (2019). Immunoglobulin D (IgD) and IgD receptor expression in diffuse large B-cell lymphoma. *Hematology, 24* (1), 544-551. doi:10.1080/16078454.2019.1642553

Douda, D. N., Khan, M. A., Grasemann, H., & Palaniyar, N. (2015). SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis induced by calcium influx. *Proc Natl Acad Sci U S A, 112* (9), 2817-2822. doi:10.1073/pnas.1414055112

Fischer, E. R., Hansen, B. T., Nair, V., Hoyt, F. H., & Dorward, D. W. (2012). Scanning electron microscopy. *Curr Protoc Microbiol, Chapter 2* , Unit 2B.2. doi:10.1002/9780471729259.mc02b02s25

Fousert, E., Toes, R., & Desai, J. (2020). Neutrophil Extracellular Traps (NETs) Take the Central Stage in Driving Autoimmune Responses.*Cells, 9* (4). doi:10.3390/cells9040915

Futosi, K., Fodor, S., & Mócsai, A. (2013). Reprint of Neutrophil cell surface receptors and their intracellular signal transduction pathways.*Int Immunopharmacol, 17* (4), 1185-1197. doi:10.1016/j.intimp.2013.11.010

Greenlee-Wacker, M. C. (2016). Clearance of apoptotic neutrophils and resolution of inflammation. *Immunol Rev, 273* (1), 357-370. doi:10.1111/imr.12453

Han, L., Zhang, X. Z., Wang, C., Tang, X. Y., Zhu, Y., Cai, X. Y., . . . Wei, W. (2020). IgD-Fc-Ig fusion protein, a new biological agent, inhibits T cell function in CIA rats by inhibiting IgD-IgDR-Lck-NF-ϰB signaling pathways. *Acta Pharmacol Sin, 41* (6), 800-812. doi:10.1038/s41401-019-0337-2

Honda, M., & Kubes, P. (2018). Neutrophils and neutrophil extracellular traps in the liver and gastrointestinal system. *Nat Rev Gastroenterol Hepatol, 15* (4), 206-221. doi:10.1038/nrgastro.2017.183

Hu, X. X., Zhang, A. J., Pan, W. W., Xin, Q. L., Chen, J. Y., Zhang, L. L., . . . Wei, W. (2021). An IgD-Fc-Ig fusion protein restrains the activation of T and B cells by inhibiting IgD-IgDR-Lck signaling in rheumatoid arthritis. *Acta Pharmacol Sin* . doi:10.1038/s41401-021-00665-w

Jorch, S. K., & Kubes, P. (2017). An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med, 23* (3), 279-287. doi:10.1038/nm.4294

Khan, M. A., Farahvash, A., Douda, D. N., Licht, J. C., Grasemann, H., Sweezey, N., & Palaniyar, N. (2017). JNK Activation Turns on LPS- and Gram-Negative Bacteria-Induced NADPH Oxidase-Dependent Suicidal NETosis.*Sci Rep, 7* (1), 3409. doi:10.1038/s41598-017-03257-z

Lelliott, P. M., Momota, M., Lee, M. S. J., Kuroda, E., Iijima, N., Ishii, K. J., & Coban, C. (2019). Rapid Quantification of NETs In Vitro and in Whole Blood Samples by Imaging Flow Cytometry. *Cytometry A, 95* (5), 565-578. doi:10.1002/cyto.a.23767

Luo, S., Li, H., Liu, J., Xie, X., Wan, Z., Wang, Y., . . . Li, X. (2020). Andrographolide ameliorates oxidative stress, inflammation and histological outcome in complete Freund's adjuvant-induced arthritis.*Chem Biol Interact, 319* , 108984. doi:10.1016/j.cbi.2020.108984

Mauracher, L. M., Posch, F., Martinod, K., Grilz, E., Däullary, T., Hell, L., . . . Thaler, J. (2018). Citrullinated histone H3, a biomarker of neutrophil extracellular trap formation, predicts the risk of venous thromboembolism in cancer patients. *J Thromb Haemost, 16* (3), 508-518. doi:10.1111/jth.13951

McInnes, I. B., & Schett, G. (2017). Pathogenetic insights from the treatment of rheumatoid arthritis. *Lancet, 389* (10086), 2328-2337. doi:10.1016/s0140-6736(17)31472-1

