Original Article
Identification of "antigen-specific" neutrophils in atherosclerosis patients that compromise vascular endothelial barrier function

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Abstract: The vascular endothelial barrier dysfunction is associated with the pathogenesis of many cardiovascular diseases, such as atherosclerosis (AS). This study aims to identify specific antigen (Ag, in short)-specific polymorpho-nuclear neutrophils (PMN) in AS patients and to investigate the role of "Ag-specific" PMN activation in causing vascular endothelial barrier dysfunction. In this study, PMNs were isolated from blood samples collected from patients with AS and analyzed with immunological approaches. Human umbilical vein endothelial cells (HUVEC) monolayers were used as a vascular endothelial barrier model. The results showed that "Ag-specific" PMNs were identified in the blood of 50 AS patients. This subset of PMN was featured as the FcγRI and specific IgG (sIgG) complexes on the cell surface; exposure to specific Ags triggered the "Ag-specific" PMNs to release proinflammatory cytokines. PMN-derived cytokine levels in the serum were positively correlated with the serum levels of sIgG in AS patients. Exposure of naive PMNs to sIgG formed FcγRI and sIgG complexes on the surface; this conferred PMNs the property to be recognized and activated by specific Ag. Stimulation of "Ag-specific" PMN activated the mitogen-activated protein kinase and the activities of nuclear factor activated T cells and promoted the gene transcription of tumor necrosis factor-α. Coculture of "Ag-specific" PMNs and HUVEC monolayers in the presence of specific Ag resulted in the HUVEC monolayer barrier dysfunction. In conclusion, "Ag-specific" PMNs were identified in AS patients. Activation of the PMNs compromised vascular endothelial barrier function. Therefore, to regulate the "Ag-specific" PMN's activities may have translational potential in the treatment of AS.

Keywords: Vascular endothelium, endothelial barrier, neutrophil, immunity, atherosclerosis

Introduction

Atherosclerosis (AS) indicates a condition that fats, cholesterol and other substances build up in and on the artery walls; it is also called plaque, which can block blood stream. The plaque may break to trigger a blood clot [1]. Although atherosclerosis is often considered a heart problem, it can affect arteries anywhere in the body [1]. Because of developing slowly and gradually, AS does not cause any symptoms in its early stage. However, when it builds up to a size large enough to block the blood

flow, partially or completely, symptoms may occur in the organs or tissues relying on the blood supply of blocked blood vessels [2]. If the block occurs in the heart, chest pain or angina may occur [3]. Although research in AS advanced rapidly in recent years, the causative factors of AS remain incompletely understood.

The AS refers to an inflammatory disease of arteries, which develops at sites with disturbed blood flow; upon endothelial activation, intimal retention of lipoproteins may occur on sites [1]. Therefore, the modified lipoproteins may con-

tribute to the AS development by the oxidized low-density lipoprotein, augment endothelial damage and trigger recruitment of leukocytes. Eventually, arterial thrombosis may form and lead to vessel occlusion [1].

Published data show many factors are associated with the AS development. Such as hypoxia, high levels of reactive oxygen species in the vascular tissues, retention of extracellular proteases, inter-endothelial cell junction-associated protein abnormality [4]. Lipid retention in the vascular intima is the essential AS pathological feature [5]. Therefore, endothelial barrier dysfunction provides a pathway for lipid getting into the intimal tissues. Tumor necrosis factor (TNF)-α is one of the factors inducing epithelial barrier dysfunction [6, 7]. Although Th1 response is associated with AS pathogenesis, it is also found that AS patients showed high Ag-specific IgE levels in the serum [8]. Yet, whether specific antigens are associated with the AS pathogenesis is to be further investigated.

PMNs are a fraction of white blood cells. It occupies about 60% circulation white blood cells and is the major part of phagocytes. PMNs play a crucial role in the defense of the human body. There are many granules in PMNs, which contain large amounts of proteases and myeloperoxidases (MPO). Upon activation, PMNs release these substances to kill microbes [9]. Activated PMNs also release proinflammatory cytokines, such as TNF-α, to induce inflammation or compromise epithelial barrier function [10]. PMNs express FcγRI, or CD64, that recognizes IgG's Fc domain [11]. Such a feature enables PMNs easy to engulf those IgG-coated bacteria or foreign antigens. The FcγRI on the surface of PMN can be bound by monomeric IgG to form complexes [12]. PMNs are also involved in the AS pathogenesis [13]. Whether can this complex be bound by specific antigen and results in PMN activation to disturb the homeostasis of vascular endothelium that contributes to AS pathogenesis needs to be investigated. Therefore, in this study, PMNs were isolated from blood samples collected from AS patients. The role of specific Ag in activating "Ag-specific" PMNs was analyzed by multiple immunological approaches. This study identified "Ag-specific" PMNs, which could compromise the vascular endothelial barrier function.

Materials and methods

Reagents

Antibodies of IgG (sc-515946), OVA (65984), MAPK (sc-374276), NFAT2 (sc-514929), Pol II (sc-55492), CD11c (sc-46676; Alexa Fluor® 488), CD66b (sc-101383; Alexa Fluor® 546), CD154 (sc-19985; Alexa Fluor® 594), Ki67 (sc-23900; Alexa Fluor 647) and FcγRI (sc-100279; Alexa Fluor® 647) were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-EpCAM antibody was purchased from abcam (Cambridge, MA). ELISA kits of IgG, TNFα and MPO were purchased from R&D Systems (Minneapolis, MN). FITC-dextran (40 kDa), lipopolysaccharide (LPS) and ChIP kit were purchased from Sigma Aldrich (St. Louis., MO). Reagents and materials for RT-qPCR, Western blotting, immunoprecipitation (IP) and chromatin IP (ChIP) were purchased from Invitrogen (Carlsbad, CA).

Patients

AS patients were recruited into this study at Guangzhou First People's Hospital. The AS diagnosis and management were carried out by our physicians following our routine procedures. Healthy control (HC) subjects were also recruited. The demographic data are presented in **Table 1**. Patients with any of the following conditions were excluded from this study, including cancer, severe organ diseases, allergic diseases, autoimmune diseases, under treatment with immune suppressors for any reasons. The experimental procedures were approved by the Human Ethic Committee at Guangzhou First People's Hospital. An informed written consent was obtained from each human subject.

PMN isolation

PBMCs were isolated from blood samples collected from AS patients or HC subjects and stained with antibodies of CD45, CD66b and CD11b (labeled with fluorochromes). CD66b+ CD11b+ PMNs were isolated by flow cytometry cell sorting (FCCS). Cell purity was greater than 95% as assessed by flow cytometry. To isolate naive PMNs, the CD154+ IgG+ PMNs were selected out the CD66b+ CD11b+ PMNs by FCCS as CD154 can be an activation cell marker in T cells [14] that we also observed in PMN.

