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Original Article

IVIG inhibits TNF-α-induced MMP9 expression and activity in monocytes by suppressing NF-κB and P38 MAPK activation

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Abstract: Matrix metalloproteinase-9 (MMP9) has been involved in inflammatory and pathologic processes of coronary artery lesions (CAL) in Kawasaki disease (KD). Intravenous immunoglobulin (IVIG), a traditional treatment for Kawasaki disease, could decrease the expressions of MMP9. The purpose of this study was to investigate the protective effect of IVIG in chemotactic migration of monocyte and the regulation of MMP9 induced by tumor necrosis factor-α (TNF-α) in U937s. Studies were carried out with real time polymerase chain reaction (RT-PCR), zymographic, Western blotting and immunofluorescence. U937s' migration was enhanced by TNF-α stimulation, while was inhibited by IVIG pretreatment. MMP9 expression and activity in U937s were also significantly enhanced by TNF-α and inhibited by IVIVG pretreatment. During inflammatory stimulus, nuclear factor kappa B (NF-κB) and P38 Mitogen-activated protein kinase (P38 MAPK) pathways play a significant role in regulating MMP9 gene expression. TNF-α induced nuclear translocation of NF-κB and P38 MAPK activation in U937s were inhibited significantly by IVIG. Furthermore, we clarified that nuclear NF-κB and P38 MAPK pathways play pivotal roles in regulating U937s' migration and MMP9 expressions using PDTC and SB203580, which were specific inhibitors of NF-κB and p38 MAPK pathways. IVIG displays striking biological effects, notably promoting monocyte migration. These effects involve the NF-κB and p38 pathways, and increased MMP9 activity. It might be a crucial mechanism of IVIG reducing the occurrence of CAL that IVIG inhibited monocytes expressing MMP9 and decreased chemotactic migration of monocyte.

Keywords: IVIG, MMP9, NF-κB, P38 MAPK, monocyte migration

Introduction

KD is an acute febrile vasculitis that preferentially affects children younger than 5 years old, which may lead to CAL [1]. Coronary artery lesions can develop in 20-25% of untreated individuals. KD is thus a major cause of acquired heart disease in children of developed countries. CAL in KD is characterized by thickness of the vessel wall inflammation and extracellular matrix (ECM) degradation. The incidence of CAL in KD can be reduced to 5-10% with IVIG therapy [2-4].

Metalloproteinases (MMPs) play vital roles in vascular remodeling, cleaving extracellular matrix components. MMPs have contributed to the high prevalence of aneurysms [5]. MMP9, a member of matrix metalloproteinase family, is known to participate in the degradation of the ECM and tissue remodeling [6]. MMP9 expressions are significantly increased in acute stage of KD patients [7]. In addition, MMP9 expression levels are much higher in coronary artery aneurysm pathology specimens [8]. Meanwhile, there are correlations between MMP9 and disease progression in the adult aneurysm, acute coronary syndrome disease [9, 10]. Arteritis in KD is characterized by proliferative inflammation that consists of marked accumulation of monocytes/macrophages; aberrant activation of those macrophages is thought to be involved in the formation of vascular lesions. Extracellular MMPs levels are involved in monocyte migration, study showed that macrophage adhesion to fibronectin was associated with increased MMP9 secretion [12].

NF-κB is a heterodimer of p65 and p50 subunits, which is inhibited by IκBα protein in an

inactive form in the cytoplasm. Activation of NF-κB requires degradation of the IκBα protein. Phosphorylation of IκB by inducing stimuli can lead to IκBα degradation, Released NF-κB dimers are further activated and translocate to the nucleus where they bind to specific DNA sequences and induce transcription of target genes [13]. NF-κB is a pivotal transcription regulation factor for proinflammatory genes that encode cytokines, chemokines and adhesion molecules [14, 15]. p38 MAPK is a member of Mitogen-activated protein kinases (MAPKs) pathway, which is critical signaling cascades that convert stimuli signals into biological responses such as cell proliferation, transformation and invasion [16].

Here we studied U937s' migration and MMP expressions in response to TNF-α and then investigated the protective effect of IVIG on U937s' migration by MMP9.

