

Sphingosine-1-phosphate (S1P) enhances glomerular endothelial cells activation mediated by anti-myeloperoxidase antibody-positive IgG

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Abstract

Cumulating evidences suggested an important role of sphingosine-1-phosphate (S1P) and its receptors in regulating endothelial barrier integrity. Our previous study revealed that the circulating S1P levels and renal expression of S1PRs correlated with disease activity and renal damage in patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). This study investigated the role of S1P and its receptors in myeloperoxidase (MPO)-ANCA-positive IgG-mediated glomerular endothelial cell (GEnC) activation. The effect of S1P on morphological alteration of GEnCs in the presence of MPO-ANCA-positive IgG was observed. Permeability assay was performed to determine endothelial monolayer activation in quantity. Both membrane-bound and soluble ICAM-1 and VCAM-1 levels were measured. Furthermore, antagonists and/or agonists of various S1PRs were employed to determine the role of different S1PRs. S1P enhanced MPO-ANCA-positive IgG-induced disruption of tight junction and disorganization of cytoskeleton in GEnCs. S1P induced further increase in monolayer permeability of GEnC monolayers in the presence of MPO-ANCA-positive IgG. S1P enhanced MPO-ANCA-positive IgG-induced membrane-bound and soluble ICAM-1/VCAM-1 up-regulation of GEnCs. Soluble ICAM-1 levels in the supernatants of GEnCs stimulated by S1P and MPO-ANCA-positive IgG increased upon pre-incubation of S1PR1 antagonist, while pre-incubation of GEnCs with the S1PR1 agonist down-regulated sICAM-1 level. Blocking S1PR2-4 reduced sICAM-1 levels in the supernatants of GEnCs stimulated by S1P and MPO-ANCA-positive IgG. Pre-incubation with S1PR5 agonist could increase sICAM-1 level in the supernatants of GEnC stimulated by S1P and MPO-ANCA-positive IgG. S1P can enhance MPO-ANCA-positive IgG-mediated GEnC activation through S1PR2-5.

Keywords: ANCA • vasculitis • sphingosine-1-phosphate

Introduction

ANCA-AAV is a group of systemic autoimmune diseases, including microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA) [1]. AAV is characterized by necrotizing inflammation of the small blood vessels, which involves GEnC injury in particular. ANCAs against MPO and proteinase 3 (PR3) are the serological hallmarks of AAV [2, 3]. What is noteworthy is that Chinese AAV patients, as demonstrated in our previous studies, are predominantly MPO-ANCA-positive [4, 5]. Moreover, cumulating evidences reveal that MPO-ANCAs can induce

direct GEnC activation and injury in AAV. Nagao *et al.* [6, 7] reported that MPO-ANCA could induce up-regulation of adhesion molecules in GEnCs both *in vivo* and *in vitro*.

S1P is a bioactive sphingolipid and key regulator in vascular inflammation. S1P is the ligand for five G-protein-coupled receptors (GPCRs) named S1PR1-5 [8, 9]. S1P and its receptors are involved in the pathogenesis of a variety of vascular inflammatory conditions including sepsis, atherosclerosis and ischaemia-reperfusion injury [10-14]. In our previous study, it was found that the levels of circulating S1P were elevated and renal expression of S1PR2-5 was up-regulated in AAV patients of active stage, and that the circulating S1P levels and the renal expression of S1PR were associated with renal involvement and disease activity of AAV [15]. Moreover, we found

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that S1P participated in ANCA-mediated neutrophil activation [16]. All these studies indicated a pathogenic role of S1P in AAV [16].

In addition, cumulating evidences suggested an important role of S1P and S1PRs in regulating endothelial barrier integrity in recent years. It was found that different doses of S1P could induce distinct biological effects on endothelial cells and that the final barrier regulating the efficacy of S1P depends on the balance of expression and activation of different S1PRs in endothelial cells. A physiological level of S1P strengthened the barrier integrity of endothelial cells through S1PR1, whereas excessive S1P resulted in disruption of endothelial integrity by activating S1PR2 and S1PR3 [17–19]. Given the potential effect of S1P on regulating endothelial barrier function, we hypothesize that S1P might participate in MPO-ANCA-positive IgG-induced GEnC activation through a S1PR-dependent way. This study aimed to investigate whether S1P contributed to MPO-ANCA-positive IgG-induced GEnC activation, and to explore the roles of different S1PRs in this process.

Materials and methods

Cell culture

Primary human GEnC (ScienCell, San Diego, CA, USA) were cultured according to the manufacturer's recommendation. After a confluent endothelial cell monolayer was formed, GEnCs were starved in basal medium supplemented with 1% foetal bovine serum (FBS) for 8 hrs. GEnC in selected wells were then stimulated for 24 hrs with 2 μ M S1P (Sigma-Aldrich, Darmstadt, Germany), which was comparable to the levels of circulating S1P in AAV patients at active stage, as demonstrated by our previous study [15].

