TIM-3 regulates the NETs-mediated dendritic cell activation in myeloperoxidase-ANCA-associated vasculitis

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Received on May 24, 2020; accepted in revised form on September 7, 2020.

Clin Exp Rheumatol 2021; 39 (Suppl. 129): S13-S20.

Key words: TIM-3, ANCA-associated vasculitis, dendritic cells, NETs, Toll-like receptor 4

Funding: this work was supported by the National Natural Science Foundation of China (81600160 and 81770038), the Fundamental Research Funds for the Central Universities (HUST: 2015ZHYX007, 2018KFYYXJJ076) and Wuhan Health and Family Planning Committee Research Project (WX17A01).

Competing interests: none declared.

ABSTRACT

Objective. *T cell immunoglobulin and mucin domain 3 (TIM-3) has been reported as an important regulatory molecule on T cells and plays a pivotal role in autoimmune diseases, but the impact on dendritic cells (DCs) is poorly explored. The formation of neutrophil extracellular traps (NETs) is considered as strongly implicated in the pathogenesis of autoimmune diseases, such as in myeloperoxidase-antineutrophil cytoplasmic autoantibody associated vasculitis (MPO-AAV). This study thus aimed to investigate the potential regulation roles of TIM-3 in the regulation of NETs-mediated DC activation in MPO-AAV.*

Methods. *Twenty untreated patients with MPO-AAV and 20 healthy controls were enrolled in this study. The expressions of TIM-3 and toll-like receptor 4 (TLR4) in peripheral blood dendritic cells were analysed by flow cytometry, and the release of NETs by neutrophils was evaluated by immunofluorescence. In animal experiments, we measured the DC activation markers after the stimulation of NETs. Furthermore, we detected the NETs-mediated DC activation after TIM-3 blockade.*

Results. *Here we found an increased spontaneous NET production in MPO-AAV patients. We also revealed a markedly reduced expression of TIM-3 and an increased expression of TLR4 on DCs of active MPO-AAV patients. We found the NETs could induce the activation of DCs and promote Toll-like receptor 4 expression on DC surface. More interestingly, the blockade of TIM-3 could further enhance the NETs-mediated DC cytokine expression.*

Conclusion. *Our results demonstrated DC surface TIM-3 plays an important role in maintaining the NETs mediated immune homeostasis in MPO-AAV, suggesting an important role in MPO-AAV development.*

Introduction

Neutrophil cytoplasmic antibody associated vasculitis (AAV) is a systemic autoimmune disease. The main pathogenesis of AAV is the disorder of immune system, which makes antineutrophil cytoplasmic antibodies (ANCA) increase abnormally. AAV can be classified by the types of autoantibodies present in peripheral circulation, one of which is myeloperoxidase-antineutrophil cytoplasmic autoantibody-associated vasculitis (MPO-AAV), in which myeloperoxidase-antineutrophil cytoplasmic antibodies (MPO-ANCA) are frequently present (1, 2). When MPO-ANCA are released, they can induce not only the activation of the neutrophils, but also the formation of neutrophil extracellular traps (NETs) (3). The generation of NETs is the unique form of death of the neutrophils. Basic researches in the recent years have reinforced that excessive NET formation is strongly implicated in AAV pathogenesis (4). NETs were first described in 2004 initially associated with an antibacterial role (5), with extracellular release of chromatin fibres and antibacterial cytoplasmic proteins, including myeloperoxidase (MPO), neutrophil elastase (NE) and others. A recent study proved that NETs mediated transfer of cytoplasmic neutrophil antigens to dendritic cells towards ANCA induction and associated autoimmunity in animals (6). Therefore, extrusion of NETs was another peculiar pathogenesis of MPO-AAV. NETs mediated DC antigen presentation provided a new link between innate and adaptive immune responses.

Immune responses are tightly regulated by the active and inhibitory receptors. Inhibitory receptors are important regulators of immune responses, helpful to maintain immune balance. When the functions of inhibitory receptors are disturbed, the tolerance of the immune

system is broken, which can induce the generation of autoantibodies and the emergence of autoimmune diseases. T cell immunoglobulin and mucin domain 3 (TIM-3) is an important inhibitory receptor, which was originally discovered on terminally differentiated Th1 cells, playing an important role in autoimmune diseases. TIM-3 and glectin-9 interaction severed as a specific down regulator of Th1 immune responses (7-9). In 2018, it was proved that germline HAVCR2 mutations altering TIM-3 characterised subcutaneous panniculitis-like T cell lymphomas with haemophagocytic lymphohistiocytic syndrome, which further highlighted the inhibitory function of TIM-3 in disease with immune hyperresponsiveness (10). TIM-3 is not only expressed on T cells, but also constitutively expressed on macrophages and DCs, and regulates the innate immune responses (11). Maurya *et al*. found an inhibitory role for TIM-3 signalling in DC activation via Bruton's Tyrosine Kinase (BTK) and c-Src, and silencing of Btk or c-Src abrogated the inhibitory effects of TIM-3 (12). TIM-3 serves as a negative regulator of DC immune responses. More interestingly, Chiba and Das demonstrated tumour-infiltrating DCs could repress nucleic acid-mediated innate immune responses through interplays between TIM-3 and high mobility group box 1 (HMGB 1) (13, 14), suggesting the regulation effect of DC surface TIM-3 in anti-tumour immunity. Because of its critical regulation roles in both innate and adaptive immune responses, TIM-3 dysregulation has been proven to correlate with the pathogenesis of a multitude of autoimmune diseases (7, 15-17).

