The pathogenic roles of S100A8/A9 and S100A12 in antineutrophil cytoplasmic antibody-associated vasculitis

Peng-Cheng Xu (nkxpc@163.com)
Tianjin Medical University General Hospital
Xue Bai
Tianjin Medical University General Hospital
Tong Chen
Tianjin Medical University General Hospital
Hao-Miao Zhang
Tianjin Medical University General Hospital
Si-Jing Wu
Tianjin Medical University General Hospital
Xia Yang
Tianjin Medical University General Hospital
Shan Gao
Tianjin Medical University General Hospital
Jun-Ya Jia
Tianjin Medical University General Hospital
Jian-Qing Jiang
Tianjin Medical University General Hospital
Tie-Kun Yan
Tianjin Medical University General Hospital

Research Article

Keywords: S100A8/A9, S100A12, ANCA, AAV

Posted Date: March 30th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1480646/v1

Abstract

Background: The significance of S100A8/A9 and S100A12 in the antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) has not been clarified. In this study, the pathogenic role of S100A8/A9 and S100A12 in AAV were investigated.

Results: Both serum and urine S100A8/A9 and S100A12 of AAV patients were excessively increased (compared with healthy controls, $p<0.001$), and were correlated with the activity and severity of disease. Serum S100A8/A9 was correlated with serum S100A12 ($r=0.728$, $p<0.001$), and there was also a relationship between urinary S100A8/A9 and S100A12 ($r=0.536$, $p=0.001$). In vitro study showed S100A8/A9 and S100A12 increased the chemotaxis index (CI) and the release of IL-1β, extended the life span, and enhanced the complement activation of ANCA-activated neutrophils. Blockade of TLR4 and RAGE inhibited the functions of S100A8/A9 and S100A12. Western-blotting showed p38 MAPK/NF-κB p65 pathway was involved in the influence of S100A8/A9 and S100A12 on ANCA-activated neutrophils. The effects of S100A8/A9 and S100A12 were reactive oxygen species (ROS)-independent because both S100A8/A9 and S100A12 did not enhance the ANCA-induced ROS and NETs generation.

Conclusion: S100A8/A9 and S100A12 not only serve as markers for assessing the disease activity and severity, but also might take part into the pathogenesis of AAV directly.

Background

Antineutrophil cytoplasmic antibody-associated vasculitis (AAV) is an autoimmune disease which is characterized by serum positive antineutrophil cytoplasmic antibody (ANCA) against myeloperoxidase (MPO) or proteinase 3 (PR3) and glomerulonephritis with pauci-immune complex deposition(1). The pathological manifestations of AAV encompass microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA)(2). In AAV, the ANCA-activated neutrophils (polymorphonuclear lymphocytes, PMNs) extrude neutrophil extracellulartraps (NETs) which was decorated by histones, MPO, PR3, neutrophil elastase (NE), as well as other cytoplasmic proteins(3).

Previous studies demonstrated the activated neutrophils can also extrude damage-associated molecular pattern (DAMP) proteins such as high mobility group box chromosomal protein 1 (HMGB-1) and some S100 family proteins(4). HMGB-1 has been reported to take part into the pathogenesis of AAV(5, 6), however, the role of S100 family proteins in AAV has not been clarified. S100A8/A9 and S100A12 both belong to S100 protein family that was first extracted from cow brain by Blake W. Moore and his colleagues in 1965(7). S100A8/A9 can stimulate renal mesangial cells to release IL-6, TNF-α and CXCL1(8). S100A12 enhances the amount of cytokines expression in a dose-dependent manner, as well as promotes the secretion of chemokines and cell adhesion molecules in normal bronchial epithelial cell(9). It has been reported that the serum levels of S100A8/A9 and S100A12 in AAV are elevated(10,

11). However, whether the S100A8/A9 and S100A12 exert pathogeneic function in AAV has not been reported yet. In the current study, we tried to investigate the role of S100A8/A9 and S100A12 in AAV.

Results

The serum and urinary levels of S100A8/A9 and S100A12 are associated with the clinical parameters of AAV

Among the 34 AAV patients, 15 were male and 19 were female, with an age of 63 (57, 68) years. Serum and urine from 34 AAV patients and 10 healthy controls were tested for the level of S100A8/A9 and S100A12. The median serum S100A8/A9 concentration in AAV was 5112 ng/ml, which was significantly increased compared to the healthy control ($p<0.001$) (Figure 1A). The median serum S100A12 concentration of AAV patients was 44.75 ng/ml, which was also significantly elevated compared to normal controls ($p<0.001$) (Figure 1B). The concentration of urinary S100A8/A9 and S100A12 were also higher than normal control (Figure 1C and 1D). Correlation analysis revealed the concentration of serum S100A8/A9 in AAV patients was positively correlated with serum level of S100A12 ($r=0.728$, $p<0.001$) (Figure 1E), and there was a moderate relationship between urinary S100A8/A9 and S100A12 ($r=0.536$, $p=0.001$) (Figure 1F).

The serum concentration of S100A8/A9 was positively correlated to the titer of ANCA of ($r=0.390$, $p=0.025$), serum ferritin ($r=0.663$, $p<0.001$), CRP ($r=0.548$, $p<0.001$), D-dimer ($r=0.417$, $p=0.016$), ESR ($r=0.363$, $p=0.038$) and RF ($r=0.395$, $p=0.021$), and negatively correlated with serum albumin ($r=-0.468$, $p=0.005$). The serum S100A8/A9 had no relationship with BVAS ($r=0.215$, $p=0.229$).

The serum S100A12 was closely related to ANCA level ($r=0.450$, $p=0.009$) and serum ferritin in AAV patients ($r=0.565$, $p<0.001$). However, there was no correlation between serum S100A12 and CRP or serum albumin ($r=0.277$, $p=0.113$ and $r=-0.164$, $p=0.355$, respectively), and serum S100A12 was not correlated with D-Dimer, RF, ESR and BVAS.

Correlation analysis also manifested that the urinary concentration of S100A8/A9 was correlated with BVAS ($r=0.350$, $p=0.439$), serum creatinine ($r=0.376$, $p=0.028$), hematuria ($r=0.416$, $p=0.016$) and the level of urinary neutrophil gelatinase-associated lipocalin (NGAL) of AAV patients. The urinary concentration of S100A12 was only correlated with serum creatinine ($r=0.373$, $p=0.030$) and had no relationship with BVAS, hematuria or urinary NGAL.