Table 1. Demographic data of human subjects

Characteristic	HC	AS
	(N = 50)	(N = 50)
Age-year	41.3 ± 5.5	42.4 ± 4.2
Male-No.	15 (60%)	13 (52%)
Body weight-kg	61.6 ± 11.1	62.2 ± 8.8
Body mass index [kg/m^2]	21.1 ± 2.6	22.2 ± 2.3
Waist to hip ratio	0.83 ± 0.08	0.84 ± 0.09
Current smoker-No.	2 (4%)	2 (4%)
Duration of disease-year	-	3.5 ± 1.5
Site of disease-No. (%)		
Coronary artery	-	38 (72%)
Aorta	-	12 (28%)
Medication-No. (%)		
No medication	50 (100%)	50 (100%)
Blood pressure		
Systolic BP (mmHg)	126 ± 8	120 ± 11
Diastolic BP (mmHg)	76 ± 6	74 ± 8
Heart rate (bmp)	78 ± 8	75 ± 9
Serum		
Hemoglobin (g/L)	133.5 ± 9.2	132.2 ± 10.5
Albumin (mg/L)	40.2 (36.8 ± 5.5)	39.6 (35.2 ± 6.6)
TC (mg/dL)	172.4 ± 7.7	172.8 ± 6.7
LDL (mg/dL)	84.5 ± 3.5	85.8 ± 6.6
HDL (mg/dL)	76.3 ± 6.6	77.1 ± 6.3
TAG (mg/dL)	92.3 ± 5.9	93.4 ± 8.6
Total IgG (mg/dL)	846.6 (785.5-1225.6)	1299.6 (815.2-1465.6)*
Total IgE (ng/mL)	23.3 (0.4-168.7)	109.6 (20.8-305.9)*
Highest sIgE (kU/L)	4.9 (0.1-26.3)	16.6 (12.4-49.8)*

TC: Total cholesterol. LDL: Low-density lipoprotein cholesterol. HDL: High density lipoprotein cholesterol. TAG: Triacylglycerols. *P<0.0001, assessed by Mann-Whitney *U* test.

Skin prick test (SPT)

SPT was performed for AS patients in the out-patient of our hospital using commercial food extract reagents (Greer Company; Taibei, China) and house dust mite (HDM; Wowu Biotech, Hangzhou, China). Food extracts included shrimp, eggs, walnuts, hazelnuts, soy, peanuts, fish and cow's milk. Saline (negative control) and histamine (positive control, 1 mg/ml) were used as control agents. SPT positive was defined when the wheal diameter was 3 mm larger than a negative control at 15 min.

Preparation of peripheral blood mononuclear cells (PBMCs)

Blood samples were collected from each human subject through the ulnar vein puncture. PBMCs were isolated from the samples by the Percoll gradient density centrifugation.

Cell culture

Cells were cultured in RPMI1640 medium. The medium was supplemented with antibiotics (penicillin, 100 U/ml; streptomycin, 0.1 mg/ml), fetal bovine serum (10%) and L-glutamine (2 mM). The medium was changed in 2-3 days. Cell viability was greater than 99% as assessed by the Trypan exclusion assay.

Activation of PMN with specific Ags

PMNs were isolated from blood samples collected from AS patients and cultured in RPMI1640 medium. To activate PMNs, specific Ags (based on the SPT results) were added to the culture at 10 µg/ml. The MPO levels in the culture were determined by ELISA and used as an indicator of PMN activation.

Flow cytometry

In the surface staining, cells collected from relevant experiments were stained with antibodies of interest or isotype IgG (labeled with fluorochromes) at 4°C for 30 min. In the intracellular staining, cells were fixed with 1% paraformaldehyde (containing 0.1% Triton X-100 to increase the cell membrane permeability) for 1 h and then stained with relevant antibodies or isotype IgG (labeled with fluorochromes) at 4°C for 30 min. After washing with phosphate-buffered saline (PBS), cells were analyzed with a flow cytometer (FACSCanto II). The data were processed with a software package flowjo. Data from isotype IgG staining were used as a gating reference.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels in the serum or culture supernatant were determined by ELISA with commercial

reagent kits following the manufacturer's instruction.

Determination of serum specific IgG (sIgG) and sIgE

Microtiter plates were coated with specific Ag proteins (20 μg/ml) at 100 μl/well. The plates were maintained at 4°C overnight, washed with PBS 3 times, incubated with 3% bovine serum albumin (BSA) for 30 min. Serum samples, or IgG standard, or IgE standard, were added to the plates (100 μl/well) and incubated for 2 hours. Biotin-labeled anti-IgG antibody or anti-IgE antibody at 1:1,000 dilution was added and incubated for 1 hour. After washing, the plates were incubated with streptavidin-peroxidase (1:1,000) at room temperature for 30 min. After washing, the solute O-phenylene diamine (substrate) was added to the plates. Optical densities were measured with a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 405 nm. The results were calculated against the standard curves.

Preparation of protein extracts

Cells (collected from relevant experiments) were lysed with RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na3VO4 and protease inhibitor cocktail; from Solarbio Life Science, Beijing, China). After centrifugation at 13,000 *g* for 10 min, the supernatant was collected and used as the cytosolic extracts. Pellets were resuspended in a nuclear lysis buffer and centrifuged for 10 min at 13,000 *g*. Supernatant was collected and used as the nuclear extracts. All the procedures were performed at 4°C.

Immunoprecipitation (IP)

Cells (collected from relevant experiments) were lysed with RIPA lysis buffer. Without the routine preclearing procedures (because one of the targets is IgG), the proteins were incubated with antibodies of interest overnight at 4°C. Immune complexes were precipitated by incubating the samples with protein G agarose beads for 2 h. The beads were collected by centrifugation at 3,000 *g* for 10 min. Proteins on the beads were eluted with an eluting buffer and analyzed by Western blotting. All procedures were performed at 4°C.

Western blotting

Protein samples (50 μg/well) were fractioned by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto a PVDF membrane. After blocking with 10% skim milk for 30 min, the membrane was incubated with antibodies of interest overnight at 4°C, washed with TBST (Tris-buffered saline containing 0.1% Tween 20) 3 times, incubated with the secondary antibodies (conjugated with peroxidase) for 2 h at room temperature, washed with TBST 3 times. Immunoblots on membrane were developed with the enhanced chemiluminescence and photographed in an imaging station.

Real-time quantitative RT-PCR (RT-qPCR)

Total RNA was extracted from cells collected from relevant experiments and converted to cDNA with a reverse transcription reagent kit following the manufacturer's instruction. The samples were amplified in a qPCR device with the SYBR Green Master Mix in the presence of TNF-α primers (gtcaacctcctctctgccat and ccaaagtagacctgcccaga). The TNF-α mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method and present as the relevant expressions.

Chromatin IP (ChIP)

Cells were collected from relevant experiments, fixed with 1% formalin for 15, lysed with RIPA lysis buffer and sonicated to shear the DNA into small pieces. The samples were precleared by incubating with protein G agarose beads for 2 h to bind pre-existing immune complexes. The beads were removed from the samples by centrifugation at 5,000 g for 10 min. Supernatant was then subjected to the IP procedures as described above. After eluting from beads, DNA was recovered from the samples with a DNA-extracting kit and analyzed by qPCR in the presence of the TNFA promoter primers (aatcagtcagtggcccagaa and gggcggggaaagaatcattc). The results were presented as fold change against the input.