We know inhibitory receptors, especially TIM-3, play a significant role in immune associated diseases. The aim of our study was to investigate the potential regulation roles of TIM-3 on the regulation of NETs-mediated DC activation in MPO-AAV. Here we detected TIM-3 expression in MPO-AAV. We also used animal experiments to clarify the potential role for NETs on DC maturation, and discussed the possible role for TIM-3 in the NETs-mediated DC activation in MPO-AAV.

Table I. Clinical characteristics.

Characteristics	MPO-AAV patients, n=20	HC, n=20
Gender (M/F)	8/12	9/11
Age (years)	55 (48-60)	53 (45-59)
Anti-MPO Ab (RU/ml)	92.52±60.45	7.6±5.4
Anti-PR3 Ab (RU/ml)	11.98±12.99	6.3±5.6
BVAS (inactive/active)	10/10	NA
Serum creatinine (μmol/L)	162.01±117.49	NA
C reaction protein (mg/L)	43.68±48.63	NA
Methylprednisolone dose (mg/day)	3 (0-20)	-

NA: not available.

Materials and methods

Patients

Twenty patients with MPO-AAV and 20 healthy individuals were recruited from Wuhan No.1 Hospital. The patients' median age was 55 (48-60). All of the patients fulfilled the revised criteria for AAV of the Chapel Hill Consensus Conference (18), with a clinical evidence of rapidly progressive glomerulonephritis, and a positive test for MPO-ANCA. Patients with other types of systemic small-vessel vasculitis, drug-induced vasculitis, systemic lupus erythematosus, malignancy-associated vasculitis or rheumatoid arthritis, and those with antiglomerular basement membrane disease vasculitis were excluded. Clinical information was obtained from the medical records, which are given in Table I. Disease activity of AAV was assessed by the Birmingham Vasculitis Activity Score (BVAS) (19). 20 age-matched healthy individuals were recruited from the Department of Medical Examination. The median age was 53 (45-59). The characteristics of healthy individuals are also listed in Table I. This research was conducted in compliance with the declaration of Helsinki and approved by the ethics committee of Wuhan No.1 Hospital.

Cytokine detection

The concentration of human sTIM-3 was detected using ELISA kits (R&D systems, Minneapolis, MN, USA). The serum levels of human TNF-α and IL-6 were detected by SEMINS IMMUNITE 1000 chemiluminescence instrument. IL-1β (eBioscience, San Diego, CA, USA) and IL-6 (eBioscience, San Diego, CA, USA) in cell culture supernatants were detected with ELISA kits.

Cell isolation

Human peripheral blood mononuclear cells (PBMCs) and neutrophils were isolated from blood cells using Percoll (Sigma Aldrich, St. Louis, MO, USA) gradients (autologous plasma, 60% Percoll and 75% Percoll). Cells between the 60% and 75% Percoll interface were neutrophils, and cells between the autologous plasma and 60% Percoll were PBMCs.

C57BL/6 mice were maintained under specific pathogen-free conditions at the animal housing facility at Wuhan No.1 Hospital. All experimental procedures were performed according to Dutch laws and international guidelines on animal experimentation and were approved by the animal ethics committee of the Wuhan No.1 Hospital. DCs were generated from bone marrow (BM) progenitors of C57BL/6 mice (20), and red blood cells were lysed with NH_4Cl. Then, cells were adjusted to 2×10^6/ml and plated on 12-well plates at 2 ml/well. They were cultured for up to 7 days in the presence of 80nM of GM-CSF (PeproTech Inc., Rocky Hill, NJ, USA) and IL-4 (PeproTech Inc., Rocky Hill, NJ, USA) at 37ºC with 5% CO_2. On day 7 of culture, flow cytometry was used to confirm the purity of DCs. Mouse neutrophils were isolated from BM cells using Percoll gradients (55, 62 and 82% Percoll in PBS). Cells between the 62% Percoll and 81% Percoll interface were extracted.

NET production and isolation

Human neutrophils were left unstimulated for 4 h at 37ºC with 5% CO_2 to spontaneously form NETosis without FBS, in which NETs were released. Identification of mouse NET fragments in this study was similar to a previously

published protocol (21). Firstly isolated neutrophils were seeded in 24-well culture plates (1×10^6/well) and stimulated with 50nM PMA (Sigma Aldrich, St. Louis, MO, USA) for 4 h at 37ºC with 5% CO_2. Then the cells were carefully washed twice with 1ml PBS, and then treated for 1 h at 37ºC with 20U/ml restriction enzyme A1uI(New England Biolabs, Hitchin, UK) in HBSS to recover large soluble NET fragments. Supernatants were collected and centrifuged at 300×g for 5 min at 4ºC to remove contaminating debris and cells. NETs were then stored at -20ºC until they were used.