Preparation of IgGs

Normal immunoglobulin (Ig)Gs and MPO-ANCA-positive IgGs were prepared from plasma of five healthy volunteers and five active MPO-ANCA-positive primary small vessel vasculitis patients, respectively. Plasma was applied to a High-Trip-protein G column on an AKTA-FPLC system (GE Biosciences, South San Francisco, CA, USA) after filtered through syringe filters (Merck Millipore, Darmstadt, Hessen, Germany). Preparation of IgGs was performed according to the methods described previously [20]. The endotoxin levels in the IgGs were below the detection limit (0.1 EU/ml) of a commercial ELISA kit (Cambrex Corporation, East Rutherford, NJ, USA). The IgG from each individual patient or healthy volunteer was prepared for subsequent anti-endothelial cell antibody (AECA) detection and GEnC stimulation. Our research was approved by the clinical research ethics committee of the Peking University First Hospital and in compliance with the Declaration of Helsinki.

Detection of AECA

The prepared IgGs were further screened for the presence of AECA through an ELISA method described previously [21]. In brief, GEnCs

were seeded onto 96-well plates until 90% confluence and then starved of serum. After that, the live GEnCs were incubated with IgG for 24 hrs and then fixed with 4% formaldehyde. After the plates were blocked with 5% bovine serum albumin (BSA), an alkaline phosphatase-conjugated rabbit anti-human IgG antibody (Sigma-Aldrich) was used to detect the bound IgG. P-Nitrophenyl phosphate (Sigma-Aldrich) was used for subsequent quantification. Each plate always had a blank control obtained from blocking solution 5% BSA, a standard positive control obtained from an active AAV patient and a negative control obtained from healthy volunteers. Results were expressed as an ELISA ratio (ER). The lower limit for positive AECA binding was defined as ER value greater than the mean + 3S.D. AECA-positive IgG was excluded with this criterion in our following experiments.

Measurements of GEnC activation

Immunofluorescence staining of zonula occluden-1 (ZO-1) and filamentous (F)-actin

As important makers for endothelial barrier function, the distributions of the tight junction scaffolding protein ZO-1 and the cytoskeletal F-actin in GEnCs were observed [22]. GEnCs were washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde after relevant treatment. Then GEnCs were permeabilized with 0.5% Triton X-100, washed in PBS, blocked by 5% BSA and then incubated with a ZO-1 antibody (Life, Carlsbad, CA, USA) at 4°C overnight. After a thorough wash in PBS, the GEnCs were incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) and conjugated rhodamine-phalloidin (Life) for 1 hr. After washing with PBS, the specimens were stained with 4',6-diamidino-2-phenylindole (DAPI) and eventually mounted with Mowiol. Confocal images were captured by a Zeiss LSM 710 confocal microscope (Zeiss, Jena, Germany) and exported by the ZEN 2012 microscopy software.

Permeability assay

We determined the permeability of GEnC monolayers using FITC-labelled BSA (Sigma-Aldrich), as described previously [23]. We grew GEnCs on the upper chamber of Costar Transwell with 0.5- μ m porous filters until confluent. After relevant stimulation for 24 hrs, we added the tracer protein FITC-albumin to the upper chamber. After incubated at 37°C for 30 min., samples were collected from both the upper and lower chambers for fluorometric analysis. Dulbecco's phosphate-buffered saline (D-PBS) and TNF- α (2 ng/ml) were used as negative and positive controls, respectively. A microplate fluorescence reader (TristarTM LB941; Berthold, Bad Wildbad, Baden-Württemberg, Germany) was used to measure fluorescence. Eventually, we use these fluorescence readings to calculate the permeability coefficient, which is indicative of vascular barrier disruption.

Measurement of membrane-bound adhesion molecule expression

Membrane-bound intercellular adhesion molecule-1 (mICAM-1) and membrane-bound vascular cell adhesion molecule-1 (mVCAM-1) expression levels were measured according to the methods described previously [6]. GEnC monolayers were pre-treated exactly as for permeability assay and then fixed with 4% formaldehyde. Fixed GEnCs were pre-incubated with 5% BSA to block non-specific binding. Biotinylated

sheep anti-human ICAM-1 or VCAM-1 antibodies were then incubated for another hour. Streptavidin-HRP (R&D, Minneapolis, MN, USA) and then substrate solution were added to detect bound antibodies. Finally, we stopped the reaction by adding sulphuric acid and determined optical density of each well immediately. As for negative controls, the primary antibody was replaced by 5% BSA.