Assessment of HUVEC monolayer barrier function

Following published procedures (Zhang R, Ge J. Proteinase-Activated Receptor-2 Modulates Ve-Cadherin Expression to Affect Human Vascular Endothelial Barrier Function. J Cell

	Egg	Soy	Fish	H-nut	Shrimp	Walnut	Milk	Peanut	HDM
Patients (%)	21 (42)	12 (24)	11 (22)	4 (8)	4 (8)	8 (16)	15 (30)	8 (16)	30 (60)

Some patients were sensitized to more than one antigen. H-nut = Hazelnut.

Biochem. 2017 Dec; 118 (12): 4587-4593), human umbilical vein endothelial cells (HUVEC; ATCC) were cultured into monolayers in the inserts of a transwell device (for immunocytochemistry, cells were seeded on a glass cover slip). The monolayers were regarded as confluence when the transepithelial electric resistance (TEER) reached or over 1000 $\Omega.cm^2$ (needs about two weeks). TEER was recorded with a volt-ohm meter using the STX-2 electrode system (Millipore, Schwalbach, Germany). To assess the permeability, 1 mg/ml FITC-dextran was added to the apical chamber at 22-h time point. After 2 h, 100 µl of media was collected from the basal chamber. And the fluorescence intensity was measured in each sample using a spectrofluorometer. The concentration of FITC-dextran in the sample was calculated using a standard curve of FITC-dextran.

Immunocytochemistry

HUVEC monolayers on cover slips were fixed with 1% paraformaldehyde for 1 h at room temperature, washed with PBS 3 times, blocked by incubating with 1% bovine serum albumin for 30 min, incubated with anti-EpCAM antibody (diluted to 1:100) overnight at 4°C, washed with PBS 3 times, incubated with FITC-labeled secondary antibody (1:300) for 2 h at room temperature, washed with PBS 3 times. The cell-loaded cover slip was mounted on a glass slide and observed with a confocal microscope (LSM 510).

Statistical analyses

Statistical data analyses were determined by Graphpad Prime version 8.0 software. Each sample was analyzed in triplicate. Data are presented as means ± SEM. Student's *t*-test was employed to calculate the statistical significance between two groups, and One-way analysis of variance (ANOVA) followed by Bonferroni test was used for multiple comparisons between different groups. Significance was set at P<0.05.

Results

Serum MPO levels are positively correlated with serum sIgG in AS patients

Published data suggest that factors from allergic response may be associated with the pathogenesis of AS [15]. Thus, we analyzed the allergy-related factors in a group of AS patients. We found that all the 50 AS patients had positive skin prick test (SPT) results to house dust specific Ag (HDM), some were also positive to food antigens (**Table 2**). The serum was isolated from blood samples collected from AS patients and healthy control (HC) subjects and analyzed by ELISA. The results showed that serum specific Ag-specific IgE (sIgE) (**Table 1**) and sIgG levels were detected in AS patients, which were higher in AS patients than that in HC subjects (**Table 1** and **Figure 1A**). As PMN activities are also involved in the AS pathogenesis [10], serum MPO, TNFα and peripheral blood PMN frequency were assessed. The results showed that although the blood PMN frequency was higher in AS patients than that in HC subjects, it was generally below 12,000 PMN/ml blood. However, serum MPO levels, TNFα levels and blood PMN counts were statistically higher in the AS group than that in the HC group (**Figure 1B-D**). A positive correlation was detected between MPO or TNFα and sIgG (**Figure 1D**, **1E**), but not between MPO and sIgE (not shown), in AS patients. The results indicate that AS patients have high serum sIgG and sIgE levels, in which the serum sIgG levels may be associated with the high PMN activities in the peripheral blood system.

Exposure to specific Ag induces AS PMN activation

Data of **Figure 1** suggest that specific Ag may induce PMN activation. To test it, CD11b⁺ CD66b⁺ PMNs (**Figure 2A**) were isolated from blood samples collected from AS patients and HC subjects; the cells were exposed to specific Ag (based on the SPT results) in the culture overnight and analyzed by flow cytometry. The
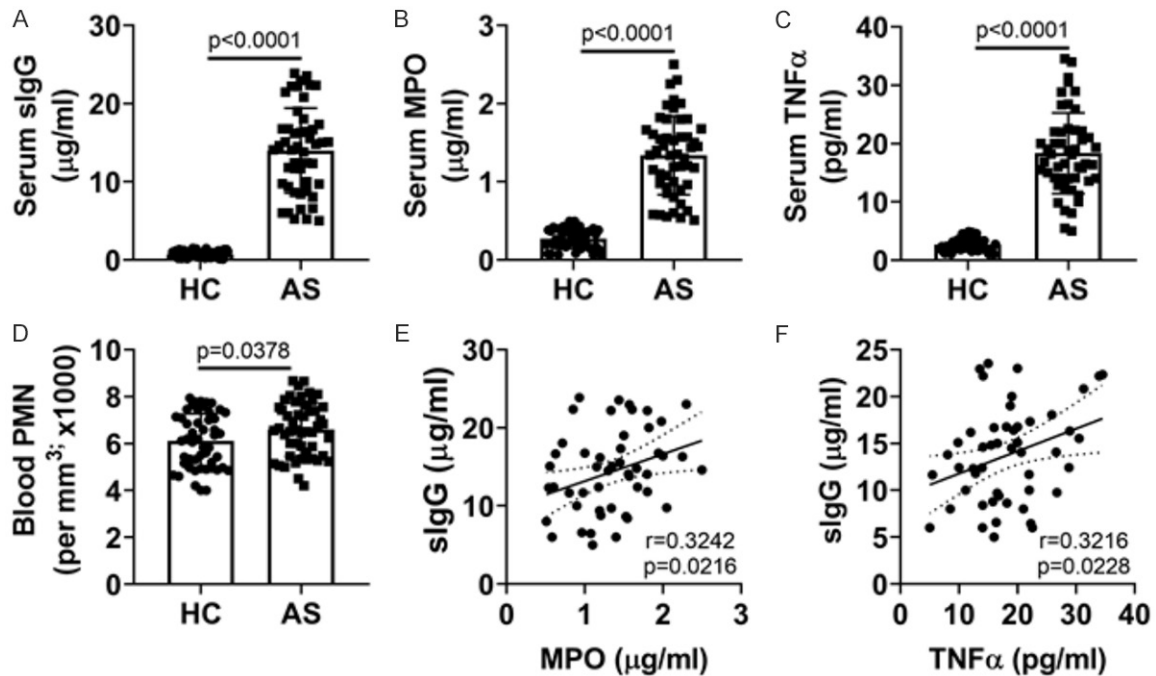
Figure 1. AS serum sIgG is associated with PMN's activities. Blood samples were collected from AS patients (n = 50) and HC subjects (n = 50). (A) serum sIgG levels. (B) serum MPO levels. (C) serum TNFα levels, (D) blood PMN counts. (E, F) positive correlation between serum sIgG and serum MPO (E), or between serum sIgG and serum TNFα (F). Data of bars are presented with median and interquartile range. Each dot presents data obtained from one sample. Statistics: The Mann-Whitney test (A-D) or Pearson correlation coefficient test (E, F). Each sample was analyzed in triplicate.

results showed that exposure to specific Ag, but not BSA (bovine serum albumin; a control Ag), markedly increased the CD154 (an activation marker of T cells [14]) and Ki67 (another activation marker) expression in PMNs (**Figure 2B**, **2C**). MPO and TNFα, two representative molecules released by PMNs upon activation, were significantly increased in the culture supernatant (**Figure 2D**, **2E**) after exposure to specific Ag in the culture. The data suggest that a fraction of PMNs in AS patients is Ag-specific; exposure to specific Ag can activate these PMNs.