Mikuls, T. R., Edison, J., Meeshaw, E., Sayles, H., England, B. R., Duryee, M. J., . . . Thiele, G. M. (2020). Autoantibodies to Malondialdehyde-Acetaldehyde Are Detected Prior to Rheumatoid Arthritis Diagnosis and After Other Disease Specific Autoantibodies.*Arthritis Rheumatol, 72* (12), 2025-2029. doi:10.1002/art.41424

Najmeh, S., Cools-Lartigue, J., Giannias, B., Spicer, J., & Ferri, L. E. (2015). Simplified Human Neutrophil Extracellular Traps (NETs) Isolation and Handling. *J Vis Exp* (98). doi:10.3791/52687

Papayannopoulos, V., Metzler, K. D., Hakkim, A., & Zychlinsky, A. (2010). Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol, 191* (3), 677-691. doi:10.1083/jcb.201006052

Remijsen, Q., Vanden Berghe, T., Wirawan, E., Asselbergh, B., Parthoens, E., De Rycke, R., . . . Vandenabeele, P. (2011). Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res, 21* (2), 290-304. doi:10.1038/cr.2010.150

Sanz, I. (2016). Editorial: IgA Responses and Future Development of Rheumatoid Arthritis. *Arthritis Rheumatol, 68* (10), 2351-2353. doi:10.1002/art.39789

Smolen, J. S., Landewé, R. B. M., Bijlsma, J. W. J., Burmester, G. R., Dougados, M., Kerschbaumer, A., . . . van der Heijde, D. (2020). EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2019 update. *Ann Rheum Dis, 79* (6), 685-699. doi:10.1136/annrheumdis-2019-216655

Söderberg, D., & Segelmark, M. (2016). Neutrophil Extracellular Traps in ANCA-Associated Vasculitis. *Front Immunol, 7* , 256. doi:10.3389/fimmu.2016.00256

Song, B., Li, X., Xu, Q., Yin, S., Wu, S., Meng, X., . . . Li, J. (2020). Inhibition of BMP3 increases the inflammatory response of fibroblast-like synoviocytes in rheumatoid arthritis. *Aging (Albany NY), 12* (12), 12305-12323. doi:10.18632/aging.103422

Sur Chowdhury, C., Giaglis, S., Walker, U. A., Buser, A., Hahn, S., & Hasler, P. (2014). Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. *Arthritis Res Ther, 16* (3), R122. doi:10.1186/ar4579

Tai, Y., Zhu, Y., Mei, D., Wang, H., Yu, Q., Hong, C., . . . Zhang, L. (2021). IgD promotes pannus formation by activating Wnt5A-Fzd5-CTHRC1-NF-κB signaling pathway in FLS of CIA rats and the regulation of IgD-Fc-Ig fusion protein. *Int Immunopharmacol, 101* (Pt A), 108261. doi:10.1016/j.intimp.2021.108261

van der Linden, M. P., van der Woude, D., Ioan-Facsinay, A., Levarht, E. W., Stoeken-Rijsbergen, G., Huizinga, T. W., . . . van der Helm-van Mil, A. H. (2009). Value of anti-modified citrullinated vimentin and third-generation anti-cyclic citrullinated peptide compared with second-generation anti-cyclic citrullinated peptide and rheumatoid factor in predicting disease outcome in undifferentiated arthritis and rheumatoid arthritis. *Arthritis Rheum, 60* (8), 2232-2241. doi:10.1002/art.24716

Wu, Y., Chen, W., Chen, H., Zhang, L., Chang, Y., Yan, S., . . . Wei, W. (2016). The Elevated Secreted Immunoglobulin D Enhanced the Activation of Peripheral Blood Mononuclear Cells in Rheumatoid Arthritis.*PLoS One, 11* (1), e0147788. doi:10.1371/journal.pone.0147788

Wu, Y., Pan, W., Hu, X., Zhang, A., & Wei, W. (2021). The prospects for targeting FcR as a novel therapeutic strategy in rheumatoid arthritis.*Biochem Pharmacol, 183* , 114360. doi:10.1016/j.bcp.2020.114360