Measurement of soluble adhesion molecule in the supernatants

Levels of soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) in the GEnC supernatants were measured using the commercial ELISA kits (R&D). A 96-well microplate was coated with the diluted ICAM-1/VCAM-1 capture antibody. After an overnight incubation and 3 times plate wash, the plate was blocked with 1% BSA to reduce non-specific binding. Then we added sample or standards to the plate. After 2 hrs incubation at room temperature and three times wash, ICAM-1/VCAM-1 detection antibody was added to each well. After another 2 hrs incubation and plate wash, streptavidin-horseradish peroxidase (HRP) was added to each well. After another incubation, substrate solution was added. Then the reaction was stopped by adding sulphuric acid. Eventually, the optical density of each well was determined immediately using a microplate reader set to 570 nm.

Expression of S1P receptors in human GEnCs

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect S1PRs expression in GEnCs. Total RNA was extracted according to the manufacturer's instructions (Thermo, Waltham, MA, USA), which was subjected to reverse-transcribed using a commercial reverse transcription system (Promega, Wisconsin, WI, USA). Quantitative real-time PCR was then performed with ABI Prism 7500 sequence detecting system (Applied Biosystems, Foster City, CA, USA), and the PCR products were size-fractionated in a 2% agarose gel. The primers for PCR amplifications were as follows: S1PR1 (Forward: 5'-CACTCTGACCAACAAGGAGATG-3', Reverse: 5'-GATGATGGGTCGCTTGAATTTG-3'); S1PR2 (Forward: 5'-AAGTCCACTCGCAATGTA-3', Reverse: 5'-AGCCA GAGAGCAAGGTATTG-3'); S1PR3 (Forward: 5'-TCTCCGAAGGTCAAGGAAGA-3', Reverse: 5'-TCAGTTGCAGAAGATCCCATTC-3'); S1PR4 (Forward: 5'-CTGAA-GACGGTGCTGATGAT-3, Reverse: 5'-CAGAGGTTGGAGCCAAAGA-3'); S1PR5 (Forward: 5'-GGTCATCGTCTGCATTACA-3', Reverse: 5'-CTAGATTCTCTAGCACGATGAAGG-3'); and β -actin (Forward: 5'-GGACCT-GACTGACTACCTCAT-3', Reverse: 5'-CGTAGCACAGCTTCTCCTTAAT-3').

Inhibition of S1P receptors

SEW2871 (SEW; Tocris, Louis, MO, USA) is a novel, potent and selective S1PR1 agonist which activates S1PR1, but does not activate S1PR2-5 at concentrations even up to 10 μ M [24]. W146 (Tocris) is a highly specific S1PR1 antagonist which displays no effect at S1PR2, S1PR3 or S1PR5 [25, 26]. JTE013 (JTE; Tocris) is a highly selective S1PR2 antagonist. Even at concentrations up to 10 μ M, JTE013 displays only 4.2% inhibition of S1PR3 and does not antagonize S1PR1 [27, 28]. TY52156 (TY; Tocris) shows submicromolar potency and a high degree of selectivity for S1P3 receptor [29, 30]. CYM50358 (CYM; Tocris) is a potent S1PR4 antagonist which displays selectivity for

S1PR4 against S1PR1, S1PR2, S1PR3 and S1PR5 [31]. A971432 is a potent and selective S1PR5 agonist which exhibits 60-fold selectivity over S1PR1, and over 1600-fold selectivity over S1PR2-4 [32].

In S1P-induced sICAM-1 expression assay, GEnCs were incubated with the aforementioned S1PR agonists or antagonists for different doses and time-points. Eventually 0.1 μ M of S1PR agonists or antagonists at 15 min. was selected for the experiments because of the highest inhibition/increase rate (Fig. S1-S2). In addition, it has been validated that these S1PR antagonists or agonists are selective for their respective receptors at the 0.1 μ M concentration according to the previous studies [25–32].

Statistical analysis

Kurtosis and skewness were used to evaluate the normality of the data (both the absolute values were <3). Differences in quantitative parameters between groups were analysed using the *t*-test, and mean \pm S.D. were presented as descriptive statistics for normally distributed data. Differences in quantitative parameters between groups were analysed using the nonparametric test, and median and interquartile range (IQR) were presented as descriptive statistics for non-normally distributed data. For normally distributed data, differences in quantitative parameters between groups were assessed with one-way ANOVA, and for non-normally distributed data, differences in quantitative parameters between groups were assessed with Kruskal–Wallis test. If $P < 0.05$, differences were considered statistically significant. Data analysis was performed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

S1P induces morphological alteration in GEnC monolayers

Double immunofluorescence staining of ZO-1 and F-actin was performed to observe the structure of the tight junction and cytoskeleton in GEnCs. The results revealed that the application of S1P or MPO-ANCA-positive IgG alone could induce the disruption of tight junction structure and the improper distribution of F-actin compared with untreated cells. Moreover, combined application of S1P and MPO-ANCA-positive IgG induced further disruption of tight junction and disorganization of cytoskeleton compared with all the above-mentioned cell groups (Fig. 1). These data suggested that S1P, with pathophysiological concentration of active AAV patients, could enhance MPO-ANCA-positive IgG-induced endothelial activation.