Identification of a triple complex FcγRI, IgG and specific Ag on the surface of AS PMNs

Published data indicate that PMNs express FcγRI, the high affinity receptor of IgG [16]. We thus infer that, in AS patients, serum sIgG may bind the FcγRI to form complexes on the surface of PMNs; it is expected that such a complex can be bound by specific Ag. To test the inference, PMNs were isolated from blood samples collected from AS patients. The PMNs were stimulated by specific Ag in the culture

overnight. After exposure to specific Ag in the culture, the CD154 expression was detected in about 30%; those exposed to BSA in the culture did not show CD154 positive (**Figure 3A**). These CD154+ PMNs were regarded as the "Ag-specific" PMNs. These cells were also FcγRI+ IgG+ specific Ag+ (**Figure 3B**, **3C**). Since these markers were stained by surface staining in flow cytometry analysis, the FcγRI, IgG and specific Ag were for sure located on the surface of the cells. Then, proteins from "Ag-specific" PMNs were analyzed by immunoprecipitation. A complex of FcγRI, IgG and specific Ag was identified (**Figure 3D**). The results demonstrate that AS patients have "Ag-specific" PMNs. These PMNs have triple complexes of FcγRI, IgG and specific Ag on the cell surface.

Formation of a triple complex of FcγRI, IgG and specific Ag on the surface of naive PMNs

To verify the triple complexes of FcγRI, IgG and specific Ag on the surface of PMNs, we collected blood samples from HC subjects. PMNs were isolated from the samples by FCCS. IgG+ PMNs were selected out first. The remained
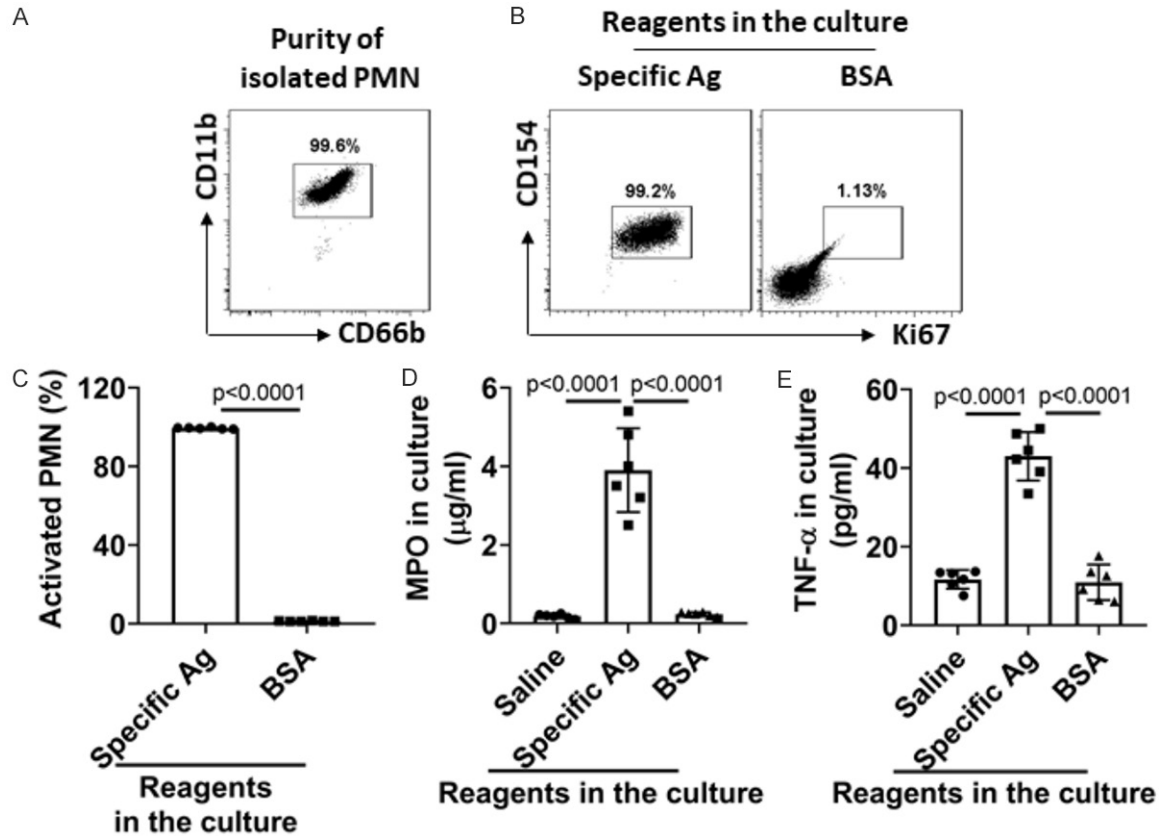
Figure 2. Specific Ag activates AS PMNs. PMNs were isolated from blood samples collected from AS patients (n = 6) by FCSS (A) and cultured (10^6 PMNs per sample) in the presence of specific Ag (based on SPT results) or BSA (a non-specific Ag) overnight. (B) gated dot plots show activated PMNs (by flow cytometry). (C) summarized data of (B). (D, E) MPO and TNFα levels in the culture supernatant (by ELISA). Data of bars are presented as mean ± SEM. Each dot presents data obtained from one sample. Statistics: The Student *t*-test (C) and ANOVA followed by the Bonferroni test (D, E).

cells were regarded as naive PMNs. In line with published data [16], we also found the FcγRI on the surface of naive PMNs (**Figure 4A**, **4B**), that was verified by Western blotting (**Figure 4C**). Exposure of PMNs to sIgG (OVA-specific IgG) resulted in complexes of FcγRI and sIgG on the surface of PMN (**Figure 4D**, **4E**). Exposure of PMNs to both sIgG and specific Ag (OVA) in the culture resulted in the formation of triple complexes of FcγRI, IgG and specific Ag on the surface of PMNs (**Figure 4F**, **4H**). The results demonstrate that, after activation, naive PMNs express FcγRI, which can be bound by sIgG and specific Ag to form a triple complex.