ANCA stimulates neutrophils to release S100A8/A9 and S100A12 in vitro

Since the serum concentrations of both S100A8/A9 and S100A12 increased in AAV and were significantly related with the ANCA levels, we tried to investigate whether the ANCA-activated neutrophils

could release S100A8/A9 and S100A12. We incubated neutrophils with ANCA-containing IgG. The supernatant S100A8/A9 was significantly increased after neutrophils were incubated with 1 mg/ml ANCA-containing IgG (compared with the group of normal IgG, 6.47±0.55 ng/ml vs. 1.56±0.19 ng/ml, p=0.011). Meanwhile, the S100A12 concentration after neutrophils were incubated with ANCA-containing IgG was also elevated (compared with the group of normal IgG, 6.7±0.43 ng/ml vs. 2.6±0.76 ng/ml, p=0.004). When the concentration of ANCA-containing IgG increased, the S100A8/A9 and S100A12 secreted by neutrophils were also enhanced (Figure 2A-B), which proved ANCA could stimulate the release of S100A8/A9 and S100A12 from neutrophils in a concentration-dependent manner.

S100A8/A9 and S100A12 induce chemotaxis and migration of neutrophils

When the concentration of S100A8/A9 in the lower transwell chamber was 5 μg/ml, the number of ANCA-induced neutrophils that migrate from the upper chamber to the lower chamber got to the maximum value and the chemotaxis index (CI) was 6.263±0.55, which was close to the positive fMLP group (6.503±0.99, p=0.99). S100A12 also has the capability of boosting the migration of neutrophils. When the concentration of S100A12 was 1 μg/ml, chemotaxis index (CI) was 5±0.73 (p=0.08 compared to fMLP group). Overall, both S100A8/A9 and S100A12 could promote the chemotaxis and migration of ANCA-induced neutrophils (Figure 3A).

S100A8/A9 and S100A12 extend the life span of neutrophils

Isolated neutrophils were treated with ANCA-containing IgG combined with different concentrations of S100A8/A9 or S100A12. The proportion of double negative cells (Annexin V-/PI-) after neutrophils were treated with ANCA-containing IgG was slightly higher than that after neutrophils were treated with normal IgG, but the difference was not statistically significant (48.57±2.14% vs. 42.97±3.29%, p=0.25). However, the proportions of Annexin V-/PI- increased after neutrophils were treated with ANCA-containing IgG combined with 1, 5 and 10 μg/ml S100A8/A9 comparing with that after neutrophils were treated with ANCA alone (58.37±1.6% and p=0.006, 61.67±4.05% and p<0.001, 57.77±2.6% and p=0.01, respectively). S100A12 also increased the proportions of Annexin V-/PI-. When neutrophils were treated with ANCA-containing IgG plus 1 μg/ml S100A12, the proportion of Annexin V-/PI- increased apparently (Compared with neutrophils treated with ANCA alone, 59.7±1.18% vs. 48.57± 2.14%, p=0.002) (Figure 3B-3K).

S100A8/A9 and S100A12 strengthen the ANCA-induced IL-1β release through TLR4 and RAGE

IL-1β plays an important role in AAV(14, 15), therefore, we investigated whether S100A8/A9 and S100A12 influenced the ANCA-induced neutrophils secretion of IL-1β. Compared with normal IgG, ANCA-containing IgG stimulated the neutrophils to release IL-1β (Figure 4A). When neutrophils were incubated with a combination of ANCA with S100A8/A9 or S100A12, the release of IL-1β was enhanced further (Figure 4A). When the receptors of TLR4 or RAGE on neutrophils were blocked, the supernatant concentrations of IL-1β decreased obviously (Figure 4A).

S100A8/A9 and S100A12 exaggerate the ANCA-triggered complement activation through TLR4 and RAGE

ANCA-containing IgG could trigger complement activation alone. After neutrophils were incubated with ANCA-containing IgG, increased supernatant C5a (15.93±2.8 ng/ml), CBb (82.6±7.5 ng/ml) and sC5b-9 (26.5±3.01 ng/ml) were detected. When neutrophils were incubated with ANCA plus 5 μg/ml S100A8/A9, the supernatant C5a got to 26.53±1.36 ng/ml (Compared with ANCA alone, $p<0.001$). The combination of ANCA and 1 μg/ml S100A12 also increased supernatant C5a concentration obviously (Compared with ANCA alone, 23.37±2.3 vs. 15.93±2.8 ng/ml, $p=0.01$). When the ANCA-activatied neutrophils were pre-incubated with the antibody of TLR4 and RAGE, the increased C5a induced by S100A8/A9 or S100A12 was inhibited significantly (Figure 4B).

S100A8/A9 and S100A12 also advanced the ANCA-induced CBb generation. The 5 μg/ml S100A8/A9 has the largest effect (CBb 164.3±43.6 ng/ml, $p=0.002$, compared with ANCA alone), while the most optimal concentration of S100A12 was 1 μg/ml (CBb 152.4±38.87 ng/ml, $p=0.009$, compared with ANCA alone). Meanwhile, the effects of S100A8/A9 and S100A12 were also inhibited after the blockade of TLR4 or RAGE (Figure 4C).

As for the sC5b-9, there was an enhanced generation of sC5b-9 when neutrophils were incubated with ANCA combined with 5 μg/ml S100A8/A9 (36.73±3.95 ng/ml, $p=0.008$, compared with ANCA alone), while S100A12 with a concentration of 1 μg/ml enhanced the ANCA-induced sC5b-9 generation (38.03±2.48 ng/ml, $p=0.002$, compared with ANCA alone). Similarly, blocking the receptors of TLR4 and RAGE reduced the effects of S100A8/A9 and S100A12 (Figure 4D).