Material and methods

Cell culture and treatment

U937 cells were obtained from the Chinese Academy of Sciences (Shanghai, China). RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Gibco (St. Louis, MO, USA). U937 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin plus streptomycin (100 U/mL and 100 μg/mL, respectively). All cultures were maintained at 37°C in a 5% CO_2 atmosphere.

U937 cells were exposed to 2 ng/ml TNF-α (PeproTech, Rocky Hill, NJ, USA) for the indicated times after 30 min treatment or no treatment with IVIG (Taibang Biological Products Co, Ltd, Shandong, China). In addition, U937 cells were exposed to 2 ng/ml TNF-α for 12 hours after 2 hours treatment with PDTC (Sigma, St. Louis, MO, USA) and SB203580 (Merck Millipore, Corp, Billerica, MA, USA).

RNA isolation and real time polymerase chain reaction (RT-PCR)

A total of 1×10^6 cells were collected. Total RNA from differently treated or untreated cells was extracted using Trizol reagent (Life Invitrogen, Leek, Netherlands). 500 ng purified RNA were used to perform reverse transcription using cDNA Synthesis Kit for RT-PCR according to the manufacturer's instructions. The RT-PCR was performed on a LightCycler-96 system with the SYBR Green approach. The sequences of the primers used were as follow: human MMP9 forward, 5'-TTTGACAGCGACAAGA-AGTGG-3' and reverse, 5'-GGCTCAGGTTCAGGGCGAGGA-3'; human β-actin forward, 5'-GATGCAGAAGGAGATCACTGC-3' and reverse, 5'-ATACTCCTGCTT-GCTGATCCA-3'. The reaction conditions were as follows: 93°C for 3 min, followed by 40 cycles of 93°C for 30 sec and 55°C for 45 sec. RNA levels of target genes were normalized by the level of β-actin. Results are expressed as a percentage of the untreated control.

Protein preparation and western blotting

The treated and untreated cells were washed twice with phosphate-buffered saline (PBS), precipitation was harvested in assay lysis buffer for 15 min on ice. Then the homogenate was centrifuged at 12,000× g for 15 min. The protein concentration was measured using BCA protein assay kit (Beyotime Biotechnology, Hangzhou, China). Total protein was extracted from control U937 cells and treated U937 cell. Protein samples (30 μg) were heated for 10 min at 100°C and subjected to 10% SDS-PAGE gel. Proteins were blotted onto PVDF membranes (Millipore, Schwalbach, Germany) for 90 min 320 mA. After transfer, the membranes were blocked in blocking buffer for 2 hours before incubation with primary antibodies at a certain concentration for 14-18 hours, Antibodies gainst nuclear factor kappa B (NF-κB) p65, phospho-p65, inhibitory factor κBα (IκBα), phospho-IκBα, p38 mitogen-activated protein kinase (MAPK), phospho-p38 MAPK, were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against MMP9, Tubulin, were purchased from Abcam (Cambridge, UK). The membranes were then washed in TBS containing 0.1% Tween-20 (TBS-Tween buffer) and incubated with secondary antibodies at a dilution of 1:2000 in 5% non-fat dry milk powder for 2 hours, Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were purchased from Beyotime Biotechnology (Hangzhou, China). Detection of bound antibodies were accomplished using substrate enhanced chemoluminescence reagent (ECL system, Amersham Bioscience, Little Chalfont,

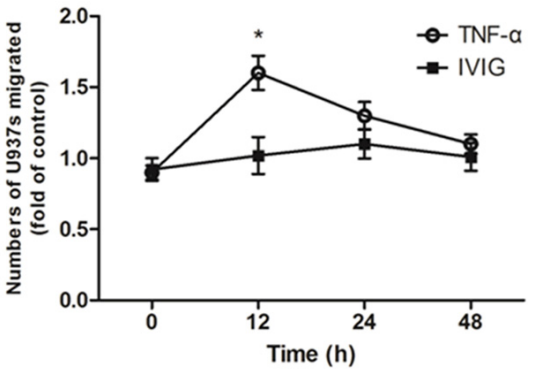

Figure 1. Number of U937 cells migrated (fold of untreated U937s). **P<0.05* compared with untreated U937 cells.