Immunofluorescence labelling and observation of NETs

NETs were observed as we previously described (22). Freshly isolated neutrophils from humans and mice were seeded on Poly-L-lysine (Sigma Aldrich, St. Louis, MO, USA) coated glass coverslips (2×10^5/well). The cells from humans were left unstimulated, and the cells from mice were stimulated with 50nM PMA (Sigma Aldrich, St. Louis, MO, USA), for 4 h at 37ºC with 5% CO_2. Then they were fixed with paraformaldehyde solution for 10 min at room temperature. NETs were stained with rabbit anti-histone H3 (citrulline R2+R8+R17) antibody (Abcam, Cambridge, MA, USA) and mouse anti-MPO antibody (Abcam, Cambridge, MA, USA), followed by incubation with Alexa Fluor 647-conjugated mouse IgG (Abcam, Cambridge, MA, USA) and Alexa Fluor 488-conjugated rabbit IgG (Abcam, Cambridge, MA, USA). DNA was stained with DAPI. Images were collected with OlymPus BX51 microscope and Qimaging camera.

BMDC and NET co-culture experiments

After 7 days of culture, non-adherent DCs were harvested. They were subsequently cultured in the presence or absence of 20% of concentrated supernatants derived from induced NETs or 5μg/ml LPS (Sigma Aldrich, St. Louis, MO, USA), with or without 2.4μg/ml anti-mouse TIM-3 mAb (R&D systems, Minneapolis, MN, USA). Co-culture supernatants were collected 18h after stimulation. And the remaining cells were acquired after surface staining on a FACS Canto II (BD Bioscience, San Jose, CA, USA).

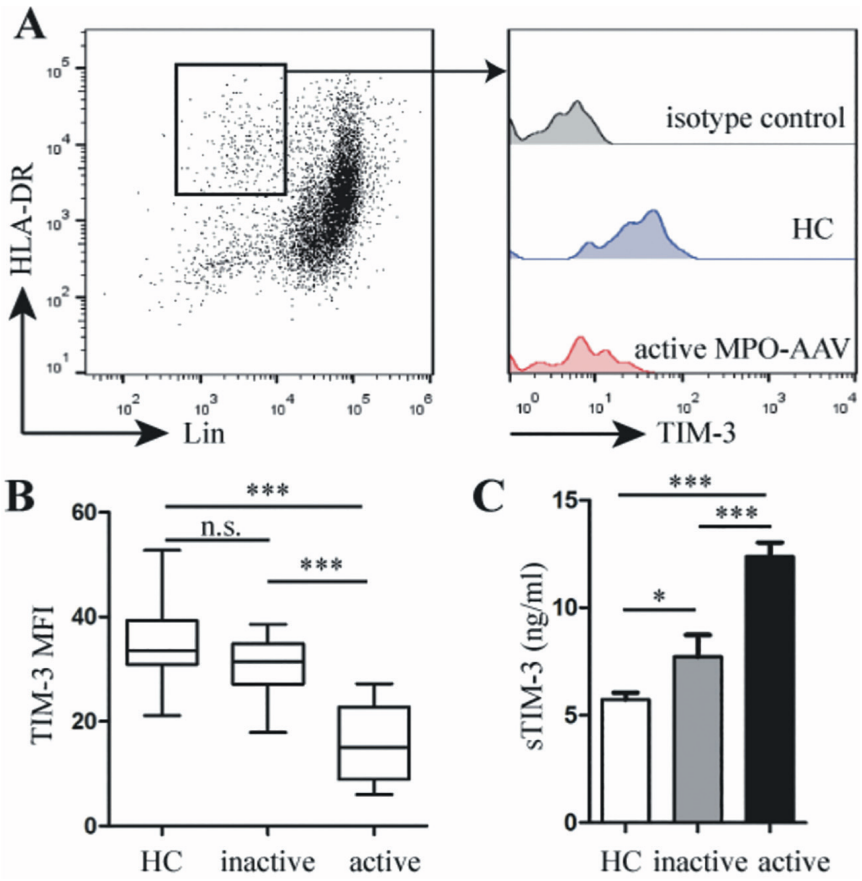

Fig. 1. The lower expression of TIM-3 in active MPO-AAV patients.
A: The representative plot of the $Lin^-HLA\text{-}DR^+$ DCs, and the representative figure of TIM-3 expression on $Lin^-HLA\text{-}DR^+$ DCs in active MPO-AAV patients and healthy controls.
B):Quantification of TIM-3 expression on $Lin^-HLA\text{-}DR^+$ DCs in active MPO-AAV patients, inactive MPO-AAV patients and healthy controls. (C) Serum from 10 inactive AAV patients, 10 active MPO-AAV patients and 20 healthy controls were collected to analyse sTIM-3 by ELISA.
*p<0.05; ***p<0.001; n.s.: no significance.
One-way analysis of variance was performed for comparisons among each group.
MFI: mean fluorescence intensity; HC: healthy controls.