S100A8/A9 and S100A12 exert pro-inflammatory effects through the p38 MAPK /NF-κBp65 pathway

In order to further determine whether the effects of S100A8/A9 and S100A12 were dependent on the activation of the MAPK/NF-κB pathway, we analyzed the intracellular expressions of the total p38 MAPK and phosphorylated p38 MAPK, as well as NF-κB p65 using western blotting. Compared with neutrophils incubated with normal IgG, neutrophils incubated with ANCA-containing IgG showed slightly elevated phosphorylation ratio of p38 MAPK (phosphorylated p38 MAPK protein/total p38 MAPK) and the

expression of NF-κB p65 increased simultaneously. Both S100A8/A9 and S100A12 could enhance the ANCA-induced p38 MAPK phosphorylation and NF-κB p65 expression, which were reduced after the blockade of TLR4 and RAGE (Figure 5).

The effects of S100A8/A9 and S100A12 do not depend on ROS

It has been reported that S100A8/A9 can inhibit neutrophils oxidative metabolism(16). Compared with normal IgG, ANCA-containing IgG significantly increased the ROS production of neutrophils (MFI, 253658±9936, $p<0.001$). When there were different concentrations of S100A8/A9 in the reaction system, the levels of ROS were obviously inhibited. S100A12 did not influence the ROS of neutrophils induced by ANCA-containing IgG.

Further, we studied whether S100A8/A9 and S100A12 could influence the ROS-dependent NETs formation (represented by the release of NE). As shown in figure 6K, S100A8/A9 had the tendency of restraining the release of NE by ANCA-activated neutrophils, and the inhibitory effect reached the maximum value when the concentration of S100A8/A9 got to 10 μg/ml (0.76±0.11, expressed as A values at 450 nm). But there was no statistical difference when comparing with the release of NE induced by ANCA alone (1.05±0.05, expressed as A values at 450 nm and $p=0.318$). S100A12 did not influence the release of NE induced by ANCA-containing IgG (Figure 6).

Discussion

As members of DAMPs, S100A8/A9 and S100A12 play crucial roles in various diseases. Previous studies have reported high levels of S100A8/A9 and S100A12 in AAV patients and the serum level of S100A8/A9 was correlated with disease relapse in PR3-AAV(17, 18). These findings prompted us to further clarify the significance of S100A8/A9 and S100A12 in AAV. In the current study, we verified the association between clinical parameters and serum levels of S100A8/A9 and S100A12 in patients with active AAV. Furthermore, we also demonstrated the association between the disease severity of AAV and the increased urine levels of S100A8/A9 and S100A12. Most importantly, we illustrated the possible pathogenic roles of S100A8/A9 and S100A12 in AAV with the in vitro experiments.

Neutrophils play an important role in the pathophysiology of AAV and this has been demonstrated both in vitro and in vivo(19). S100A8/A9 and S100A12 are primarily released from activated or necrotic neutrophils and involved in pathogenesis of various disease(20). Since ANCA is an activator of neutrophils, it is reasonable to speculate that the ANCA-activated neutrophils are important sources of the increased serum S100A8/A9 and S100A12 in AAV. In the current study, we confirmed that the serum levels of both S100A8/A9 and S100A12 were positively correlated with the serum ANCA levels in vivo, and the ANCA-containing IgG could stimulate neutrophils to release S100A8/A9 and S100A12 in a dose-dependent manner in vitro.

In AAV, ANCA-activated neutrophils migrate across endothelial cells and cause inflammation in AAV(21). Thus, all factors which can enhance the chemotaxis and migration of neutrophils might increase the severity of AAV. Previous studies had reported S100A8/A9 and S100A12 induced neutrophils chemotaxis and adhesion(22, 23). In the present study, we demonstrated that S100A8/A9 and S100A12 dramatically enhanced the chemotaxis of ANCA-activated neutrophils in the transwell experiment. This indicated that in AAV the S100A8/A9 and S100A12 in the local area of tissue damage would attract more ANCA-activated neutrophils and further aggravate tissue damage.

Dysregulation of neutrophils survival may contribute to the pathogenesis of AAV. Some studies have reported the infiltration and accumulation of unscavenged apoptotic neutrophils in perivascular tissues of the vessel walls and the delayed spontaneous apoptosis of neutrophils in AAV(24, 25). However, the exact mechanisms have not been clarified. In our study, we found S100A8/A9 and S100A12 extend the survival and decrease apoptosis of neutrophils. This indicated S100A8/A9 and S100A12 might contribute to neutrophils accumulation in regions of inflammation through inhibiting neutrophils apoptosis in AAV.

As an important pro-inflammatory cytokine, IL-1β plays a vital role in autoimmune disease. Previous studies had demonstrated that neutrophils could be stimulated by ANCA to express mRNA and protein for IL-1β(26). On the other hand, S100A8/A9 was once reported to stimulate peripheral blood mononuclear cells (PBMCs) to product IL-1β(27). In this study, we found S100A8/A9 and S100A12 could exaggerate the release of IL-1β through binding on the TLR4 and RAGE on neutrophils. This signified S100A8/A9 and S100A12 had pro-inflammatory function in AAV.

The activation of the complement system via the alternative pathway plays a crucial role in the development of AAV(28, 29). In the present study, we found that S100A8/A9 and S100A12 increased the level of complement factors in the supernatant of ANCA-activated neutrophils. These increased complement factors could be attenuated by TLR4 or RAGE inhibitors, which illustrated that S100A8/A9 and S100A12 can promote the activation of the alternative complement pathway through TLR4 or RAGE signaling pathway.

According to previous studies, the TLR4 and RAGE signaling can activate the MAPKs and NF-κB, and subsequently enhance the transcription of pro-inflammatory mediators(30, 31). MAPKs are crucial regulators of a series of cellular process, such as the proliferation and differentiation of cells(32). Our data showed that S100A8/A9 and S100A12 increased the expression of phosphorylated p38 MAPK and NF-κB p65. After the blockade of TLR4 and RAGE, the expressions of phosphorylated p38 MAPK and NF-κB p65 were also reduced. Taken together, our results indicated that TLR4/RAGE-p38 MAPK-NF-κB p65 signaling pathways were involved in the effects of S100A8/A9 and S100A12 on neutrophils.