S1P induces increased endothelial permeability of GEnC monolayers

To determine the effect of S1P on monolayer permeability in GEnCs, a transwell system and a FITC-labelled BSA were used. The results demonstrated that compared with untreated cells, monolayer permeability increased significantly in GEnCs stimulated by S1P and MPO-ANCA-positive IgG (4.47 \pm 0.20% versus 3.25 \pm 0.10%,

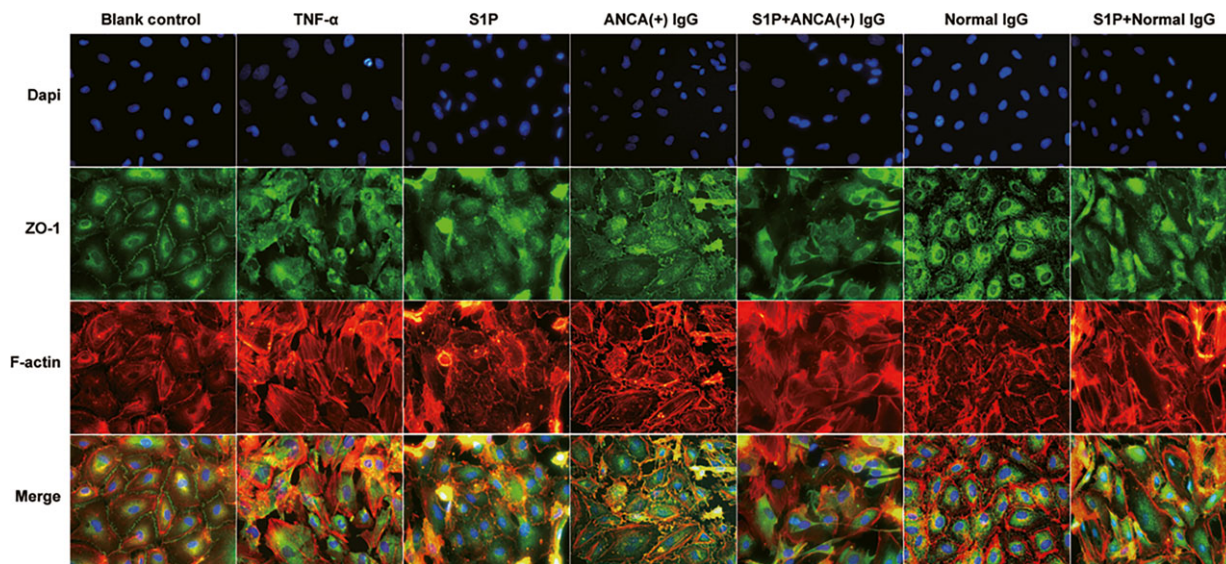


Fig. 1 S1P could induce alterations in cellular morphology of GEnCs in the presence of MPO-ANCA-positive IgG.

$P < 0.001$). Monolayer permeability also increased in GEnCs stimulated by S1P or MPO-ANCA-positive IgG alone ($3.83 \pm 0.04\%$ versus $3.25 \pm 0.10\%$, $P < 0.001$; $3.85 \pm 0.12\%$ versus $3.25 \pm 0.10\%$, $P < 0.001$, respectively). Moreover, compared with the above cells, monolayer permeability still increased significantly in GEnCs stimulated by S1P and MPO-ANCA-positive IgG ($4.47 \pm 0.20\%$ versus $3.25 \pm 0.10\%$, $P < 0.001$; $4.47 \pm 0.20\%$ versus $3.83 \pm 0.04\%$, $P < 0.001$; $4.47 \pm 0.20\%$ versus $3.85 \pm 0.12\%$, $P < 0.001$, respectively) (Fig. 2). These data suggested that S1P enhanced MPO-ANCA-positive IgG-mediated increasing of GEnC permeability.

S1P up-regulates membrane-bound adhesion molecule expression on GEnC

Adhesion molecules such as ICAM-1 and VCAM-1 play an important role in neutrophil adhesion to endothelium, which is essential in the pathogenesis of AAV [22]. We detected mICAM-1 and mVCAM-1 expression levels using a cell ELISA assay, and we found that compared with untreated cells, cells stimulated by S1P or MPO-ANCA-positive IgG alone, the mICAM-1 levels increased significantly in GEnCs stimulated by S1P and MPO-ANCA-positive IgG (1.65 ± 0.01 versus 1.41 ± 0.053 , $P < 0.001$; 1.65 ± 0.01 versus 1.55 ± 0.09 , $P < 0.05$; 1.65 ± 0.01 versus 1.52 ± 0.05 , $P < 0.01$, respectively. Data was shown as OD values). The levels of mVCAM-1 also increased significantly in GEnCs stimulated by S1P and MPO-ANCA-positive IgG compared with unstimulated cells, cells stimulated by S1P or MPO-ANCA-positive IgG alone (1.15 ± 0.03 versus 0.74 ± 0.10 , $P < 0.001$; 1.15 ± 0.03 versus 1.06 ± 0.13 , $P < 0.05$; 1.15 ± 0.03 versus 1.00 ± 0.09 , $P < 0.01$, respectively. Data were shown as OD values) (Fig. 3). Collectively, these data illustrate that S1P enhanced MPO-ANCA-positive IgG-mediated mICAM-1 and mVCAM-1 up-regulation on GEnCs.