Flow cytometry

Fluorescent-conjugated monoclonal antibodies to mouse CD86, MHCII, CD11c and to human CD3, CD4, CD8, CD25, CD127, CD45, TIM-3, TLR4 were all from BD pharmingen. Fluorescent-conjugated monoclonal antibodies to human Lin (CD3, CD14, CD19, CD20 and CD56) and HLA-DR were from Beckman Coulter. Cells were stained for extracellular markers for 15min at 4ºC in PBS with 2% FBS. Then cells were acquired on a FACS CantoII and analysed using the FlowJo software.

Statistics

All values were expressed as mean ± standard deviation (SD). Student's t test was used for comparisons between two groups. Significance for the differences among three groups was determined using One-way analysis of variance. Two-tailed p-values <0.05 were considered significant. For statistical analysis, GraphPad Prism 5.0 was used (Graphpad software, USA).

Results

Lower expression of TIM-3 on DC surface in active MPO-AAV patients

We firstly detected the frequency of different T cell subtypes in peripheral blood of MPO-AAV patients by flow cytometry. Among them, the population of Treg cells in MPO-AAV patients declined markedly compared with healthy controls (Supplementary Fig. S1). We also detected the expression of TIM-3

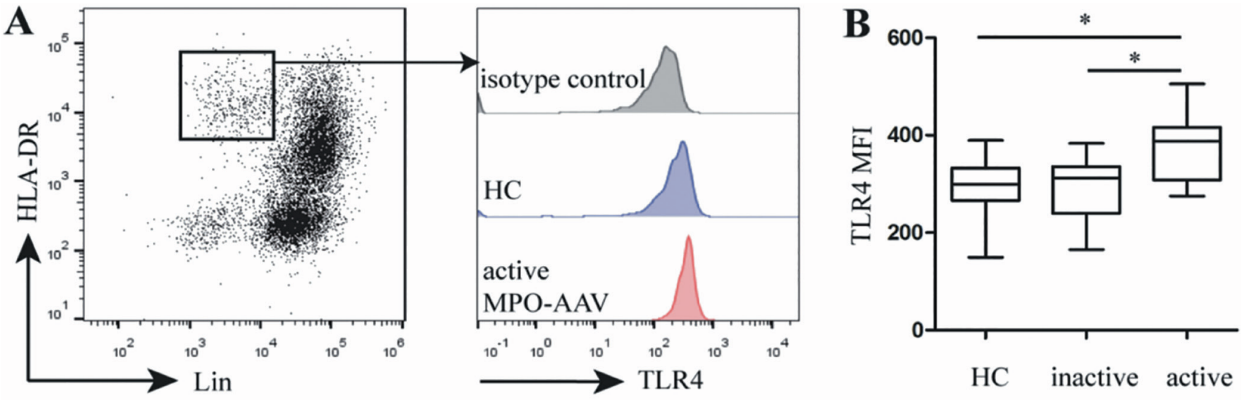

Fig. 2. The higher expression of TLR4 in active MPO-AAV patients.
A: The representative plot of the Lin⁻HLA-DR⁺ DCs, and the representative figure of TLR4 expression on Lin⁻HLA-DR⁺ DCs in active MPO-AAV patients and healthy controls.
B: Quantification of TLR4 expression on Lin⁻HLA-DR⁺ DCs in healthy controls, inactive MPO-AAV patients and active MPO-AAV patients. *p<0.05. One-way analysis of variance was performed for comparisons among each group. MFI: mean fluorescence intensity; HC: healthy controls.