It was noteworthy that S100A8/A9 inhibited the ANCA-induced ROS generation of neutrophils. This was correspondent with the previous report which demonstrated that S100A8 reduced reactive oxygen species generated by activated leukocytes through its thiol-scavenging capacity(33). Correspondingly, S100A8/A9 had a tendency to inhibit the generation of NETs which was ROS-dependent. However,

S100A12 did not inhibit the ANCA-induced ROS generation of neutrophils and did not have a tendency to inhibit the generation of NETs. So the pro-inflammatory effects of both S100A8/A9 and S100A12 did not depend on ROS. In addition to the above experimental results, we also found that S100A8/A9 and S100A12 can aggravate the damage of NETs produced by ANCA stimulation to vascular endothelial cells and more validated experiments are required in the future.

Some limitations of this study should be mentioned. First, our samples of patients are relatively small, thus larger cohort will be needed to explore the relationship between the level of S100A8/A9 and S100A12 and the prognosis of the AAV. Second, due to the characteristics of AAV in the Chinese population, all patients enrolled in our study were MPO-ANCA positive. Third, our experiments were done in vitro, therefore the results might be slightly affected by the experimental conditions. More persuasive in vivo study is needed in future.

In conclusion, the serum and urine levels of S100A8/A9 and S100A12 in AAV patients were elevated and correlated with the activity of disease. Besides, S100A8/A9 and S100A12 might take part into the pathogenesis of AAV directly. Both S100A8/A9 and S100A12 can exaggerate the inflammatory effects of ANCA in a ROS-independent manner.

Materials And Methods

Patients

Patients' serum and urine were obtained from 34 AAV patients with positive myeloperoxidase (MPO)-ANCA. All of these 34 patients, diagnosed in Tianjin Medical University General Hospital, fulfilled the 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides(2). Serum and urine from 10 healthy donors were obtained as normal controls. The research was in compliance of the declaration of Helsinki and the protocol was approved by the institutional review board of Tianjin Medical University General Hospital (IRB2018-202-01). Informed consent was obtained from all individual participants included in the study.

Clinical and laboratory data

Clinical data included following: gender, age and the score of Birmingham Vasculitis Activity Score (BVAS). Laboratory data included as following: hemoglobin, serum creatinine level, the level of ANCA, C-reactive protein (CRP), complement 3 (C3) and 4 (C4), erythrocyte sedimentation rate (ESR), ferritin, D-dimer, rheumatoid factor (RF), albumin, proteinuria, hematuria, urinary neutrophil galetinase associated lipocalin (NGAL). Proteinuria was defined as the total urine protein was above the upper normal limit (0.15g/24h).

Immunoassays of S100A8/A9 and S100A12

Concentrations of S100A8/A9 (439707, biolegend) and S100A12 (CSB-E13095h, cusabio) in serum and urine from participants were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions.

Neutrophil isolation of peripheral blood

Neutrophils from healthy donors were isolated by density centrifugation (12). Briefly, a double gradient was formed by layering Histopaque 1077 (10771, Sigma-Aldrich) on an equal volume of Histoque 1119 (11191, Sigma-Aldrich). Heparinized blood donated by healthy volunteers was carefully layered onto the upper gradient. After 20 min centrifugation at 300×g, neutrophils between two Histopaque medium were carefully collected. Cells were washed by addition of 10 ml of isotonic phosphate buffered saline (PBS). Neutrophil pellet was resuspended with red blood cell lysis buffer (R1010, Solarbio) to lyse red blood cell. Neutrophils were then washed twice and resuspended in an appropriate volume of RPMI1640 medium. The concentration of neutrophils was adjusted to 1×10^6/ml.

Production of S100A8/A9 and S100A12 by neutrophils stimulated with ANCA

Neutrophils (1×10^6/ml) were primed with 2 ng/ml TNF-α (H8916, Sigma-Aldrich) at 37°C for 15 min and cultured with normal control IgG from healthy donors and different concentration of ANCA-containing IgG purified from AAV patients at 37°C for 24 hr. The concentrations of S100A8/A9 and S100A12 were detected by ELISA as above.

Neutrophil migration and chemotaxis

A sterile 24-well transwell plate with a pore size of 3 μm was used. Cells were pre-stimulated with ANCA-containing IgG (1 mg/ml) for 1 hr and were inoculated in the upper chamber. Then the upper chamber was moved to experimental well with different concentrations of S100A8/A9 (Sino Biological) and S100A12 (Cusabio) in a 37°C, 5% CO2 cell incubator for 2 hr. Five fields of view were randomly selected to take pictures under a 100×optimal microscope. Chemotaxis Index (CI) was calculated by dividing the total number of cells in the lower chamber of each experimental group by the total number of cells in the lower chamber of the blank control group.

Apoptosis of neutrophils

Isolated neutrophils from healthy donors were primed with TNF-α and treated with ANCA, S100A8/A9 or S100A12 for 12hr at cell incubator. Cells were collected at 1000 rpm and washed twice with PBS. The PMNs pellet was resuspended with binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCL, 2.5 mM

$CaCL_2$). Apoptosis of neutrophils was assessed with Annexin V-FITC/PI-PE Kit (FX018, 4A Biotech) by flow cytometry. The results were analysed by FlowJo 10.4.0.

Assessment of IL-1β and complement factors released by neutrophils

TNF-α-primed neutrophils were stimulated for 2 hr with normal control IgG, ANCA-containing IgG, S100A8/A9, S100A12, S100A8/A9+ANCA, S100A12+ANCA respectively. In the group of blocking TLR4 or RAGE, the antibody of Toll-like receptor 4 (TLR4) (312802, Biolegend) or Receptor for Advanced Glycosylation Endproducts (RAGE) (ab37647, Abcam) was added and incubated for 30 min before adding S100A8/A9 and S100A12. The supernatant was collected to detect the concentration of IL-1β (437007, biolegend), complement 5a (C5a) (JL10644, Shanghai Jianglai Biological Technology Co., Ltd.), complement Bb (CBb) (JL19313, Shanghai Jianglai Biological Technology Co., Ltd.) and soluble complement 5b-9 (sC5b-9) (JL18355, Shanghai Jianglai Biological Technology Co., Ltd.).