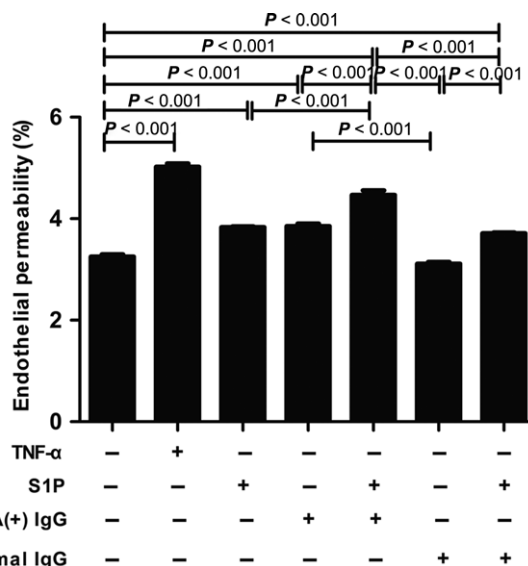
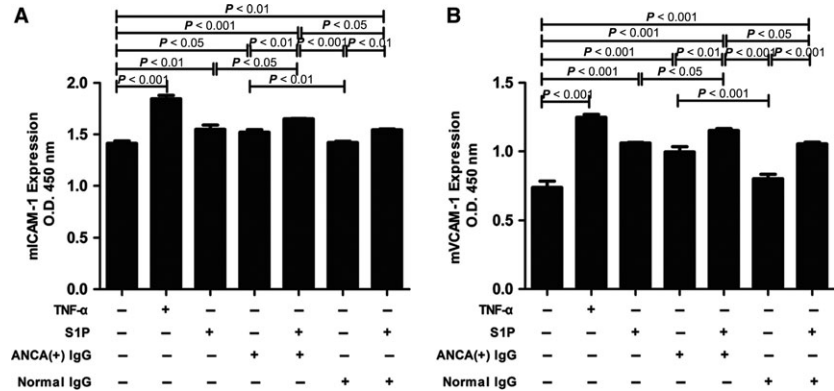


Fig. 2 S1P could induce increased endothelial permeability of GEnC monolayers in the presence of MPO-ANCA-positive IgG. Bars represent mean \pm S.D. of repeated measurements of five independent experiments or donors.

S1P increases soluble adhesion molecule level in the supernatants

Soluble adhesion molecules such as sICAM-1 and sVCAM-1 levels in the supernatants were measured with commercial ELISA kits. It was found that compared with unstimulated cells, cells stimulated by S1P or MPO-ANCA-positive IgG alone, the levels of sICAM-1 increased significantly in GEnCs treated with S1P and

Fig. 3 S1P could up-regulate membrane-bound adhesion molecule expression levels of GEnC in the presence of MPO-ANCA-positive IgG. **A.** S1P could up-regulate mICAM-1 expression on GEnC in the presence of MPO-ANCA-positive IgG. **B.** S1P could up-regulate mVCAM-1 expression on GEnC in the presence of MPO-ANCA-positive IgG. Bars represent mean \pm S.D. of repeated measurements of five independent experiments or donors.



MPO-ANCA-positive IgG (1271.93 ± 185.33 pg/ml versus 597.02 ± 72.86 pg/ml, $P < 0.001$; 1271.93 ± 185.33 pg/ml versus 915.89 ± 20.22 pg/ml, $P < 0.001$; 1271.93 ± 185.33 pg/ml versus 907.61 ± 73.18 pg/ml, $P < 0.01$, respectively). The levels of sVCAM-1 also increased significantly in GEnCs stimulated by S1P and MPO-ANCA-positive IgG compared with unstimulated cells, cells stimulated by S1P or MPO-ANCA-positive IgG alone (1276.77 ± 109.68 pg/ml versus 432.65 ± 13.33 pg/ml, $P < 0.001$; 1276.77 ± 109.68 pg/ml versus 961.92 ± 32.85 pg/ml, $P < 0.001$; 1276.77 ± 109.68 pg/ml versus 896.66 ± 57.13 pg/ml, $P < 0.001$, respectively) (Fig. 4). Collectively, these data reveal that S1P enhanced MPO-ANCA-positive IgG-induced sICAM-1 and sVCAM-1 up-regulation in the GEnC supernatants.