Specific Ags activate sensitized PMNs to trigger production of proinflammatory cytokines

Data reported by **Figures 2**-**4** indicate that sIgG binds the FcγRI to form complexes to make

PMNs sensitized. Therefore, upon binding of specific Ag to the complex of FcγRI and sIgG, PMNs may be activated. To corroborate the data, PMNs were isolated from blood samples collected from AS patients and exposed to specific Ag or BSA (a non-specific antigen) in the culture overnight. We found that exposure to specific Ag markedly increased the MPO levels in the culture supernatant (**Figure 5A**). The results indicate that exposure to specific Ag can activate PMNs; these PMNs should be specific Ag-specific. To this end, after exposure to specific Ag overnight, the PMNs were analyzed by flow cytometry. CD154 (an activating marker) positive cells were gated first (**Figure 5B**). We found that, more than 98% CD154+ PMNs also showed specific Ag on the cell surface (**Figure 5C**), indicating that specific Ags bound to the sIgG/FcγRI complexes on the surface of PMN. To verify this, AS PMNs were treated with
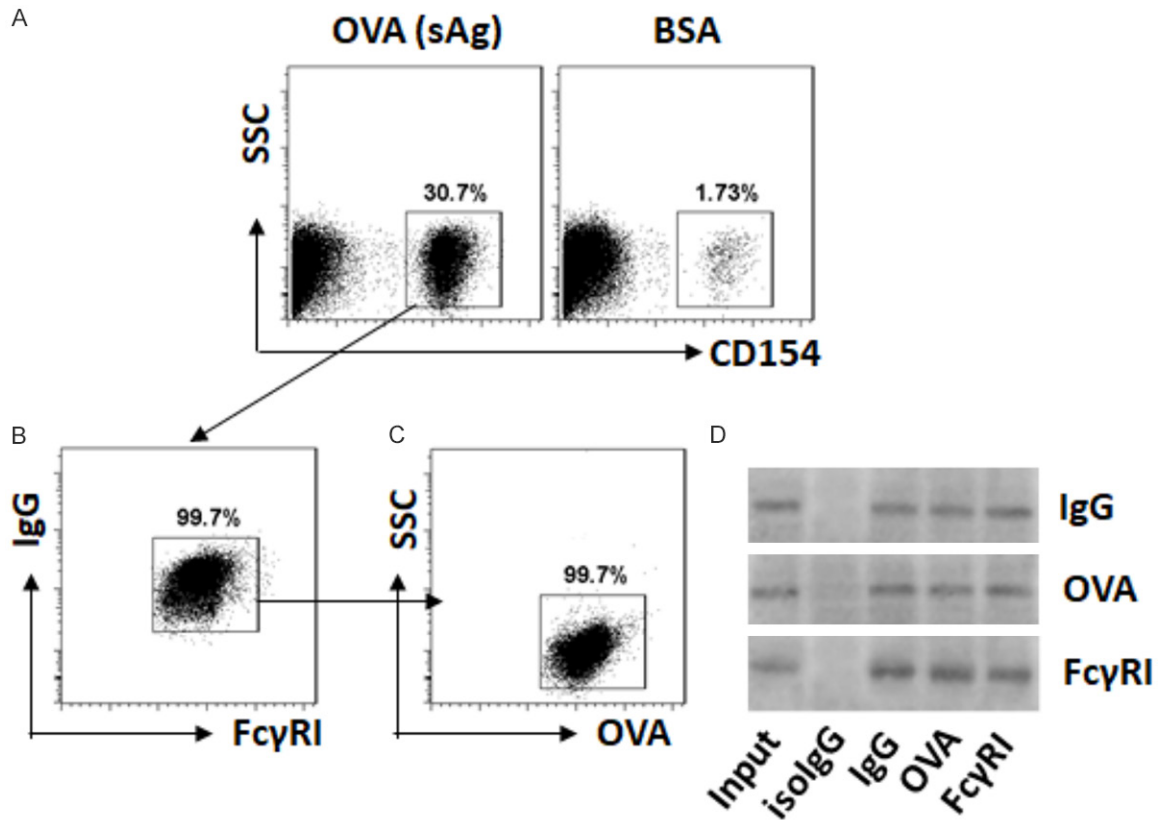
Figure 3. Identification of a triple complex on the surface of Ag-specific PMNs. PMNs were isolated from blood samples collected from AS patients (sensitized to eggs), cultured (10^6 PMNs per sample) and exposed to OVA (OVA; 5 μg/ml) or BSA (5 μg/ml; a non-specific Ag) in the culture overnight. A. Gated cells are Ag-specific PMNs (CD154 serves as an activation marker). B, C. Gated dot plots indicate positive staining of IgG, FcγRI and OVA (OVA) on the surface of Ag-specific PMNs. D. Immunoblots show a triple complex of FcγRI/IgG/OVA (OVA) in Ag-specific PMNs. The data represent 6 independent experiments.

proteinase K in the culture to remove sIgG from the surface of AS PMNs (**Figure 5D**) and then exposed to specific Ag in the culture overnight. Indeed, neither the MPO release (**Figure 5A**) nor the specific Ag on the cell surface of PMN (**Figure 5E**) was detected. Furthermore, naive PMNs were prepared and exposed to sIgG to form the sIgG/FcγRI complexes on the surface of PMN. The cells were then exposed to specific Ag in the culture overnight; it markedly induced MPO release from PMNs. The results demonstrate that exposure to specific Ag can activate "Ag-specific" PMNs and trigger PMNs to release proinflammatory cytokines into the micro environment.

Mite activates the mitogen-activated protein kinase pathway to promote TNF-α production in PMNs