Western blot analysis

Neutrophils were collected and added to RIPA lysis solution together with the protease inhibitor PMSF. After 30min incubation on ice, supernatants were extracted with a 10,000 rpm centrifugation. Denatured PMNs protein extract was subjected to SDS-PAGE and transferred to nitrocellulose membranes, which were then blocked 1 hr at room temperature with 5% skim milk. After incubated with primary antibodies against p-p38 MAPK/t-p38 MAPK, NF-κB p65 (Abcam, Cambridge, USA) and GAPDH (A19056, ABclonal) overnight at 4°C, the horseradish peroxidase-conjugated mouse anti-rabbit monoclonal antibody was used to detect binded primary antibodies. The membranes were exposed with chemiluminescence imaging system and the results were performed with Image J software system (NIH,USA).

Flow cytometry for oxidative respiratory burst of neutrophils

The measurement of oxidative activation of neutrophils was based on ROS-dependent oxidation of dihydrorhodamine 123 (DHR123) to rhodamine 123 (R123), which is a cationic green fluorescent dye and can derive the uncharged non-fluorescent dye DHR123(13). DHR123 was added to the TNF-α primed neutrophils suspension to the final concentration of 5 μg/ml. Neutrophils were then incubated with ANCA-containing IgG (1 mg/ml), normal control IgG (1 mg/ml), S100A8/A9+ANCA IgG and S100A12+ANCA IgG at 37°C for 1hr. The samples were assessed by flow cytometry analysis and the production of ROS was represented by mean fluorescence intensity (MFI) of FITC gating channel.

Induction of netting neutrophils by S100A8/A9 and S100A12 with ANCA

Neutrophils (1×10^6/ml) were primed with 2 ng/ml TNF-α at 37°C for 15 min then incubated with normal control IgG (1 mg/ml), ANCA-containing IgG (1 mg/ml), S100A8/A9 heterodimer protein or S100A12 protein at 37°C for 24 hr. In order to detect the production of NETs, neutrophils were centrifuged 5 min at 1500rpm and the supernatant was collected. The concentration of neutrophil elastase (NE) (JL12352, Shanghai Jianglai Biological Technology Co., Ltd.) was determined by ELISA.

Statistical analysis

Different experiments were performed at least three times. The normal distribution of quantitative data was tested by *Kolmogorov*-Smirnov test. Multiple sets of continuous variables which were normally distributed were evaluated using one-way ANOVA analysis. For two-independent groups that were not normally distributed, Mann-Whitney U test was applied. A Spearman rank correlation was used for correlation data. Statistical significance was set at p value <0.05. GraphPad Prism 8.0.1 software for windows (GraphPad Software, California, USA) was used for data analysis.

Abbreviations

AAV, ANCA-associated vasculitis; ANCA, anti-nutrophil cytoplasmic antibody; MPO, myeloperoxidase; PR3, proteinase 3; MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis; PMN, polymorphonuclear lymphocytes; NETs, neutrophil extracellular traps; NE, neutrophil elastase; DAMP, damage-associated molecular pattern; HMGB1, high mobility group box protein 1; BVAS, birmingham vasculitis activity score; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; NGAL, neutrophil galetinase associated lipocalin; PBS, phosphate buffered saline; CI, chemotaxis index; TLR4, toll-like receptor 4; RAGE, receptor of advanced glycation endproducts; PMSF, phenylmethylsulfonyl fluoride; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROS, reactive oxygen species; MFI, mean flourescence intensity; fMLP, formylmethionyl leucyl phenylalanine; PBMC, peripheral blood mononuclear cells

Declarations

Ethics approval and consent to participate

Our study complies with the Declaration of Helsinki. The Institutional Ethical Committee of Tianjin Medical University General Hospital has approved the research protocol. All subjects provided written informed consents.

Consent for publication:

Not applicable

Availability of data and materials:

Raw data used during the current study are available from the corresponding author on reasonable request for non-commercial use.

Competing interests

Author Xue Bai, Author Peng-Cheng Xu, Author Tong Chen, Author Hao-Miao Zhang, Author Si-Jing Wu, Author Xia Yang, Author Shan Gao, Author Jun-Ya Jia, Author Jian-Qing Jiang and Author Tie-Kun Yan declare that they have no conflict of interests.

Funding

This work was supported by four grants of National Natural Science Fund (Grant No. 82170726, No. 81900125, No. 82000669, and No. 81570630) and three grants of Tianjin Research Program of Application Foundation and Advanced Technology (Grant No. 20JCQNJC00570, No.15JCQNJC10700 and No.17JCQNJC10200). The funders have no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Peng-Cheng Xu had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Xue Bai, Peng-Cheng Xu, Tong Chen.

Acquisition of data. Hao-Miao Zhang, Si-Jing Wu, Xia Yang, Shan Gao.

Analysis and interpretation of data. Jun-Ya Jia, Jian-Qing Jiang, Tie-Kun Yan.

Acknowledgements

The authors thank all patients who participated in this study.

References

1. Schonermarck U, Csernok E, Gross WL. Pathogenesis of anti-neutrophil cytoplasmic antibody-associated vasculitis: challenges and solutions 2014. Nephrol Dial Transplant 2015;30 Suppl 1:i46-52.
2. Jennette JC, Falk RJ, Bacon PA et al. 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. Arthritis Rheum 2013;65:1-11,.