UK). For sequential detection of different antigens, antibodies were removed from the blot by incubating the membrane in stripping buffer for 45 min, with subsequent washing in TBS-Tween buffer.

Immunofluorescence

To measure activity of NF-κB signal pathway furtherly, the translocation of p65 was detected by immunofluorescence. The ells were washed in PBS and fixed with 4% paraformaldehyde in PBS for 30 min and then permeabilized for 10 min with 0.1% Triton X-100 at room temperature. Cells were blocked with 3% BSA and incubated with primary antibodies anti-p65 overnight. Secondary antibody FITC-conjugated anti-rabbit immunoglobulins were incubated for 1 hour at room temperature. Cells were washed three times with PBS between incubations. Images were acquired by fluorescence microscope with appropriate filter sets.

Gelatin zymography

U937 cells were incubated in serum-free medium, and treated with or without IVIG, and then stimulated with TNF-α for 12 hours. Cell culture Supernatants were collected. The protein concentration was measured using BCA protein assay kit. Samples containing 15 μg of protein were loaded on SDS-polyacrylamide gels, containing 0.1% gelatin (Sigma, St. Louis, MO, USA), 8% separating gel and 5% stacking gel. Electrophoresis was performed under non-reducing conditions for 2.5 hours at 80 v. After electrophoresis, Gels were washed twice for 45 min in 2.5% Triton X-100. Then, Gels were preincubated in reaction buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$). Thereafter, the gel was placed in the incubation medium for 42 h at 37°C. Gelatinolytic activity was visualized as clear areas in a Coomassie Blue-stained gel.

In vitro monocyte chemotaxis assay

To examine the bioactivity of MMP9 in U937s, we performed U937 monocyte chemotaxis assays. U937 cells were treated with TNF-α for 12 hours after 30 min no treatment, 30 min treatment with IVIG, 2 hours treatment with PDTC or 2 hours treatment with SB203580. 600 μl RPMI-1640 medium supplemented with 10% FBS and 10 ng/ml MCP-1 were added to the bottom wells of chambers, apolycarbonate 5 mm membrane was placed over there agents. U937 cells at 1×10^5 cells/mL cultured in 200 μl RPMI-1640 medium without 10% FBS were added to the top wells and incubated for 12 hours. Each test group was assayed in quadruplicate. Three high-power (3×400) fields were counted in each replicate well, and the results are expressed as the fold of cell number in contrast to untreated U937 cells group.

Statistical analysis

All data presented are mean ± standard deviation. Data were analyzed using the statistical program SPSS 17.0. $P<0.05$ was considered to be statistically significant. All experiments were repeated at least three times.

Results

Effect of IVIG on U937s migration in U937 cells stimulated with TNF-α

We first investigated the effect of TNF-α and IVIG on monocyte migration to MCP-1, TNF-α enhanced U937s migration in, in contrast, pretreatment of U937 cells with IVIG significantly inhibited MCP-1 directed transmigration (**Figure 1**).

Effect of IVIG on MMP9 production in U937 cells stimulated with TNF-α

We explored the possible role of MMP2 and MMP9 in TNF-α-enhanced migration. Here, MMP2 and MMP9 expressions and activities were quantified by RT-PCR and gelatin zymogra-