Differential activation of S1PRs mediates the S1P-induced endothelial activation

First, RT-PCR was performed to confirm the expression of S1PRs in GEnCs, and we found that GEnCs express all the five types of S1P receptors, that is S1PR1-5 (Fig. S4). To further determine the role of S1PRs through which S1P exert its effect, GEnCs were pre-incubated

with various S1PR antagonists or agonists for 15 min. before stimulation with S1P and MPO-ANCA-positive IgG, and the sICAM-1 level in the supernatants was measured. Pre-incubation of GEnCs with the S1PR1 antagonist W146 significantly increased sICAM-1 levels in the supernatants of GEnCs stimulated by S1P and MPO-ANCA-positive IgG. The sICAM-1 levels in the supernatants of GEnCs stimulated by S1P and MPO-ANCA-positive IgG were 1228.88 ± 118.63 pg/ml, which increased to 1427.92 ± 81.58 pg/ml upon pre-incubation with S1PR1 antagonist W146 (compared with that without the antagonist, $P < 0.01$, with the increase rate of $16.20 \pm 6.64\%$). On the contrary, pre-incubation of GEnCs with the S1PR1 agonist SEW significantly decreased sICAM-1 level in the supernatants of GEnCs stimulated by S1P and MPO-ANCA-positive IgG, with the inhibition rate of $13.02 \pm 7.93\%$.

Pre-treatment with S1PR2 antagonist JTE significantly reduced sICAM-1 level in the supernatants of GEnCs stimulated by S1P and MPO-ANCA-positive IgG, with the inhibition rate of $24.04 \pm 6.55\%$.

The sICAM-1 levels reduced from 1368.44 ± 277.54 pg/ml in the supernatants of GEnCs stimulated by S1P and MPO-ANCA-positive IgG to 1006.27 ± 83.09 pg/ml, upon pre-incubation with S1PR3 antagonist TY, with the inhibition rate of $26.47 \pm 6.07\%$.

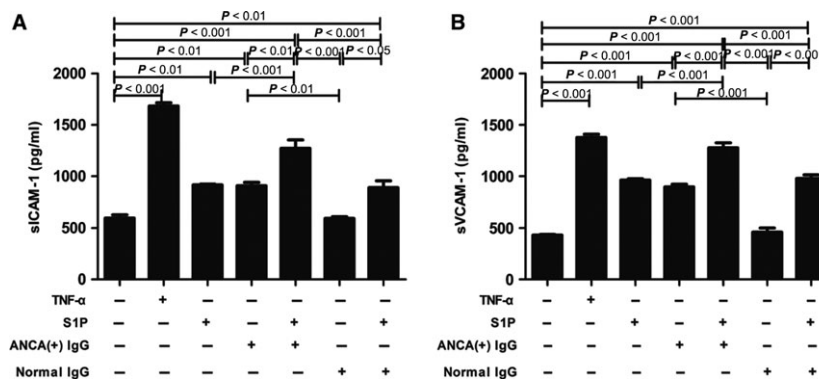


Fig. 4 S1P could up-regulate soluble adhesion molecule expression levels in the supernatants of GEnC in the presence of MPO-ANCA-positive IgG. **A.** S1P could up-regulate sICAM-1 level in the supernatants of GEnC in the presence of MPO-ANCA-positive IgG. **B.** S1P could up-regulate sVCAM-1 level in the supernatants of GEnC in the presence of MPO-ANCA-positive IgG. Bars represent mean \pm S.D. of repeated measurements of five independent experiments or donors.

Pre-treatment with S1PR4 antagonist CYM significantly decreased sICAM-1 level in the supernatants of GEnCs stimulated by S1P and MPO-ANCA-positive IgG, with the inhibition rate of $21.96 \pm 5.60\%$.

For S1PR5 agonist, sICAM-1 level in the supernatants was 1249.18 ± 111.50 pg/ml in the supernatants of GEnCs stimulated by S1P and MPO-ANCA-positive IgG, which increased to 1502.25 ± 40.11 pg/ml upon pre-incubation with S1PR5 agonist A97, with the increase rate of $20.26 \pm 3.21\%$ (Fig. 5, Fig. S3).

Collectively, these data revealed that S1P enhanced MPO-ANCA-positive IgG-mediated sICAM-1 up-regulation of GEnCs through S1PR2–5, whereas S1PR1 induced sICAM-1 down-regulation of GEnCs stimulated by S1P and MPO-ANCA-positive IgG. Differential activation of S1PRs mediated the S1P-induced endothelial activation in the presence of MPO-ANCA-positive IgG.

Discussion

In this study, we observed that S1P could enhance MPO-ANCA-positive IgG-induced GEnC activation. Furthermore, we found that activation of S1PR2–5 was implicated in the S1P-

induced endothelial activation in the presence of MPO-ANCA-positive IgG.