Wu, Y. J., Chen, W. S., Chen, H. S., Dai, X., Dong, J., Wang, Y., . . . Wei, W. (2017). The immunoglobulin D Fc receptor expressed on fibroblast-like synoviocytes from patients with rheumatoid arthritis contributes to the cell activation. *Acta Pharmacol Sin, 38* (11), 1466-1474. doi:10.1038/aps.2017.105

Zhang, J., Hu, X., Dong, X., Chen, W., Zhang, L., Chang, Y., . . . Wei, W. (2020). Regulation of T Cell Activities in Rheumatoid Arthritis by the Novel Fusion Protein IgD-Fc-Ig. *Front Immunol, 11* , 755. doi:10.3389/fimmu.2020.00755

Figure legends

Φιγυρε1.Τηε εξπρεσσιον οφ ΦςδP, ΐτH3, ανδ σIγΔ ιν PA πατιεντς.

(A) We stained for neutrophils using immunofluorescence in peripheral blood from RA patients. Neutrophils express FcδR (red). DAPI serves as a nuclear DNA counterstain (blue). The scale bar denotes 3μm. (B, C)The expression of FcδRon neutrophils from healthy controls (HC) (n=13), RA patients(n=25). (D, E) The plasma CitH3 and IgD levels in HC (n=13) and RA patients (n=44). (F)Spearman correlation analyses were shown for the association of CitH3 with the plasma level of sIgD (n=44). (G)Spearman correlation analyses were shown for the association of CitH3 with the expression of FcδR(n=25).

Figure2.Effects of IgD-activated neutrophils in healthy controls (HC).

Neutrophils were collected from HCperipheral blood and treated with IgD(0.1, 0.3, 1, 3, and 10μg/ml) within 6h. After cell culture, neutrophils were obtained. The effects of IgD on neutrophils and NETs formation (MPO^+CitH3^+ in neutrophils) were compared between different treatment groups. (A, B) FcδR expression on neutrophils was assayed by flow cytometry. (C,D) Expression of NETs in neutrophils was assayed by flow cytometry. (E) The protein expressions of (F)MPO, (G)NE, and (H) CitH3 in neutrophils from HC were monitored by western blot. Data were expressed as the mean ± SEM (n=5). $^*P$ <0.05 and $^{**}P$ <0.01 *vs.*control.

Figure3.Effects of IgD-Fc-Ig (DG) on IgD activated neutrophils in healthy controls (HC).

Effects of DG on neutrophils fromHC induced by IgD. Neutrophils were incubated with IgD (3μg/ml) and different concentrations of DG (1, 3, and 10μg/ml) for 6h. The effects of DG on neutrophils and NETs formation (MPO^+CitH3^+) in neutrophils were compared between different treatment groups. (A**,** B) FcδR expression on treated neutrophils was assayed by flow cytometry. (C**,** D) NETs formation in treated neutrophils was assayed by flow cytometry. Q-PCR was conducted to determine the expression levels of gene (E) MPO, (F) PAD4, and (G) NE. Data were expressed as mean±SEM(n=5). $^{**}P < 0.01$ *vs.* control,$^{\#}P <$ 0.05 and$^{\#\#}P < 0.01$ *vs.* IgD (3μg/mL) group.

Figure 4. Effects of IgD-Fc-Ig(DG) on the morphology of neutrophils and NETs formation.

Imaging flow cytometry, confocal immunofluorescence microscopy, and scanning electron microscopy assays were performed to observe the morphology of neutrophils and NETs formation after treatment of IgD and DG. In imaging flow cytometry assay,(A, B)representative example images of cells, NETsin IgD(3μg/ml) group with strong positive staining(green is MPO, red is CitH3) were presented. And the DG (10μg/ml) could weaken the fluorescence intensity and reduce the NETs formation (MPO^+CitH3^+ in neutrophils). NETs were imaged by confocal microscopy, neutrophils were stained for (C)MPO(green), (D)NE(red), and (E)CitH3(red).DNA was labeled with DAPI. The scale bar denotes 5μm. (F) Scanning electron microscopy showed NETs generated from neutrophils treated with IgD and DG. Data were expressed as the mean ± SEM (n=5).$^*P$ <0.01 *vs.*Control, $^{\#\#}P$ <0.01 *vs.* IgD (3 μg/ml) group.