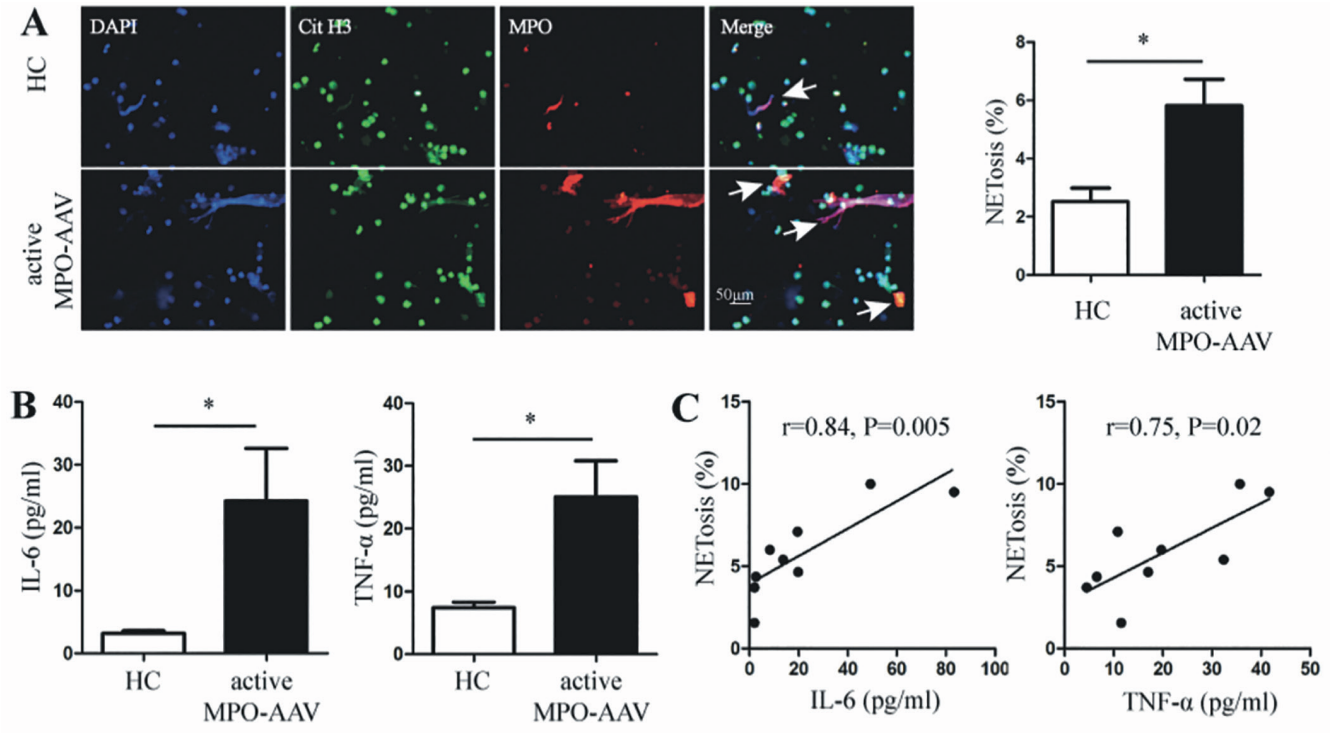

Fig. 3. Serum cytokine expression and spontaneous circulating NETs forming in MPO-AAV patients.
A: Neutrophils (1×10^6) from MPO-AAV patients and healthy controls were left unstimulated for 4 h at 37 ˚C with 5% CO_2 without serum. The results were expressed as a percentage of total PMN number. Representative figure was demonstrated. DNA stained blue, citrullinated histone H3 stained green and MPO stained red. Three colours were merged by software Photoshop CS5 (Adobe, USA).
B: Serum from 20 AAV patients and 20 healthy controls was collected to analyse IL-6 and TNF-α by ELISA.
C: Correlations between percentage of NETosis and cytokine (IL-6 and TNF-α) levels were analysed. *p<0.05.
Student's t test was used for comparisons between groups. Pearson correlation coefficient test was used to examine the correlation.

on different T cell subtypes, but we did not find the difference between MPO-AAV patients and healthy controls (Suppl. Fig. S2).

TIM-3 was not only expressed on T cells, but also constitutively expressed on dendritic cells, so we gated Lin⁻HLA-DR⁺ DCs among PBMCs and evaluated the expression of TIM-3 on Lin⁻HLA-DR⁺ DCs in MPO-AAV patients and healthy controls. We found the expression of TIM-3 on Lin⁻HLA-DR⁺ DCs in active MPO-AAV patients significantly lower than that in healthy controls (Fig. 1A-B).

To further evaluate the role of TIM-3 in MPO-AAV patients, we also detected the level of serum soluble TIM-3 (sTIM-3). In our results, we found a higher expression of sTIM-3 in active MPO-AAV patients than inactive patients or healthy controls (Fig. 1C).