According to the study by Nagao *et al.*, anti-MPO antibody activates endothelial cells *via* moesin. Moesin, with the full name of membrane-organizing extension spike protein, shares certain similar sequences with those on the N-terminal region of the MPO heavy chain [7]. Furthermore, MPO-ANCA from patients with active AAV had high reactivity to the aforementioned sequences in human [33]. Although MPO is not expressed in endothelial cells, anti-MPO antibody could activate GEnCs by recognizing moesin [34]. In our previous study, we also confirmed the binding of anti-MPO antibody to moesin in human GEnCs using various methods. Furthermore, binding of anti-MPO antibody to moesin was confirmed to up-regulate adhesion molecules and to induce hyperpermeability of human GEnCs [35].

Recently, a number of studies have documented that alterations in intercellular junctional integrity and cytoskeletal organization interact can regulate endothelial barrier homeostasis dynamically. Disruption of tight junction leads to gap formation and increased endothelial permeability, whereas assembly of tight junction enhances barrier integrity. Therefore, assembly of tight junction which consists of a

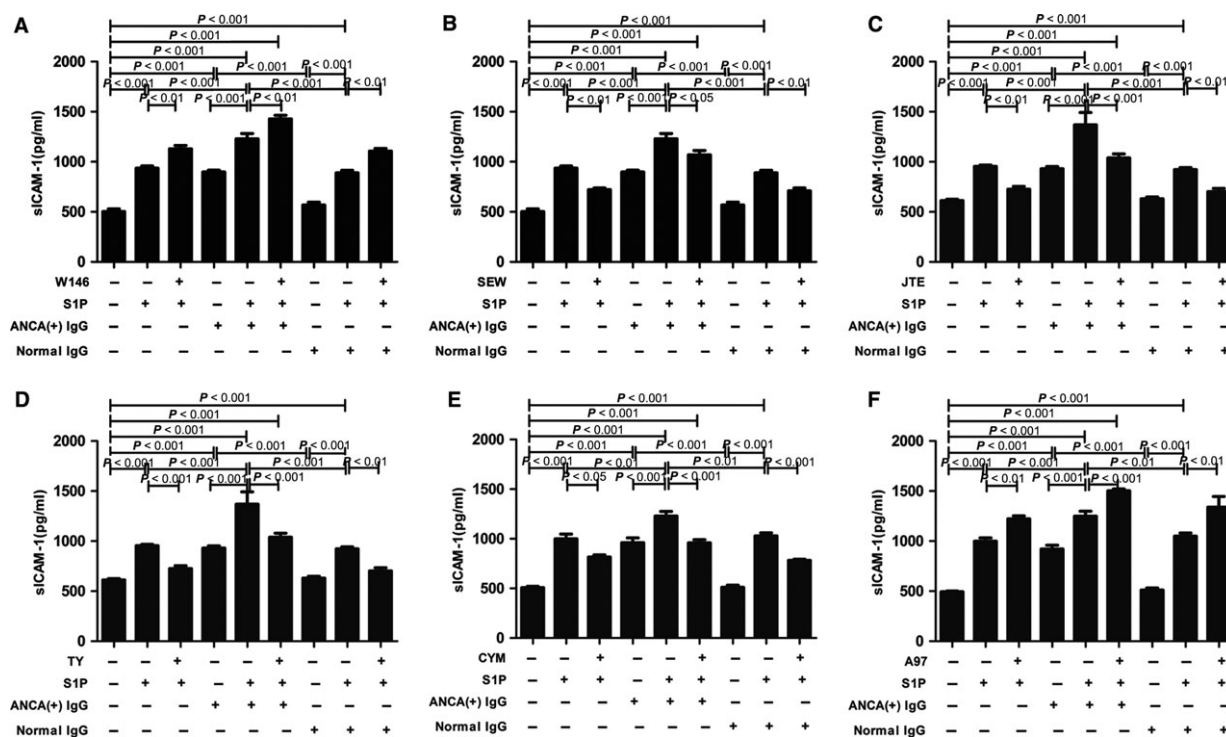


Fig. 5 Differential activation of S1PRs mediated the S1P-induced sICAM-1 up-regulation of GEnCs in the presence of MPO-ANCA-positive IgG. **A.** S1PR1 antagonist W146 significantly increased sICAM-1 level in the supernatants of GEnC stimulated by S1P plus MPO-ANCA-positive IgG. **B.** S1PR1 agonist SEW significantly reduced sICAM-1 level in the supernatants of GEnC stimulated by S1P plus MPO-ANCA-positive IgG. **C.** S1PR2 antagonist JTE significantly reduced sICAM-1 level in the supernatants of GEnC stimulated by S1P plus MPO-ANCA-positive IgG. **D.** S1PR3 antagonist TY significantly reduced sICAM-1 level in the supernatants of GEnC stimulated by S1P plus MPO-ANCA-positive IgG. **E.** S1PR4 antagonist CYM significantly reduced sICAM-1 level in the supernatants of GEnC stimulated by S1P plus MPO-ANCA-positive IgG. **F.** S1PR5 agonist A97 significantly increased sICAM-1 level in the supernatants of GEnC stimulated by S1P plus MPO-ANCA-positive IgG. Bars represent mean \pm S.D. of repeated measurements of five independent experiments or donors.

complex of proteins such as occludin, claudin and junctional adhesion molecule (JAM) is essential for the maintenance of endothelial barrier function [36–39]. In our current study, we found that S1P induced disruption of ZO-1-indicated tight junction and disorganization of phalloidin-indicated cytoskeleton, therefore increasing glomerular endothelial permeability in the presence of MPO-ANCA-positive IgG. However, the key proteins of tight junction involved in this process and the underlying mechanism require further investigation.