Tumor necrosis factor (TNF)-α plays a critical role in the AS pathogenesis [17]. PMNs are one of the sources of TNF-α [18]. Thus, we tested the role of specific Ag antigen in TNF-α production by AS PMNs. After exposure of AS PMNs to specific Ag in the culture for 24 h, TNF-α was detected in culture supernatant, of which the TNF-α levels were markedly higher after exposing to specific Ag in the culture (**Figure 6A**). We also assessed the TNF-α mRNA levels in PMNs. However, the TNF-α mRNA levels in PMNs were not significantly altered after 24 h-culture (**Figure 6B**). The results indicate that exposure of PMN to specific Ag (but not BSA, not shown) for 24 h only triggers PMNs to release the preformed TNF-α. To elucidate if exposure to specific Ag has long-term effects on TNF-α expression in PMNs, after exposure to specific Ag in the culture for 24 h, PMNs were washed and cultured with fresh medium without the presence of specific Ag. We found that the TNF-α mRNA levels in PMNs were start increasing at 36 h and continuously increased at 48 h and 72 h, and slightly declined at 96 h (**Figure 6B**).
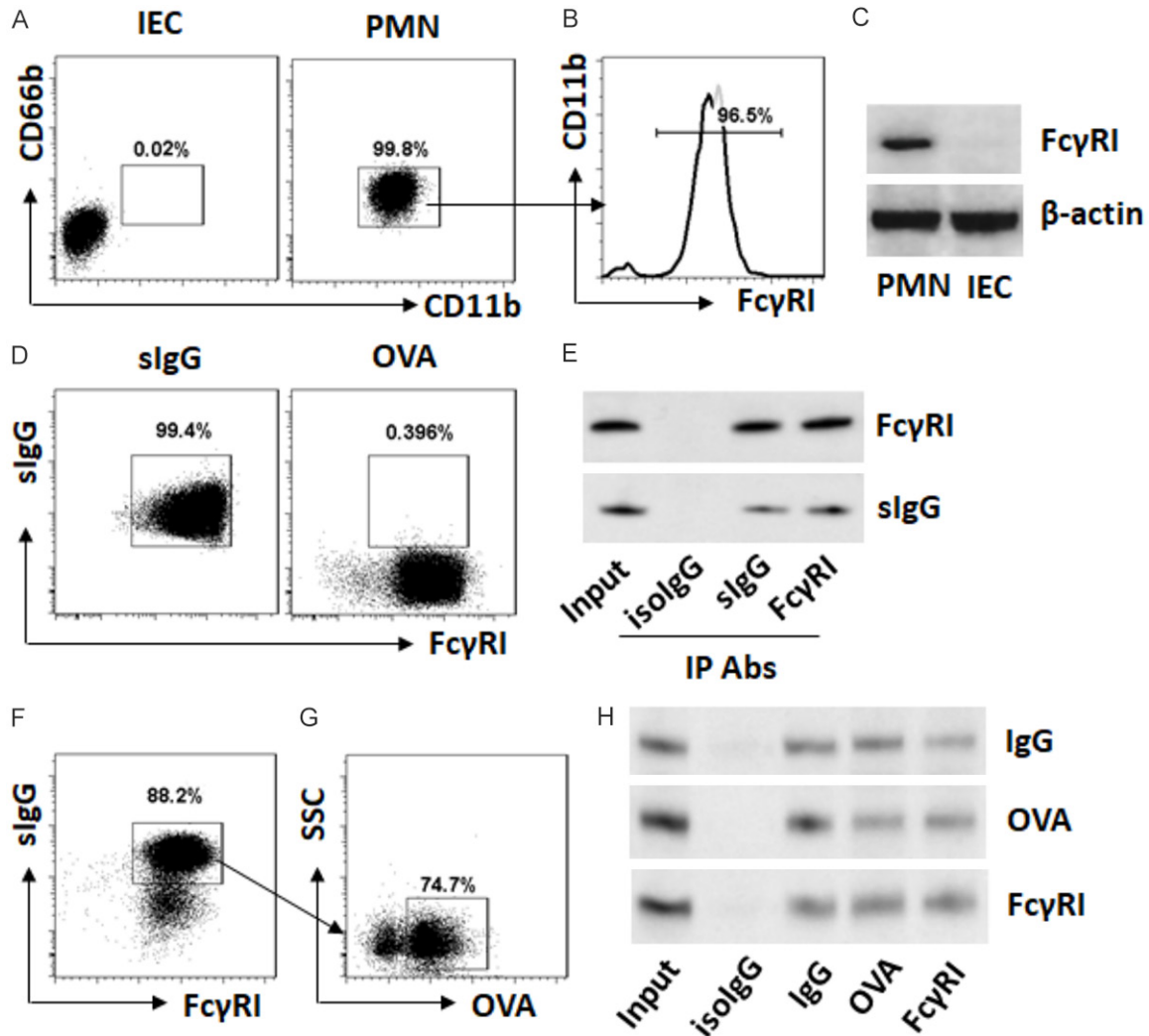
Figure 4. Formation of the FcγRI, IgG and OVA triple complexes on the surface of PMN in an in vitro experiment. (A, B) naive PMNs were prepared from blood samples collected from HC subjects and stimulated by LPS (10 µg/ml) in the culture overnight. Gated dot plots show isolated CD11b⁺ CD66b⁺ PMNs. Gated histograms show the FcγRI on the surface of PMNs (by surface staining). (C) immunoblots show the FcγRI protein in PMN's extracts. (D, E) naive PMNs were exposed to sIgG (OVA-specific IgG; 1 µg/ml) or OVA in the culture overnight. Gated dot plots show the FcγRI and sIgG on the surface of PMN. Immunoblots show a complex of FcγRI and sIgG in protein extracts of PMN. (F-H) PMNs were exposed to sIgG and OVA in the culture overnight. (F) gated dot plots show the FcγRI and sIgG on the surface of PMN. (G) gated dot plots show OVA staining on the surface of gated PMNs in panel (F). (H) a triple complex of FcγRI, sIgG and OVA in protein extracts of PMN. The data represent 6 independent experiments.

The TNF-α protein levels in culture supernatant were also increased in parallel to the changes of TNF-α mRNA in PMNs (**Figure 6A**). Further analysis showed that exposure to specific Ag increased p38 MAPK phosphorylation (**Figure 6C**), NFAT2 (the TNF-α gene transcription factor) and Pol II levels at the *TNFA* promoter locus (**Figure 6D, 6E**). Exposure of PMNs to BSA in the culture did not alter the TNF-α expression (**Figure 6A-E**). The results demonstrate that exposure to specific Ag can trigger "Ag-specific" PMNs to release preformed TNF-α and generate new TNF-α in PMNs.

Ag-specific PMN-derived TNF-α compromises vascular endothelial barrier functions

It is recognized that endothelial barrier dysfunction is a critical risk factor associating with the AS pathogenesis [4]. TNF-α plays a role in the induction of endothelial barrier dysfunction [19]. AS PMNs produced significantly more TNF-α after exposure to specific Ag in the culture (**Figure 7A**). We therefore inferred that "Ag-specific" PMN-derived TNF-α might compromise the vascular endothelial barrier functions. To test this, human umbilical vascular
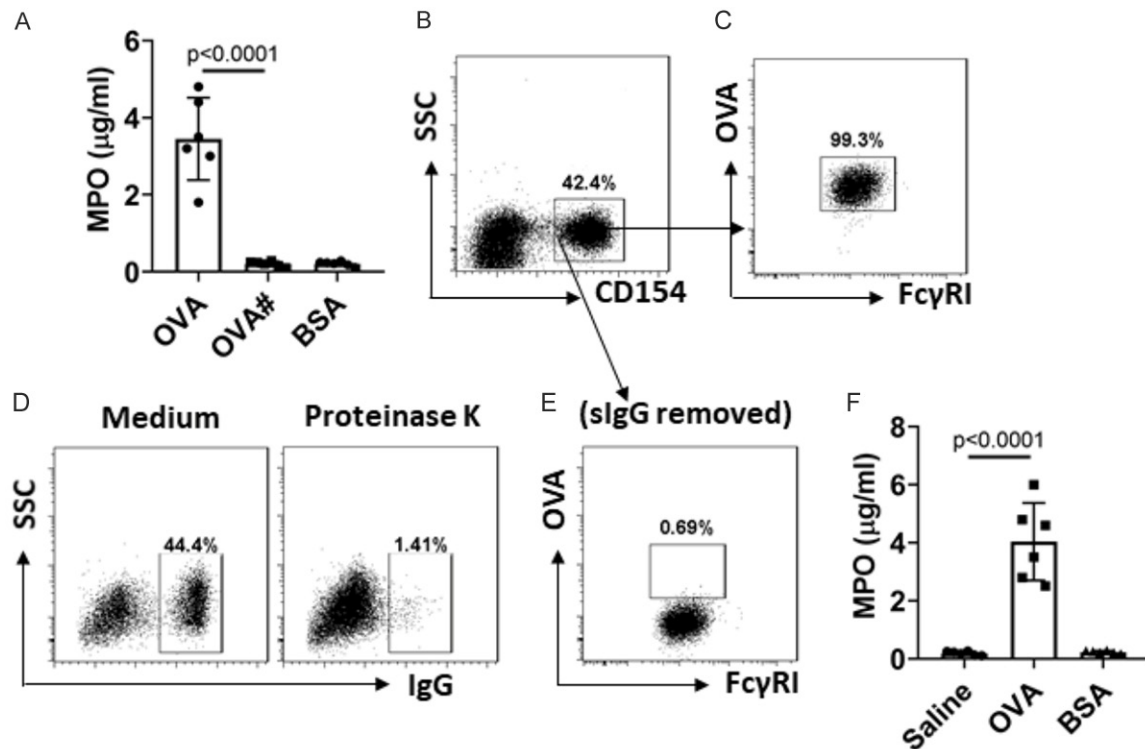
Figure 5. OVA (a specific Ag) activates Ag-specific PMNs. A. PMNs were prepared with blood samples collected from AS patients. The cells were exposed to OVA in the culture overnight. Bars show MPO levels in the culture supernatant (by ELISA). B. Gated dot plots show CD154+ PMN frequency after exposure to OVA in the culture overnight. C. Gated dot plots show the CD154+ PMNs also have the FcγRI positive staining on the surface of AS PMNs. D. AS PMNs were treated with proteinase K (#; 1.25 μg/ml) for 15 min in the culture. Gated dot plots show IgG-positive staining on the surface of PMN. E. Gated dot plots show OVA-positive staining on the surface of PMNs. F. Bars indicate MPO levels in culture supernatant after constructing the sIgG/FcγRI complexes on the surface of naive PMNs and exposure to OVA (OVA) in the culture. The data represent 6 independent experiments. Data of bars are presented as mean ± SEM. Each dot in bars present data obtained from one sample. Statistics: The Student *t*-test.