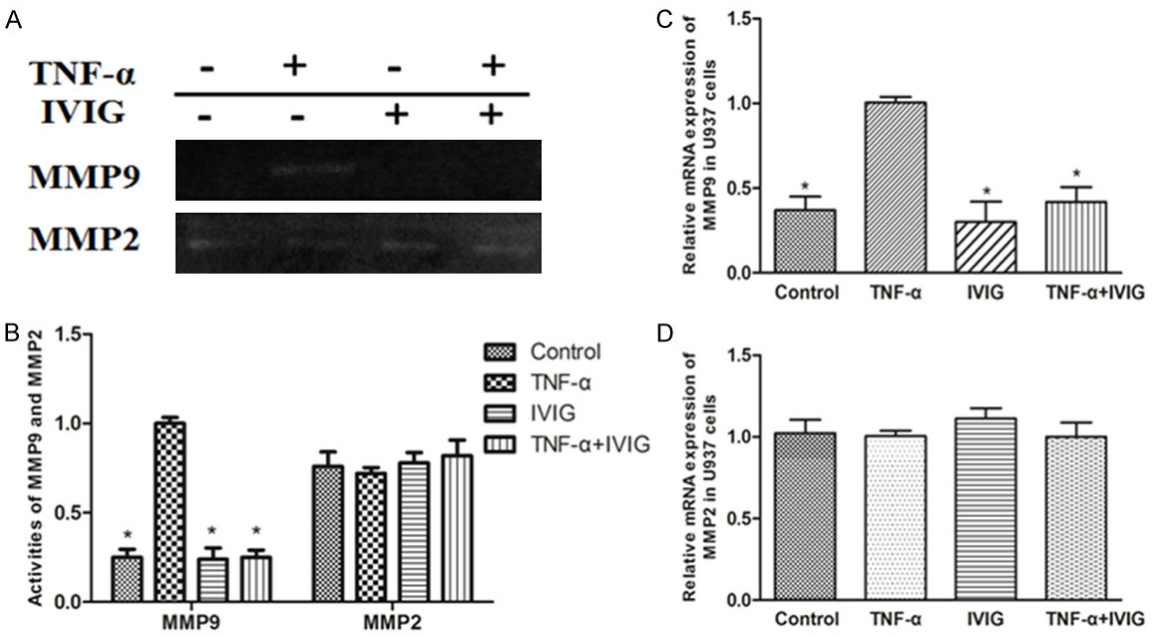

Figure 2. The activities and expressions of MMP9 and MMP2 in U937 cells induced by TNF-α and IVIG. A. The activities of MMP9 and MMP2 were determined by gelatin zymography. B. Quantification activities of MMP9 and MMP2 in each group. C. The levels of mRNA for MMP9 were determined by RT-PCR. D. The levels of mRNA for MMP9 were determined by RT-PCR. Results are expressed as mean ± SEM (n = 3). **P*<0.05 compared with the U937 cells induced by TNF-α.