Higher expression of TLR4 on DC surface in active MPO-AAV patients

We knew immune responses were regulated by opposing positive and nega-

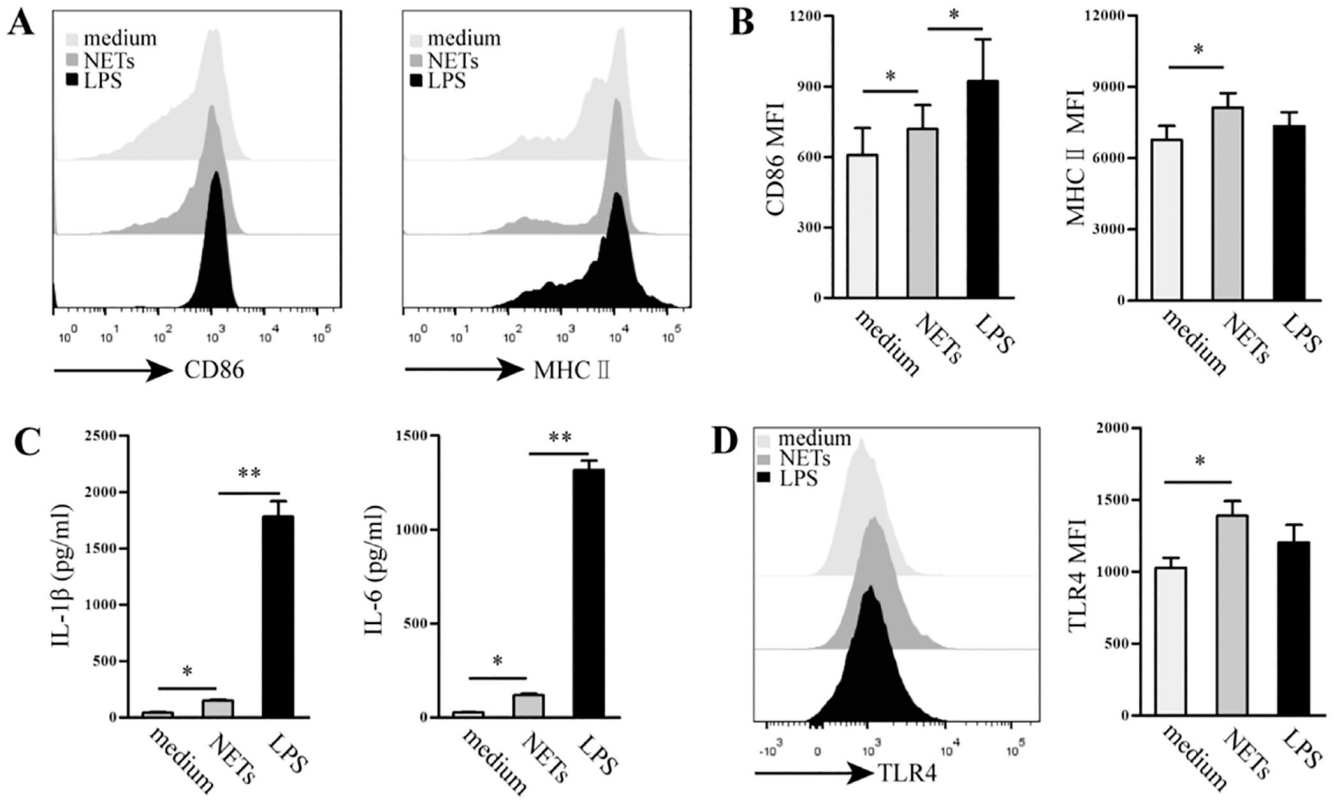

Fig. 4. NETs enhanced the maturation of BMDCs and TLR4 expression. NET fragments were added to immature BMDCs. As a positive control, cells were treated with 5μg/ml LPS.
A: After 18 h of incubation, the expressions of CD86 and MHCII were analysed by flow cytometry. **B**: Statistic results of the expressions of CD86 and MHCII. **C**: IL-1β and IL-6 were quantified in the culture supernatants by ELISA. **D**: The expression of TLR4 was analysed by flow cytometry. Data represent the results of six independent experiments. Low grey histogram represented unstimulating group, high grey histogram represented 20% NETs stimulating group, black histogram represented LPS stimulating group.
***p*<0.01; **p*<0.05, significant differences by the One-way analysis were indicated.

tive signals triggered by the interaction of active or inhibitory receptors. We also evaluated $Lin^{-}HLA\text{-}DR^{+}$ DC surface TLR4 expression in MPO-AAV patients and healthy controls. We observed a significantly higher expression of TLR4 in active MPO-AAV patients compared with inactive MPO-AAV patients or healthy controls (Fig. 2A-C).

Higher spontaneous production of NETs and higher expressions of pro-inflammation cytokines in MPO-AAV

Chronic inflammatory stimulation plays an important role in the pathogenesis of autoimmune diseases. Recent studies had displayed that NETs participated in endothelial cell damage (23). In order to prove the pathogenic role of NETs in active MPO-AAV, we examined the release of spontaneous NETs by peripheral blood neutrophils, and we also evaluated the levels of IL-6 and TNF-α in serum. We found that active MPO-AAV neutrophils displayed an increased propensity to form NETs spontaneously compared to control neutrophils isolated from healthy controls (Fig. 3A). And there was no significant difference between patients sampled before the onset of induction therapy for active disease and patients sampled after (Suppl. Fig. S3). Compared with healthy controls, the expressions of IL-6 and TNF-α were significantly increased (Fig. 3B). More interestingly, the expressions of IL-6 and TNF-α in active MPO-AAV patients were correlated with the percentage of spontaneous NETosis (Fig. 3C). These results suggested that NETosis could be a prominent characteristic in MPO-AAV.

NETs increase the maturation of DCs and promote TLR4 expression

Since NETosis was a prominent characteristic in MPO-AAV. We sought to examine whether induced NETs could promote DC maturation. We sheared NETs from PMA-activated neutrophils by using the restriction enzyme A1uI, thereby forming larger fragments of NETs. To offer direct evidences for the role of NETs on DC maturation, we generated BMDCs from bone marrow of wild type C57BL/6 mice. Afterwards we co-cultivated BMDCs with NET fragments, and we used LPS as the positive control. We could see that NET fragment stimulation increased the expressions of CD86 and MHC II on DC surface (Fig. 4A-B). Moreover, the assessment of DC secreted proinflammatory cytokine expression after NET fragment stimulation revealed that NETs could significantly increase the levels of IL-1β and IL-6 (Fig. 4C). A set of experiments showed the effect of NETs on BMDC maturation.