Figure 5. Effects of IgD-Fc-Ig(DG) on arthritis index and IgD, CitH3, CCP2 levels of AA rats.

SD male rats were immunized with CFA on D0. Then the rats were treated with DG, IgG1-Fc, and etanercept by subcutaneous injection administration. (A)The time points of DG fusion protein administrated. (B-C) Effects of IgD-Fc-Ig on the arthritis index of AA rats. (C-G) Effects of IgD-Fc-Ig on serum levels of sIgD, CitH3, and CCP2 of AA rats. Data were expressed as the mean ± SEM (n=8).$^{**}P < 0.01$ *vs.* normal, $^{\#}P$ <0.05*vs.* AA model.

Figure 6. Effects of IgD-Fc-Ig(DG) on ankle joints and spleens histopathology of AA rats. (A-F**)** HE staining pathology and Safranin O-fast green cartilage staining of each group of rat ankle joints. Original magnification x5. Synovial cell proliferation is represented by—, inflammatory cell infiltration was represented by —, vascular pannus formation is represented by -, cartilage erosion is represented by -. Comparison of pathologic modification of rat ankle joints between groups**.** (G)Immunofluorescence was used to assess the expression of MPO in the ankle joints of rats. DG decreased MPO expression in the ankle joints of arthritic rats. (H-L)A photomicrograph of spleen histopathology showed red pulp congestion—, geminal center-, marginal zone— , and peripheral arterial lymphatic sheath-. Comparison of pathologic modification

of rat spleen between groups. Data were expressed as the mean +- SEM (n=5). $^{*}P < 0.05$ and $^{**}P$ <0.01, *vs* . AA model.

Figure 7. Effects of IgD-Fc-Ig(DG)on the neutrophils functions of AA Rat.

Isolated neutrophils from rat peripheral blood in different groups were measured with flow cytometry. And samples from the spleens were lysed and detected with qPCR analysis. (A, B) ROS production by the rat neutrophils from different groups. (C)Apoptosis rates for neutrophils were analyzed by flow cytometry with annexin V-FITC/PI apoptosis analysis. (D) The percentage of apoptotic cells. Neutrophils from the DG treated groups weree compared with the AA model group. Q-PCR was conducted to determine the expression levels of gene (F) MPO, (G) PAD4, and (H) NE. Etanercept group was set as a positive control. Data are mean +- SEM(n=5). $^{**}P$ <0.01 vs control,$^{\#}P$ <0.05 and$^{\#\#}P$ <0.01 vs AA model.

Figure 8. Effects of IgD and IgD-Fc-Ig(DG) on neutrophil-FLS interaction.

RA FLS was incubated with NETs from human neutrophils stimulated by IgD in the presence or absence of DG for 48 hours. AA rat FLS was incubated with AA rat neutrophils for 18 hours. (A) NETs and neutrophils are cultured separately with FLS from RA patients and AA rats. After co-culture, (B, C)flow cytometry evaluated surface expression of MHCII in RA FLS treated with NETs by staining HLA-DR.The proliferation of RA FLS(D) and AA FLS (E) were performed. Data were expressed as the mean +- SEM(n=5). $^{*}P$ <0.05 and $^{**}P < 0.01$ *vs.*Control; $^{\#}P$ <0.05 and$^{\#\#}P$ <0.01 *vs.*IgD(3μg/ml);$^{*}P$ <0.05 *vs.* Normal,$^{\#\#}P$ <0.01 *vs.* AA.

Figure 9. Possible mechanisms of NETs mediated RA pathogenesis

IgD induces neutrophils to formation NETs. Neutrophils stimulated by IgD exhibited increased NET formation in *vitro* , associated with enhanced myeloperoxidase (MPO), neutrophil elastase (NE), peptidyl arginine deiminase 4 (PAD4), and citrullinated histone 3 (CitH3) expression in neutrophils. NETs also promote the activation and proliferation of FLS, leading to the increase of MHCII in FLS. This cascade contributes to the RA autoimmune responses. And IgD-Fc-Ig may inhibit the activation of the neutrophil and generation of NETs in*vivo* and in *vitro* .

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