3. Kessenbrock K, Krumbholz M, Schonermarck U et al. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med 2009;15:623-5.
4. Pinegin B, Vorobjeva N, Pinegin V. Neutrophil extracellular traps and their role in the development of chronic inflammation and autoimmunity. Autoimmun Rev 2015;14:633-40.
5. Wang C, de Souza AW, Westra J et al. Emerging role of high mobility group box 1 in ANCA-associated vasculitis. Autoimmun Rev 2015;14:1057-65.
6. Ma YH, Ma TT, Wang C et al. High-mobility group box 1 potentiates antineutrophil cytoplasmic antibody-inducing neutrophil extracellular traps formation. Arthritis Res Ther 2016;18:2.
7. Moore BW. A soluble protein characteristic of the nervous system. Biochem Biophys Res Commun 1965;19:739-44.
8. Pepper RJ, Wang HH, Rajakaruna GK et al. S100A8/A9 (calprotectin) is critical for development of glomerulonephritis and promotes inflammatory leukocyte-renal cell interactions. Am J Pathol 2015;185:1264-74.
9. Zhang Z, Han N, Shen Y. S100A12 promotes inflammation and cell apoptosis in sepsis-induced ARDS via activation of NLRP3 in fl ammasome signaling. Mol Immunol 2020;122:38-48.
10. Pepper RJ, Hamour S, Chavele KM et al. Leukocyte and serum S100A8/S100A9 expression reflects disease activity in ANCA-associated vasculitis and glomerulonephritis. Kidney Int 2013;83:1150-8.
11. Brown KL, Lubieniecka JM, Armaroli G et al. S100A12 Serum Levels and PMN Counts Are Elevated in Childhood Systemic Vasculitides Especially Involving Proteinase 3 Specific Anti-neutrophil Cytoplasmic Antibodies. Front Pediatr 2018;6:341.
12. Xu PC, Lin S, Yang XW et al. C-reactive protein enhances activation of coagulation system and inflammatory response through dissociating into monomeric form in antineutrophil cytoplasmic antibody-associated vasculitis. BMC Immunol 2015;16:10.
13. Kiani-Esfahani A, Tavalaee M, Deemeh MR, Hamiditabar M, Nasr-Esfahani MH. DHR123: an alternative probe for assessment of ROS in human spermatozoa. Syst Biol Reprod Med 2012;58:168-74.
14. Rider P, Carmi Y, Guttman O et al. IL-1alpha and IL-1beta recruit different myeloid cells and promote different stages of sterile inflammation. J Immunol 2011;187:4835-43.
15. O'Brien EC, Abdulahad WH, Rutgers A et al. Intermediate monocytes in ANCA vasculitis: increased surface expression of ANCA autoantigens and IL-1beta secretion in response to anti-MPO antibodies. Sci Rep 2015;5:11888.
16. Sroussi HY, Lu Y, Zhang QL, Villines D, Marucha PT. S100A8 and S100A9 inhibit neutrophil oxidative metabolism in-vitro: involvement of adenosine metabolites. Free Radic Res 2010;44:389-96.
17. Page TH, Chiappo D, Brunini F et al. Danger-associated molecular pattern molecules and the receptor for advanced glycation end products enhance ANCA-induced responses. Rheumatology (Oxford) 2021. Epub ahead of print.

18. Pepper RJ, Draibe JB, Caplin B et al. Association of Serum Calprotectin (S100A8/A9) Level With Disease Relapse in Proteinase 3-Antineutrophil Cytoplasmic Antibody-Associated Vasculitis. Arthritis Rheumatol 2017;69:185-93.
19. Xiao H, Heeringa P, Hu P et al. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. Journal of Clinical Investigation 2002;110:955-63.
20. Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol 2009;86:557-66.
21. Calderwood JW, Williams JM, Morgan MD, Nash GB, Savage CO. ANCA induces beta2 integrin and CXC chemokine-dependent neutrophil-endothelial cell interactions that mimic those of highly cytokine-activated endothelium. J Leukoc Biol 2005;77:33-43.
22. Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. J Immunol 2003;170:3233-42.
23. Rouleau P, Vandal K, Ryckman C et al. the calcium-binding protein S100A12 induces neutrophil adhesion, migration, and release from bone marrow in mouse at concentrations similar to those found in human inflammatory arthritis. Clinical Immunology 2003;107:46-54.
24. Kettritz R, Wilke S, von Vietinghoff S, Luft F, Schneider W. Apoptosis, proliferation and inflammatory infiltration in ANCA-positive glomerulonephritis. Clin Nephrol 2006;65:309-16.
25. Abdgawad M, Pettersson A, Gunnarsson L et al. Decreased neutrophil apoptosis in quiescent ANCA-associated systemic vasculitis. PLoS One 2012;7:e32439.
26. Brooks CJ, King WJ, D J Radford, D Adu MM, Savage CO. IL-1β production by human polymorphonuclear leucocytes stimulated by anti-neutrophil cytoplasmic autoantibodies: relevance to systemic vasculitis. Clin Exp Immunol 1996;106:273-9.
27. Nicaise C, Weichselbaum L, Schandene L, Gangji V. Phagocyte-specific S100A8/A9 is upregulated in primary Sjögren's syndrome and triggers the secretion of pro-inflammatory cytokines in vitro. Clin Exp Rheumatol 2017;35:129-36.
28. Xiao H, Schreiber A, Heeringa P, Falk RJ, Jennette JC. Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies. Am J Pathol 2007;170:52-64.
29. Xiao H, Dairaghi DJ, Powers JP et al. C5a receptor (CD88) blockade protects against MPO-ANCA GN. J Am Soc Nephrol 2014;25:225-31.
30. Vijay K. Toll-like receptors in immunity and inflammatory diseases: Past, present, and future. Int Immunopharmacol 2018;59:391-412.
31. Kierdorf K, Fritz G. RAGE regulation and signaling in inflammation and beyond. J Leukoc Biol 2013;94:55-68.

32. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res 2002;12:9-18.
33. Zhao J, Endoh I, Hsu K, Tedla N, Endoh Y, Geczy CL. S100A8 modulates mast cell function and suppresses eosinophil migration in acute asthma. Antioxid Redox Signal 2011;14:1589-600.