In this study, we also found that S1P contributed to the up-regulation of both membrane-bound and soluble ICAM-1/VCAM-1 expression levels of GEnCs in the presence of MPO-ANCA-positive IgG. ICAM-1 and VCAM-1, not only are biomarkers for endothelial cell activation and injury [40], but also might promote neutrophil adhesion and damage to endothelial cells. Evidences have revealed that neutrophils adhere only in response to the local expression of adhesion molecules on the endothelial surface and release of cytokines along the basal side of endothelium [41, 42]. Therefore, we speculate that in the presence of MPO-ANCA-positive IgG, S1P-induced ICAM-1/VCAM-1 up-regulation may promote neutrophil adhesion to GEnCs, which may aggravate the endothelial barrier dysfunction.

How MPO-ANCA and S1P interact to cause GEnCs activation in the *in vitro* setting is not fully clear yet. Moesin, which can be recognized by MPO-ANCA in GEnCs, is previously described as a cytoskeletal protein that belongs to the ezrin–radixin–moesin (ERM) family [43]. Recently, it was reported that S1P could cause acute and potent ERM activation. S1P was confirmed to activate moesin at nanomolar concentrations within a few minutes of treatment. Multiple protein kinase C (PKC) isoforms were shown to be responsible for moesin phosphorylation in response to S1P [44–46]. Therefore, we speculate that moesin recognized by MPO-ANCA could be further activated by S1P, which might cause enhanced GEnC activation *in vitro*.

All the five types of S1P receptors, S1PR1–5, have high affinity for S1P [8]. Recent studies revealed that the balance of the expression and activation of different S1PRs in endothelial cells plays a key role in regulating endothelial integrity. Physiological concentrations of S1P preserve endothelial barrier function by activating S1PR1, whereas excessive S1P induced endothelial malfunction by activating S1PR2 [17]. S1PR3 promotes leucocyte rolling, while leucocyte rolling is enhanced in endothelial-specific S1PR1 knockout mice [47]. In the present study, it was found that under pathophysiological concentration of S1P in active AAV patients, the activation of S1PR2–5 dominates the S1P-induced MPO-ANCA-positive IgG-mediated endothelial activation, whereas S1PR1 exerts opposite effect during this process. It indicated that the imbalance between different S1PR activation might participate in the development of AAV.

Regarding the role of S1PRs in regulating vascular integrity, most studies concentrated on S1PR1–3, while the effect of S1PR4 and S1PR5 on endothelial barrier function is far from clear. Our previous study found that compared with normal controls, the expression levels of S1PR4 and S1PR5 in AAV patients in glomeruli were up-regulated. Moreover, correlation analysis revealed that the S1PR4 expression level correlated with the proportion of cellular crescents in renal specimens from AAV patients [15]. In this current study, we demonstrated that activation of S1PR4 and S1PR5 participated in

S1P-induced MPO-ANCA-positive IgG-mediated endothelial activation, which extends our knowledge about the function of S1PR4 and S1PR5.

Recent studies demonstrated that the S1PRs can regulate small GTPases of the Rho family differentially, especially Rho and Rac, which are essential in cytoskeletal rearrangement, therefore participate in maintenance/disruption of cell barrier integrity [39, 48]. Activation of S1PR1 promotes cytoskeletal rearrangement in a Rac GTPase-dependent manner, which is necessary for S1P-induced endothelial barrier enhancement. On the contrary, blockade of S1PR2 or S1PR3 significantly inhibited RhoA activation, therefore alleviating endothelial barrier disruption [19, 49, 50]. Whether similar mechanisms exist in AAV remains further exploration.

Conclusions

In conclusion, S1P was found to be able to enhance MPO-ANCA-positive IgG-mediated GEnC activation through S1PR2–5. The current findings are of help to figure out the pathogenic role of S1P in AAV, thus providing potential clues for intervention strategies.

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Conflict of interest

There is no conflict of interest to declare.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Dose effect of S1P on sICAM-1 expression in the supernatants of GEnCs.

Figure S2. Dose effect of S1PR agonists or antagonists on S1P-induced sICAM-1 expression.

Figure S3. Effect of S1PR agonists on sICAM-1 level in the supernatants of GEnC stimulated by S1P plus MPO-ANCA-positive IgG.

Figure S4. Expression of S1PR1–5 in GEnCs were measured by RT-PCR.

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