vein endothelial cell (HUVEC cell) monolayers were prepared in the inserts of transwell device, "Ag-specific" PMNs were prepared and cultured in the basal chambers. Upon exposing to specific Ag in transwell device, the transepithelial resistance (TEER) of HUVEC monolayers dropped markedly and the permeability was elevated significantly, which were abrogated by the presence of anti-TNF-α antibodies (**Figure 7B**, **7C**). The results demonstrate that "Ag-specific" PMN-derived TNF-α can compromise the vascular endothelial barrier functions.

Discussion

In this study, we identified the "Ag-specific" PMNs in AS patients. The "Ag-specific" PMNs are characterized as there are complexes of FcγRI and sIgG on the cell surface; exposure to specific Ag antigens activate "Ag-specific"

PMNs and induces the cells to release preformed cytokines and to generate new proinflammatory cytokines, such as TNF-α, to be released into the micro environment. The activation of "Ag-specific" PMNs compromises the vascular endothelial barrier functions as shown by an *in vitro* endothelial monolayer model.

The present data show that PMNs are associated with AS. It is not long that investigators recognize that PMNs are associated with the pathogenesis of AS. In the last decades, Doring and Drechesler et al showed evidence that PMNs activated endothelial cells, chemoattracted leukocytes, specifically monocytes, to AS lesion sites to induce endothelial cell dysfunction, initiated or/and aggravated inflammation in the local blood vessel tissues [20, 21]. The neutrophil extracellular traps (NET), a complex structure of nuclear chromatin and proteins of nuclear, granular, and cytosolic origin,
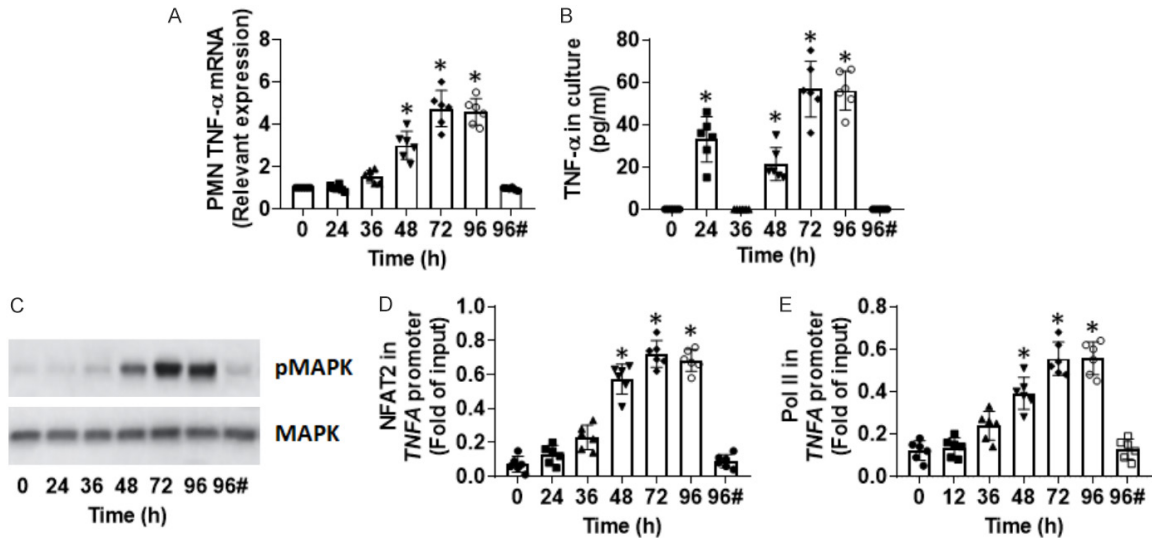
Figure 6. OVA binding regulates TNF-α release and TNF-α generation in PMNs. PMNs were isolated from blood samples collected from AS patients and cultured in the presence of OVA (BSA was used as a control). PMNs or culture supernatant were collected at indicated time points (denoted on the x axis) and analyzed. A. Bars show TNF-α mRNA levels in PMNs (by RT-qPCR). B. Bars show TNF-α protein levels in culture supernatant (by ELISA). C. Immunoblots show phosphorylated MAPK and total MAPK levels in PMNs. D, E. Bars show NFAT2 and Pol II levels in the *TNFA* promoter locus (by ChIP). Data of bars are presented as mean ± SEM. Each dot presents data obtained from one sample. The data represent 6 independent experiments. Each sample was analyzed in triplicate. *, P<0.01 (ANOVA followed by the Dunnett's test), compared with dose 0. #, PMNs were exposed to BSA instead of OVA.
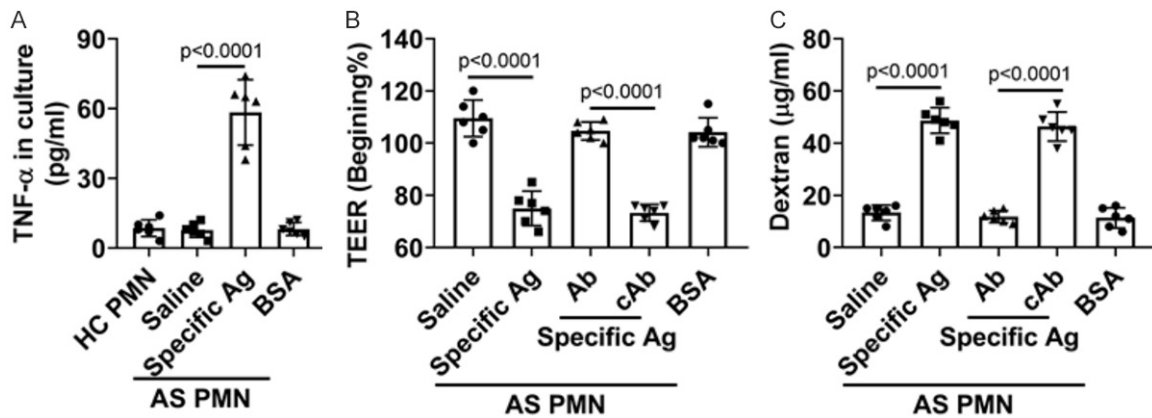