Figures

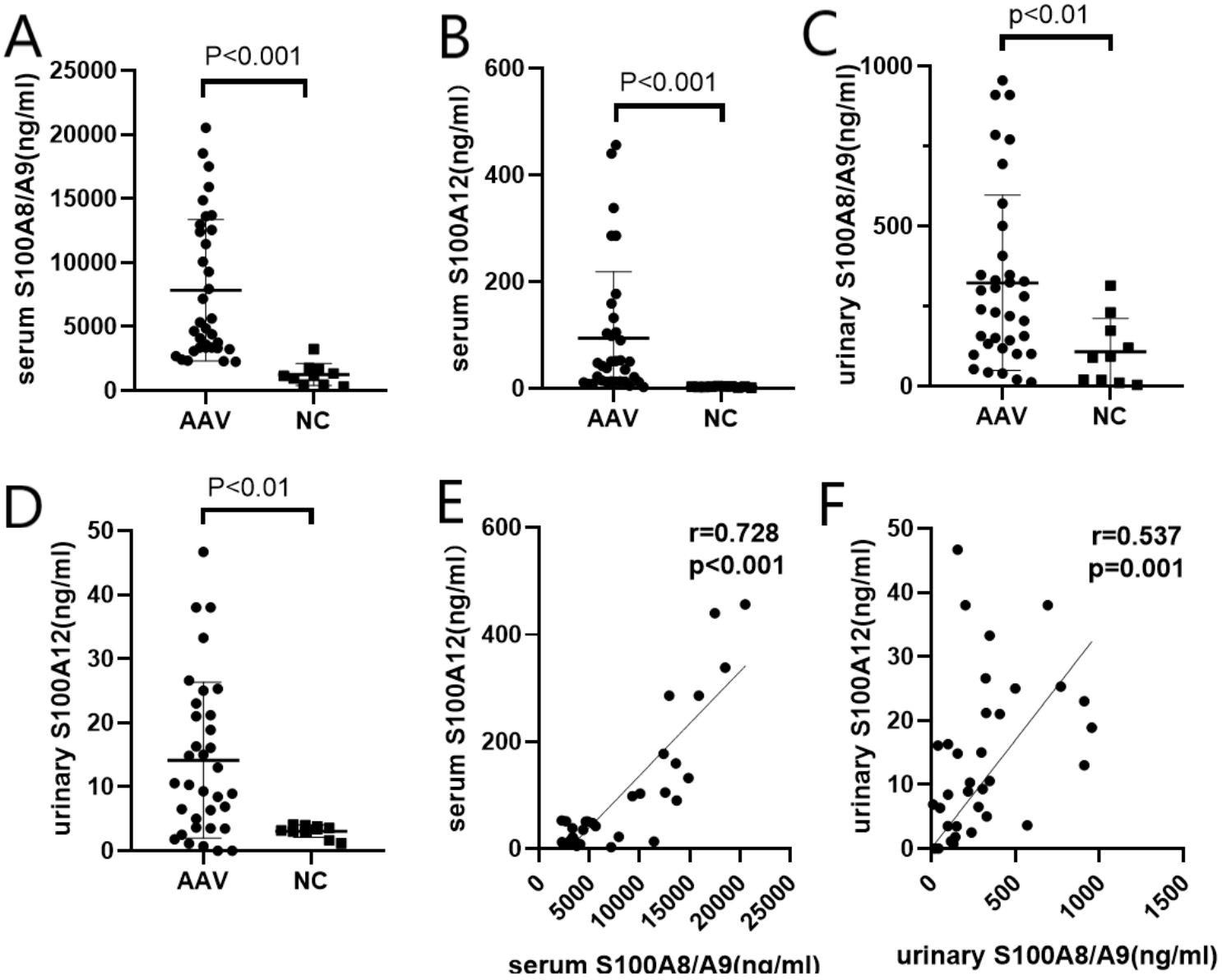

Figure 1

Levels of serum and urinary S100A8/A9 and S100A12 in AAV patients and normal controls. A: Comparison of the serum concentration of S100A8/A9 between AAV and NC. B: Comparison of the serum concentration of S100A12 between AAV and NC. C: Comparison of the urinary concentration of S100A8/A9 between AAV and NC. D: Comparison of the urinary concentration of S100A12 between AAV

and NC. E: The relationship between serum S100A8/A9 and serum S100A12 in AAV. F: The relationship between urinaryS100A8/A9 and urinary S100A12 in AAV. NC: normal controls.

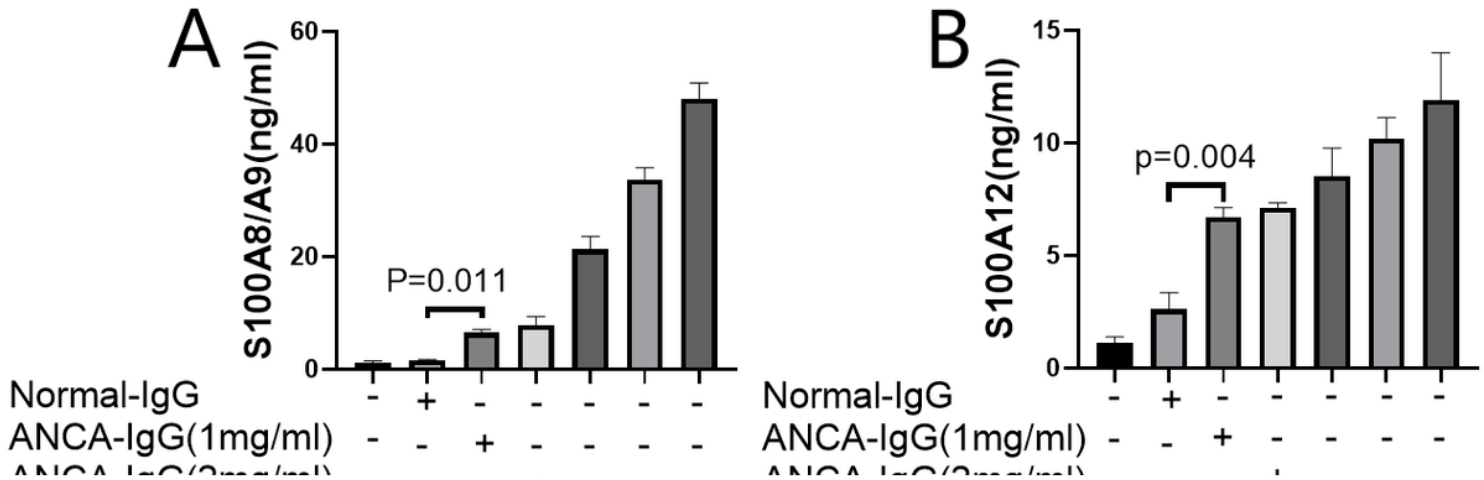

Figure 2

ANCA IgG stimulates neutrophils to release S100A8/A9 (A) and S100A12 (B) concentration-dependently.

Figure 3

Influence of different concentrations of S100A8/A9, S100A12 on the chemotaxis and cell death of neutrophils. A: The chemotaxis index of ANCA-activated neutrophils treated with different concentrations of S100A8/A9 and S100A12. B: Effects of S100A8/A9 and S100A12 on the cell death of ANCA-activated neutrophils. C-K: Representative images of the flow cytometry analysis. C: Neutrophils were incubated with PBS. D: Neutrophils were incubated with normal-IgG. E: Neutrophils were incubated with ANCA-IgG alone. F-H: Neutrophils were incubated with ANCA combined with 1, 5, 10 μg/ml S100A8/A9 respectively. I-K: Neutrophils were incubated with ANCA combined with 1, 5, 10 μg/ml S100A12 respectively.