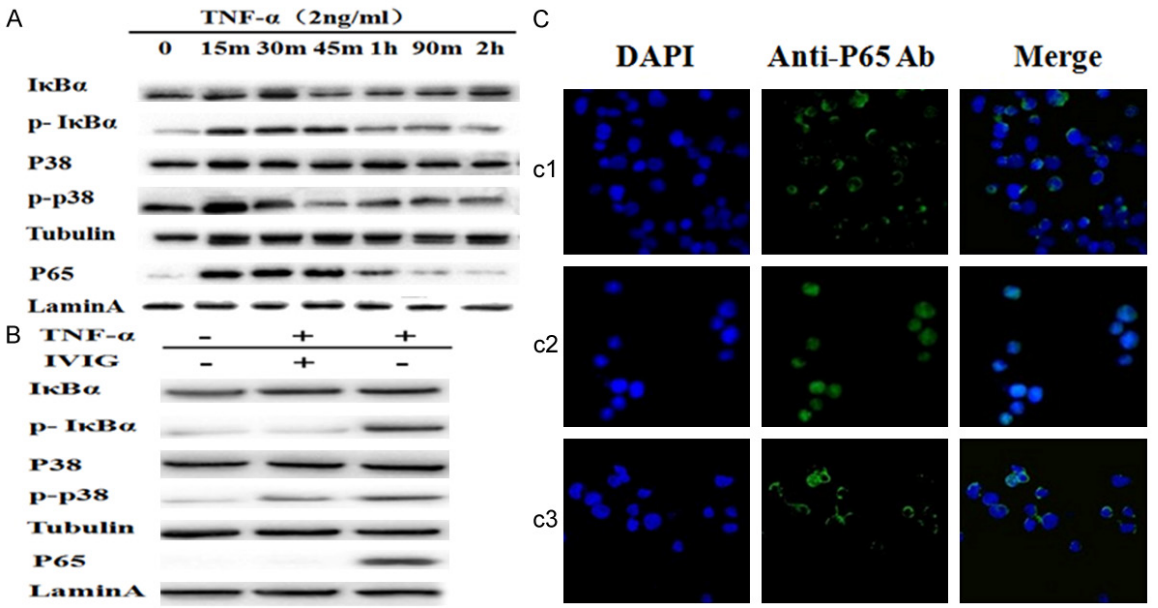

Figure 3. TNF-α stimulation induced the phosphorylation of NF-κB and P38 MAPK pathways, IVIG pretreatment suppressed the phosphorylation of NF-κB and P38 MAPK pathways. A. U937 cells were stimulated with 2 ng/ml TNF-α for the indicated time periods. B. The cells were untreated or treated with 20 mg/ml IVIG for 30 min and then stimulated with 2 ng/ml TNF-α for 15 min. C. Nuclear translocation of p65 was measured by immunofluorescence among none treatment (c1), TNF-α stimulation (c2) and IVIG pretreatment (c3), Magnification 20×10.

phy. As shown in **Figure 2A** and **2B**, TNF-α-treated group exhibited higher MMP9 activity relative to the untreated group, whereas IVIG pretreatment significantly reduced MMP9 activ-