As TLR4 is an important mediator of the excessive inflammatory responses during MPO-AAV, we also measured TLR4 expression after NET fragment stimulation. We found a significantly increased expression of TLR4 after

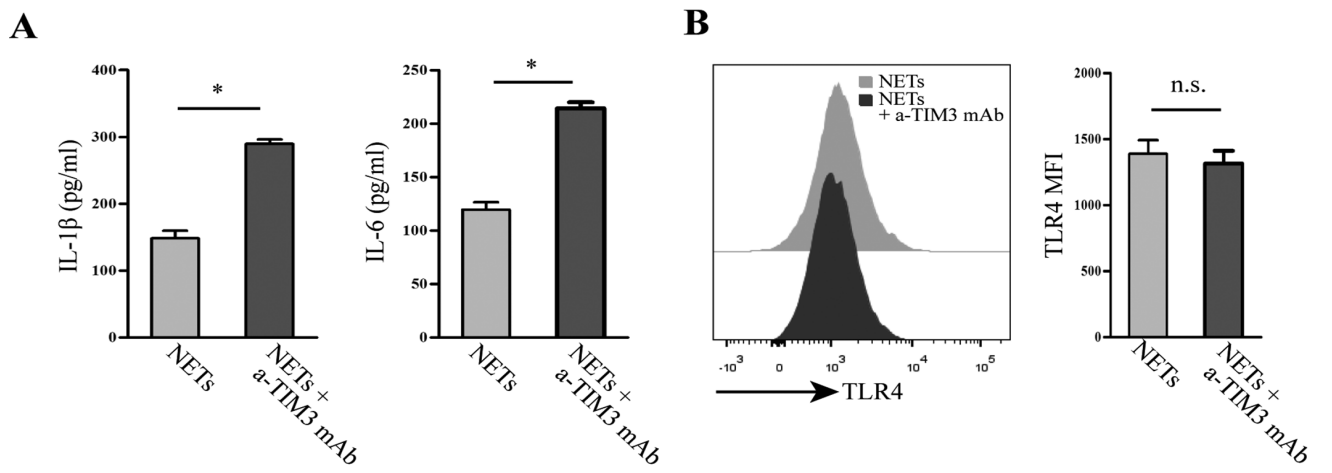

Fig. 5. The change of BMDC activation after anti-TIM-3 treatment. NET fragments were added to immature BMDCs for 18 h.
A: IL-1β and IL-6 were quantified in the culture supernatants by ELISA. **B**: The expression of TLR4 was analysed by flow cytometry. Data represent the results of six independent experiments. Low grey histogram represented 20% NETs stimulating group, high grey histogram represented 20% NETs stimulating with a-TIM-3 mAb treated group. *p<0.05; n.s.: no significance; significant differences by the Student's t test were indicated.

NET stimulation (Fig. 4D). TLR4 was an important receptor for damage associated pattern molecule, TLR4 expression could regulate the transduction of downstream signalling pathways.

TIM-3 inhibited NETs induced BMDC cytokine expression

A set of experiments showed the effect of NETs on BMDC maturation. We also used anti-TIM-3 antibody as a blocking antibody to block the effect of TIM-3. We found that the secretion of cytokines by BMDCs was increased than that of not set with anti-TIM-3 antibody group (Fig. 5A). In brief, induced NETs promoted BMDC maturation and cytokine secretion, and TIM-3 blockade enhanced NETs induced BMDC cytokine secretion. We also measured the level of TLR4 after TIM-3 blockade, unfortunately, TIM-3 blockade did not further increase NETs mediated DC surface TLR4 expression (Fig. 5B).

Discussion

ANCA-associated vasculitis is a relapsing-remitting autoimmune and inflammatory disease leading to necrotic inflammation of small-sized blood vessels and capillaries. In the present study, we characterised the expressions of TIM-3 and TLR4 on DCs with MPO-AAV and discussed the role of TIM-3 in disease homeostasis. In our results, we observed a lower expression of TIM-3 in active patients. We also found increased TLR4 expression in active MPO-AAV patients. We further confirmed and extended the finding about the increased level of NETs in the circulation of patients with active MPO-AAV. Moreover, NETs could facilitate the maturation of DCs and promote TLR4 expression, blockade of TIM-3 led to further DC cytokine expression in the NETs-mediated immune responses.

In recent years, a unique type of cell death of neutrophil granulocytes, which was called NETosis, has been discovered that is characterised by the active release of chromatin fibres. NETs can kill viruses, bacteria, fungi and parasites and are universally acknowledged to prevent microorganism dissemination. NETs can also stick to the endothelium and cause tissue damage during sepsis (24). Moreover, NETs can modulate sterile inflammation through turning on the transcription of gene encoding IL-6 and pro-IL-1β (25). We also detected higher expressions of IL-6 and IL-1β in MPO-AAV patients. Taken together, NETs could contribute to the pathogenesis of immune related diseases. A recent human study demonstrated that the activity of DNase I, a crucial regulator of NETs, was lower in MPO-ANCA-associated MPA and SLE sera (26, 27). In these diseases, the NETs enhanced, followed by releasing more intracellular materials, which resulted in the increase of free target autologous antigen in serum. When autoantigenic could not be effectively removed, immune tolerance was broken. In our results, we observed an increased spontaneous NETs forming in unstimulated neutrophils of MPO-AAV patients with active disease, and these results were consistent with the results of Soderberg *et al*. (28). Taken together, NETs played a prominent role in MPO-AAV. We also observed a significant correlation of NETs percentage with levels of IL-6 or IL-1β in active disease, which suggested NETs participated in inflammatory processes. However, we need more studies to explore.