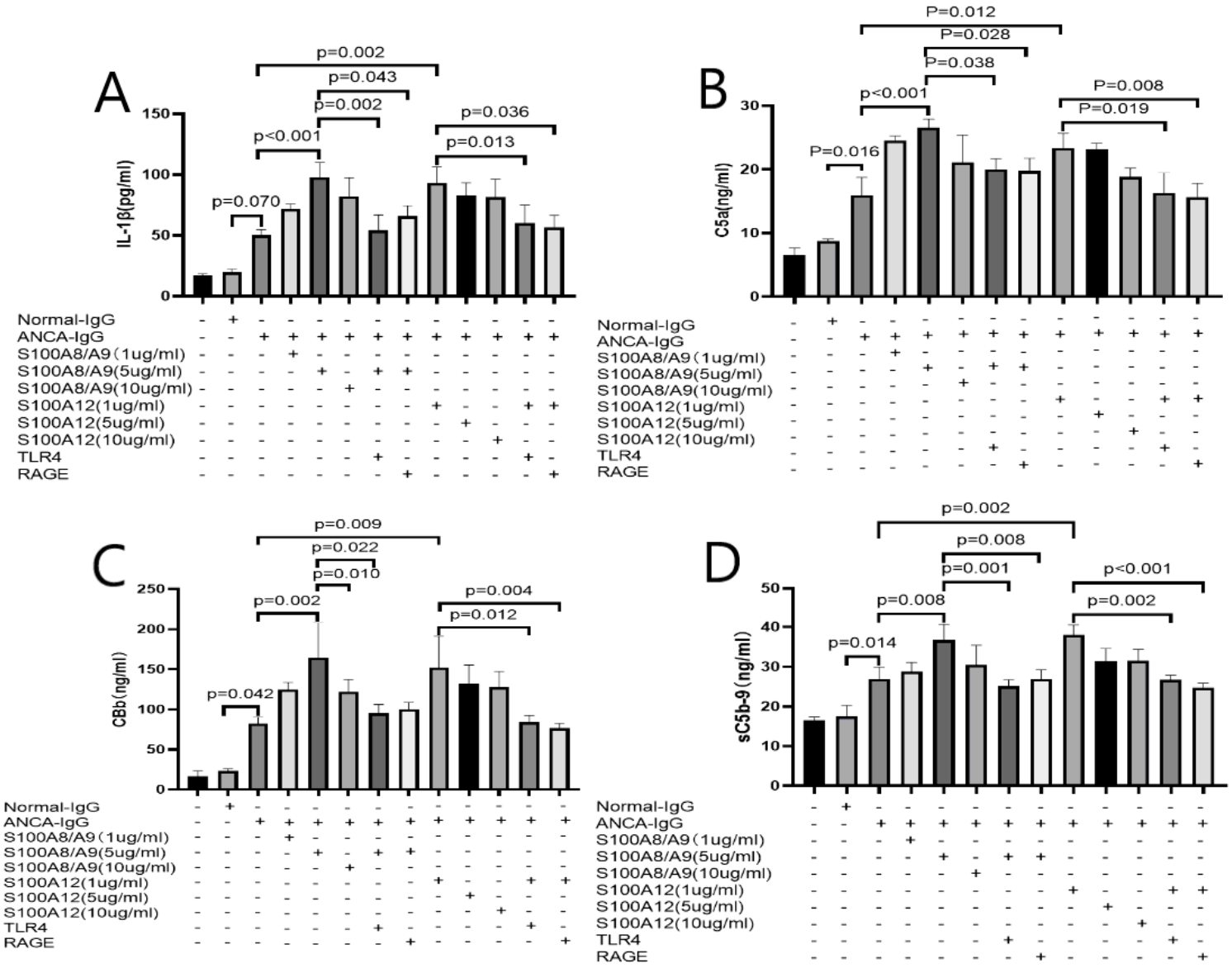

Figure 4

S100A8/A9, S100A12 enhanced the release of IL-1β and complement activation of ANCA-stimulated neutrophils through TLR4/RAGE. A: Effects of S100A8/A9 and S100A12 on the release of IL-1β from ANCA-activated neutrophils. B-D: Effects of S100A8/A9 and S100A12 on the supernatant concentrations of C5a, CBb and sC5b-9 of ANCA-activated neutrophils.

Figure 5

S100A8/A9 and S100A12 played their pro-inflammatory effects through p38 MAPK/NF-κB p65 pathway. Original blots are presented in Supplementary Figure 1 and 2. A: S100A8/A9 and S100A12 induced p38 MAPK phosphorylation and NF-κB p65 expression. B: The phosphorylation ratios of p38 after neutrophils were stimulated with a combination of ANCA and S100A8/A9 or S100A12. C: The activation of NF-κB

p65 after neutrophils were stimulated with a combination of ANCA and S100A8/A9 or S100A12. D: The phosphorylation of p38 MAPK and the activation of NF-κB p65 after the blockade of TLR4 and RAGE. E: The phosphorylation ratio of p38 after blocking TLR4 and RAGE. F: The expression of NF-κB p65 after blocking TLR4 and RAGE.

Figure 6

Effects of S100A8/A9 and S100A12 on the production of ROS and NETs in AAV. A-I: Representative images of influences of S100A8/A9 and S100A12 on ANCA-induced production of ROS. Neutrophils were incubated with different stimulators and analyzed using flow cytometry. A: Neutrophils were incubated with PBS. B: Neutrophils were incubated with normal-IgG. C: Neutrophils were incubated with ANCA. D-F: Neutrophils were incubated with ANCA combined with 1, 5, 10 μg/ml S100A8/A9 respectively. G-I: Neutrophils were incubated with ANCA combined with 1, 5, 10 μg/ml S100A12 respectively. J: The mean flourescence intensity of ROS after neutrophils were incubated with different stimulators. K: Effects of S100A8/A9 and S100A12 on ANCA-induced formation of NETs. NE: neutrophil elastase.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials.pdf