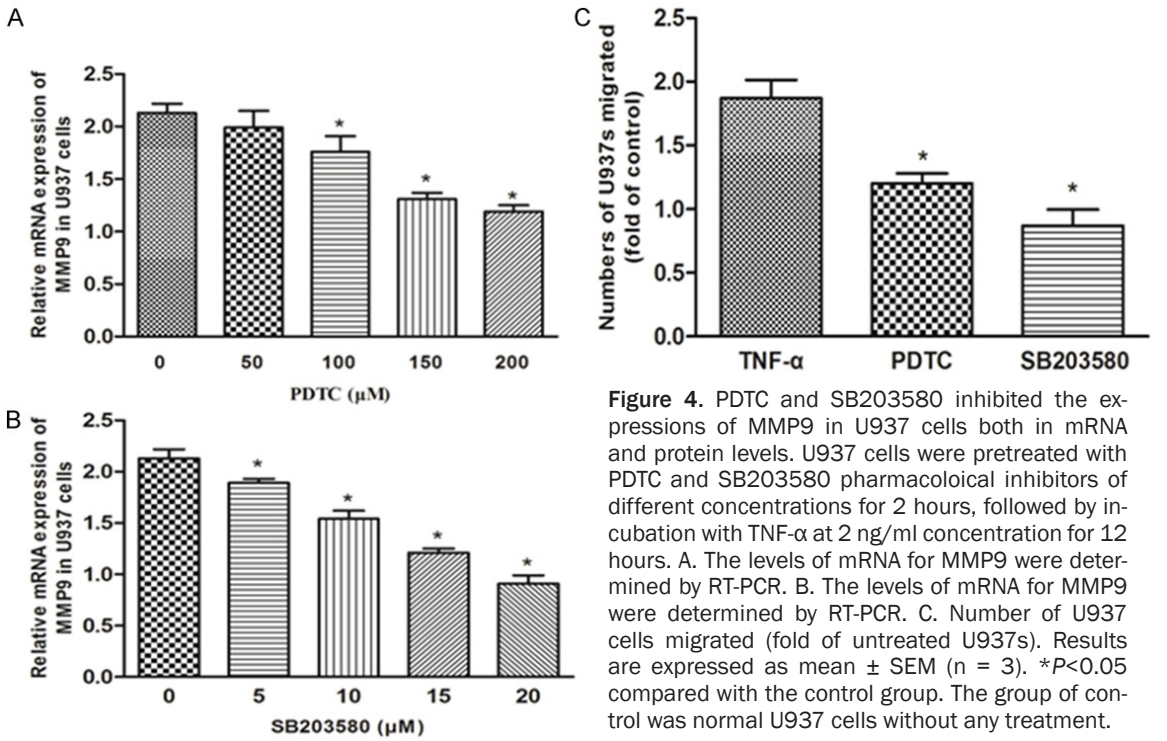

Figure 4. PDTC and SB203580 inhibited the expressions of MMP9 in U937 cells both in mRNA and protein levels. U937 cells were pretreated with PDTC and SB203580 pharmacoloical inhibitors of different concentrations for 2 hours, followed by incubation with TNF-α at 2 ng/ml concentration for 12 hours. A. The levels of mRNA for MMP9 were determined by RT-PCR. B. The levels of mRNA for MMP9 were determined by RT-PCR. C. Number of U937 cells migrated (fold of untreated U937s). Results are expressed as mean ± SEM (n = 3). **P*<0.05 compared with the control group. The group of control was normal U937 cells without any treatment.

ity relative to TNF-α-treated group. However, we observed no difference in MMP2 activities both in the TNF-treated and IVIG pretreatment groups relative to the control group. Furthermore, we showed the same results in mRNA level by RT-PCR (**Figure 2C** and **2D**). This effect of IVIG on MMP9 secretion is unlikely to be sole mechanism underlying the observed effect of TNF-α on monocyte migration.

NF-κB and MAPKs pathways were affected by treatment of IVIG in U937 cells stimulated with TNF-α

To investigate how TNF-α and IVIG regulate U937s' migration and MMP9 expressions, we investigated changes in NF-κB and MAPKs pathways in U937 cells using western blot and immunofluorescence. The time course of phosphorylation of NF-κB and MAPKs pathways induced by TNF-α were examined. Exposure of U937 cells to 2 ng/ml TNF-α led to an increase of p65 in nuclear and an increase in phosphorylation of IκBα, p38 MAPK in a time-dependent manner. Both increases of p65 in nuclear and the phosphorylation of IκBα and p38MAPK were observed at 15 min after TNF-α stimulation (**Figure 3A**). It demonstrated that NF-κB and MAPKs signal pathways were activated in the early stage of TNF-α stimulation in U937 cells.

Furthermore, to confirm whether NF-κB and MAPKs pathways participate in the process of IVIG suppressing U937s' migration and MMP9 expressions, we examined the effects of IVIG on the activation of NF-κB and MAPKs pathways at 15 min after TNF-αstimulation. As shown in **Figure 3B**, **3C**, IVIG suppressed p65 translocation to nuclear and the phosphorylation of IκBα and p38 MAPK in TNF-α-stimulated U937 cells, whereas it had no effect on the protein expression level of IκBα and p38 MAPK. Therefore, our results demonstrate that NF-κB and MAPKs pathways may participate in the process of IVIG inhibiting MMP9 expressions.

U937s migration and MMP9 expressions are countered by NF-κB and MAPKs pathways inhibition

We then examined whether TNF-α-induced U937s' migration and MMP9 production indeed occurred through NF-κB and p38 MAPK. We used specific NF-κB and p38 MAPK pathways inhibitors: PDTC and SB203580. U937 cells were pretreated with PDTC and SB203580 pharmacoloical inhibitors for 2 hours, followed

by incubation with TNF-α at 2 ng/ml concentration for 12 hours (**Figure 4**). Our data reveal that NF-κB and p38 MAPK activities inhibition significantly blocked MMP9 production in U937 cells. Therefore, our results demonstrated that TNF-α-stimulated U937s' migration and MMP9 expressions were dependent on the NF-κB and p38 MAPK pathways, and NF-κB and MAPKs pathways may participate in the process of IVIG inhibiting U937s' migration and MMP9 expressions. As expected, NF-κB and p38 inhibition abrogated the ability of TNF-α to enhance monocyte migration. It highlighted the prominent role of these pathways in TNF-α-enhanced and IVIG-prevented U937s migration.

Discussion

We provide new evidence for a major effect on IVIG on monocyte migration. We found that IVIG decreased monocyte migration towards MCP-1 induced by TNF-α, an effect associated with suppressing NF-κB and p38 signaling pathway and with MMP9 secretion.