We know that if immune tolerance was broken, autoantibodies can emerge. Although the mechanisms of autoantibody formation in AAV remain unclear, DCs play an important role in this process. DCs are crucial antigen-presenting cells, after damaging of tissue or invasion of microbial, they may capture and transfer those information from the outside world to the intracellular, leading DCs to become more mature (29). Our findings provided evidences for the ability of NETs to increase the expression of DC costimulatory molecules, CD86 and MHC II, as well as the secretion of IL-6 and IL-1β. In line with this, NETs could efficiently trigger DC maturation.

Immune responses are tightly regulated by active and inhibitory receptors (IRs) (30). Chevalier *et al*. first investigated several IRs in circulating DCs

from blood of urothelial cancer (UCs). They found high expressions of BTLA and TIM-3, which could potentially mediate the decrease of DC functions and the escape from immune surveillance for tumour cells (31). Contrary to tumours, in autoimmune diseases, which have excessive immune activation, what about the role of IRs on DCs? FcγRIIb is an inhibitory FcR important in the maintenance of tolerance on DC surface. Weink *et al*. firstly observed correlation between FcγRIIb expression on DCs and disease activity, which demonstrated the importance of DC surface IRs in the maintenance of balance in autoimmune diseases (32). TIM-3 is another IR on DCs. It is a member of *TIM* gene family encoding cell surface receptors (33). TIM-3 protein is specially expressed on Th1 cells and negatively regulates Th1 responses (9). In our experiments, we discovered higher TIM-3 expression on DCs than on T cells, we also observed a lower expression of TIM-3 on DCs of active MPO-AAV patients, which was to say TIM-3 played a crucial role in disease pathogenic processes. We knew, besides the full-length, membrane-anchored form, TIM-3 also existed as a soluble form. Shedding of the TIM-3 ectodomain was found in human $CD14^+$ monocytes after LPS stimulation (34) and TIM-3 expression T cells in patients with graft-versus-host disease after allogeneic haematopoietic cell transplantation (35). Researchers found such a process abrogated TIM-3 mediated signalling transduction. Production of sTIM-3 may interfere with a TIM-3-TIM-3 ligand (TIM-3L) interaction (36). In our results, we found high expression of sTIM-3 in serum of MPO-AAV patients, which clarified that the higher expression of sTIM-3 might be a consequence of the lower expression of surface TIM-3.

DC surface TIM-3 plays an important role in maintaining immune balance. However, the detail mechanisms remain largely unknown. Maurya *et al*. found TIM-3 inhibited DC activation and maturation by blocking the NF-κB pathway. After TIM-3 became tyrosine phosphorylated, it then sequentially bound and activated the nonreceptor tyrosine kinases Bruton's tyrosine Kinase (BTK) and c-Src. Activation of BTK-Src signalling in turn triggered the secretion of quite a few inhibitory factors from DCs that inhibited the NF-κB pathway and subsequent activation and maturation of DCs (12). Yand *et al*. demonstrated blockade and/or downregulation of TIM-3 led to increased macrophage activation, whereas TIM-3 overexpression in macrophages significantly suppressed TLR-mediated immune responses. Cross-talk between the TIM-3 and TLR4 pathway makes TLR4 an important contributor to TIM-3-mediated negative regulation of the immune responses. And TIM-3 signalling inhibited LPS-TLR4-mediated NF-κB activation by increasing PI3K-AKT phosphorylation and A20 activity (37). Accumulating data have suggested the involvement of TIM-3 in the pathogenesis of many immune-related diseases, cancers and viral infections (13, 15, 38). Here, for the first time, we provide evidences for involvement of TIM-3 in MPO-AAV. NET stimulation led to increased DC activation and TLR4 expression, and blockade of TIM-3 enhanced NETs induced DC activation. TIM-3 played an important role in maintaining the NETs mediated immune homeostasis in MPO-AAV. Unfortunately, the expression of TLR4 could not be regulated by anti-TIM-3 antibody treatment. The reason may be that there were other TLRs or pattern recognition receptor (PRRs) on DC surface to mediate damage associated molecular patterns (DAMPs) signalling transduction. This negative regulatory role of TIM-3 indicated that TIM-3 associated pathway might be a new target for intervention in the treatment of MPO-AAV. Inhibiting the TIM-3 pathway in antigen-presenting cells could be a therapeutic approach to stimulate anti-tumour immune responses (13). Conversely, stimulation of the pathway may also ameliorate autoimmune diseases.

Our results demonstrated that DC surface TIM-3 plays an important role in maintaining the NETs mediated immune homeostasis in MPO-AAV, suggesting targeting TIM-3 signalling pathway could be an effective therapeutic intervention in MPO-AAV patients. Furthermore, future studies with more AAV patients and in vivo experiments are needed to be set up. Experimental animal model of MPO-AAV and mouse experiments are also needed to clarify the possible value of TIM-3 therapy.

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