Figure 7. Ag-specific PMN-derived TNF-α compromises vascular endothelial barrier function. A. PMNs were isolated from blood samples collected from HC subjects and AS patients. Bars indicate TNF-α levels in culture supernatant. B, C. HUVEC monolayers were prepared in a transwell device with "Ag-specific" PMNs in the basal chambers. TEER was recorded before and 24 h after the addition of Saline or specific Ag or BSA (a non-specific Ag) to the culture (5 µg/ml). Permeability to FITC-dextran (MW = 40 kDa) was assessed between 22-24 h after addition of the reagents. B. Bars indicate the TEER change. C. Bars indicate FITC-dextran in basal chambers. Data of bars are presented as mean ± SEM. Each dot presents data obtained from one sample. Statistics: ANOVA + Bonferroni test. The data represent 6 independent experiments.

can be detected in AS lesions of both human and mice [1]. PMN-derived proteases and reactive oxygen species can induce advanced AS plaques to abrupt [22]. The present data provide a novel aspect of PMN activities associating with AS pathogenesis, of which a fraction of

PMNs can be specifically activated by specific Ag antigens.

We found that, in AS patients, the PMN's activities were positively correlated with the serum sIgG levels. This prompted us to investigate into

the relation between immune responses and PMN's activities in AS patients. We found that a complex of FcγRI and sIgG on the surface of PMN. It was reported previously that PMNs expressed FcγRI. For example, Zhao et al found that PMNs expressed FcγRI, which was significantly increased in acute coronary syndrome patients [23]. Minar et al found that PMNs in Crohn's disease (CD) patients expressed higher levels of FcγRI than those without CD. The FcγRI expression levels were significantly correlated with the CD mucosal injury [24]. Our recent study also showed similar "Ag-specific" PMNs in tonsils with frequent acute inflammation [25]. It is proposed that high-affinity human IgG receptor FcγRI promotes the IgG-mediated inflammation [26]. The FcγRI expression on PMN can be an indicator of bacterial infection [11]. In line with those previous studies, our data also show that the FcγRI expression on PMN is significantly higher in AS patients.

We observed that sIgG bound to FcγRI to form complexes on the surface of AS PMNs. This phenomenon was reproduced in an *in vitro* experiment. By exposing naive PMNs to sIgG, a complex of FcγRI and sIgG was formed on the surface of the PMNs. Others also found that monomeric IgG bound FcγRI [27]. IgG is one of the major fractions of the five immunoglobulins in the body. Besides the anti-infection function, another major function of IgG is to facilitate the elimination of foreign substances, such as macromolecular antigens. sIgG forms complexes with specific Ag. Such complexes can bind to FcγRI on the surface of phagocytes. The complexes are then phagocytosed by the phagocytes. Our data show another aspect of the interaction between IgG and FcγRI. We found the complexes of FcγRI and sIgG on PMNs of AS patients. The fact indicates that besides binding the complexes of specific Ag/IgG, FcγRI on AS PMNs also can bind sIgG to form complexes on the cell surface.

The data show that specific Ags can bind to the complexes of FcγRI and sIgG on AS PMNs. In this study, we used OVA as the specific Ag. The PMNs were isolated from AS patients with high serum levels of OVA-sIgG. Previous reports suggested that the pre-bound monomeric IgG prevented FcγRI from binding immunocomplexes [12]. Our results showed another aspect of this phenomenon. The *in vitro* experimental results

showed that the sIgG-pre-saturated FcγRI on PMNs were still bound by specific Ags. Others also found this phenomenon [27]. This may be because of binding FcγRI by its Fc domain, sIgG still leave its Fab domain open, which specific Ags can bind to. The present data show that specific Ags indeed can bind to the complexes of FcγRI and sIgG on AS PMNs to form triple complexes.

Upon exposure to specific Ag, PMNs with complexes of FcγRI and sIgG on the surface were activated and released preformed TNF-α into culture supernatant. Furthermore, the data show that exposure to specific Ag has a long-term effect on PMNs to drive PMNs to generate new TNF-α through the MAPK-NFAT2 pathway. TNF-α is a proinflammatory cytokine that is involved in many inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis as well as AS [17, 28]. Our data show that exposure to specific Ag can induce AS PMNs to release TNF-α into culture supernatant. PMNs have TNF-α receptors. Exogenous TNF-α can activate PMNs and up regulate the FcγRI expression in PMNs. Thus, the autocrine TNF-α may in turn to activate PMNs to expand the TNF-α effects. TNF-α can activate the NF-κB pathway to increase transcytosis of low-density lipoprotein across endothelial cells [17], induce vascular endothelial inflammation [17]. Blocking TNF-α sources can prevent AS plaque from rupture [29]. Our data provide novel information to this study area by showing that "Ag-specific" PMNs are a TNF-α source in AS patients.

Lipid retention in the vascular intima tissues plays an essential role in the AS development. Endothelial barrier dysfunction provides the opportunity to allow lipids to get into the vascular intima [30]. Thus, vascular endothelial barrier dysfunction is a most important risk factor in the AS development [30]. To elucidate the causative factors of vascular endothelial barrier dysfunction is of importance in the prevention or/and alleviation of AS. Many factors, such as reactive oxygen species, hypoxemia, extracellular proteases, inter-endothelial cell junction-associated protein abnormality [4]. Our data provide a novel causative factor of vascular endothelial barrier dysfunction. Activation of "Ag-specific" PMN can induce vascular endothelial barrier dysfunction.

Prompted by published data that allergic response may be involved in the AS pathogenesis [15], we screed the sensitization status of AS patients to common food antigens and HDM. The results showed all the AS patients positively responded to the tested antigens. The response includes high serum sIgE and sIgG levels and positive SPT. However, none of the AS patients complained allergic symptoms; this may be the allergic status was in the subclinical stage [31] or the sIgG masks the sIgE effects on inducing allergic response [32].

In summary, the present study identified the "Ag-specific" PMNs in AS patients. The "Ag-specific" PMNs have complexes of FcγRI, sIgG and specific Ags on the cell surface. Exposure of "Ag-specific" PMNs to specific Ags triggers the cells to release preformed proinflammatory cytokines and start to generate new proinflammatory cytokines, such as TNF-α. The activation of "Ag-specific" PMNs compromises the vascular endothelial barrier functions.

Acknowledgements

Disclosure of conflict of interest

None.

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