Peripheral blood monocytes/macrophages play vital roles in the pathogenesis and vascular inflammation of Kawasaki disease by regulating matrix metalloproteinases, producing amounts of pro-inflammatory cytokines and chemokines [17]. In response to proinflammatory signals, circulating blood monocytes adhere transiently to the activated vascular endothelium before crossing the vessel wall. The histopathological findings of coronary artery in KD comprise panvasculitis with infiltration of mononuclear cells [18]. Therefore, the increase of cytokines secreted by monocytes/macrophages, the adhesion to the vessel wall of monocytes/macrophages might lead to vascular inflammation and aggravate vascular damages. Experimental strategies reducing the macrophage population in the aneurysm wall, through macrophage depletion and cytokine inhibition, decrease MMP activity and attenuate aneurysm formation in mouse models of AAA. Our data clearly showed that TNF-α stimulation led to U937 cells migration to monocyte MCP-1, while IVIG pretreatment attenuated migration. IVIG had been used to treat inflammatory and autoimmune disorders.

A number of mechanisms for the immunomodulation and anti-inflammatory actions of IVIG therapy have been described, including blockade effects of Fc receptor, inhibition or neutralization of cytokines and growth factors, modulation of adhesion molecules and cell receptors, anti-complement effects, modulation of regulatory T cells, activation of regulatory macrophages through the FcgRIIB receptor and accelerated clearance of autoantibodies [19]. In the present study, we examined the effects of IVIG on U937s' migration and MMP9 expressions, which participated in the pathogenesis of KD. J Leukoc Biolpreviously demonstrated thatIVIG treatment may block MCP-1 activity and decrease monocyte adhesing to the vessel wall, thus alleviating KD vasculitis [20]. Infiltration of macrophages in the peripheral nerves is involved in the mechanisms underlying the effect of IVIG therapy in Chronic inflammatory demyelinating polyneuropathy [21]. Ichiyama et al. previously demonstrated that IVIG can partially inhibit TNF-α-induced inflammatory cytokines production by suppressing the activation of NF-κB pathway in HCAECs and monocytes [22, 23]. It was investigated that NF-κB were activated in peripheral monocytes/macrophages and T cells during acute Kawasaki disease, while IVIG could inhibit NF-κB activation [24]. Our results were consistent with previous reports, and showed that NF-κB and p38 MAPK pathways participated in suppressing MMP9 production of IVIG in TNF-α-stimulated U937 cells. Moreover, we further clarified p38 MAPK pathway participated in the modulations of IVIG. Previous study also reported the activity of IVIG might be regulated by inhibiting p38 MAPK pathways in THP-1 cells [25].

Many studies of aneurysm have supported-MMP9 play a pivotal role in the degradation of the ECM and pathogenesis of the diseases. Monocyte migration and secretion of MMP9 are crucial for the initiation and progression of atherosclerosis and rheumatoid arthritis [26, 27]. MMP9 gene expression is chiefly regulated by transcriptional factors (for example, NF-κB and AP-1) via the PI3K/AKT pathway [28]. Previous studies demonstrated that TNF-α and IL1B could induce MMP9 expression via the NF-kB pathway indifferent types of cells [29]. In a murine model of Kawasaki disease, IVIG may inhibit the inflammatory cell infiltration and alleviate coronary artery by suppressing the overexpression of MMP9 and overactivation of NF-κB pathway [30]. In this study, IVIG can inhibit MMP9 expression, IVIG inhibited the

nuclear translocation of p65 in TNF-α-treated U937 cells and down regulated IκBα phosphorylation and MMP9 proteins expression. We further demonstrated that SB203580 inhibited p38 phosphorylation and suppressed MMP9 protein expression in U937 cells. A number of studies showed that MAPK signal pathways participated in MMP9 expressions and functions [27, 31, 32]. Ashida et al. reported that the ERK pathway is involved in monocyte adhesion, while the p38 pathway would be involved in cell migration [33].

Overall, our results help to explain the effects of IVIG on monocyte migration and support the therapeutic potential of IVIG in decreasing CAL incidence.

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Disclosure of conflict of interest

None.

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