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ADAM10 inhibits the interaction between IL-17 and HMGB1 in Buerger's disease

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Abstract. – OBJECTIVE: Buerger's disease is a rare disease that causes critical limb ischemia; however, the underlying pathophysiological mechanism remains unclear. Therefore, we investigated the interaction between interleukin (IL)-17 and high-mobility group protein B 1 (HMGB1) and determined whether A disintegrin and metalloproteinase 10 (ADAM10) inhibit this interaction.

PATIENTS AND METHODS: The study population included 15 patients with Buerger's disease and 10 healthy donors without a history of giving peripheral blood samples. Cytokine levels were measured using a luminex multiplex assay in plasma. Flow cytometry was used to analyze the subtypes of helper T (Th) cells among peripheral blood mononuclear cells (PBMCs). The effect of ADAM10 on PBMCs was analyzed *in vitro*.

RESULTS: The levels of inflammatory cytokines and production of pathogenic Th cells were found to be higher in Korean patients with Buerger's disease. IL-17 treatment induced HMGB1 associated molecules. HMGB1 also induced IL-17 and Th17 associated transcription factors in Buerger's patients. We observed that ADAM10 regulates the interaction between IL-17 and HMGB1 via advanced glycation end products (RAGE)/nuclear factor-kappa B (NF-kB) pathway in patients with Buerger's disease.

CONCLUSIONS: This study suggests that IL-17 and HMGB1 cytokines contribute to the pathogenesis of Buerger's disease. These results indicate that ADAM10 alleviates inflammation in Buerger's disease via the HMGB1 and RAGE/NF-κB signaling pathway and provides insights into the molecular basis of and a potential therapeutic strategy for Buerger's disease.

Key Words:
Buerger's disease, IL-17, HMGB1, ADAM10.

Abbreviations

HMGB1: high-mobility group protein B 1; ADAM10: A disintegrin and metalloprotease 10; Th: helper T; PBMCs: peripheral blood mononuclear cells; RAGE: advanced glycation end products; NF-kB: nuclear factor-kappa B; PTA: percutaneous transluminal angioplasty; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction.

Introduction

Buerger's disease, also known as thromboangiitis obliterans, is a nonatherosclerotic inflammatory disorder of unknown etiology that affects small and medium arteries of the extremities[1,2]. Although smoking is considered to be the most significant risk factor for Buerger's disease, it is not the sole cause, and the other potential causes are still unknown. Recently, the concept of "a susceptible patient" or "autoimmune disease" has emerged. Since not all smokers develop Buerger's disease, so it has been suggested that tobacco may trigger an immune response in susceptible persons, or may unmask a clotting defect in them, either of which could induce an inflammatory reaction in the vessel walls[3]. Abnormalities in immunoreactivity are believed to drive the inflammatory process. Patients with thromboangiitis obliterans have been shown to have increased cellular immunity to type I and III collagens when compared to patients with atherosclerosis[4]. In addition, high titers of anti-endothelial cell antibodies have been detected in patients with this disorder[5]. However, understanding of

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the immunological mechanisms involved in the progression of vascular tissue inflammation, and hence the pathophysiology of this disease, is still limited.

Autoreactive helper T (Th) cells can induce tissue damage and inflammation, leading to various types of immune diseases. Recent studies have suggested that Th cell subtypes, including Th1, Th2, and Th17, mediate systemic immunity and progression of autoimmune diseases[6,7]. Th17 cells have been broadly implicated in autoimmune diseases, and autospecific Th17 cells have been shown to be highly pathological. Interleukin-17 (IL-17) is expressed in Th17 cells and is a cytokine that is actively being studied in relation to autoimmune diseases. High-mobility group box 1 (HMGB1), secreted by activated macrophages and monocytes, induces an inflammatory response, and is involved in increasing the levels of pro-inflammatory cytokines that mediate lymphocyte infiltration and inflammatory responses[8]. De Caridi et al[9] demonstrated that plasma HMGB1 levels were higher in patients with Buerger's disease, suggesting that this protein could be a potential therapeutic target for treating this condition. HMGB1 is also known to contribute to Th 17 cell activation in certain inflammatory diseases[10,11]. Despite the recognition of the important roles of IL-17 and HMGB1 in Buerger's disease, no studies have assessed their interactions and roles in disease development and progression so far. In addition, A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) play an inhibitory role in the progression of Buerger's disease, as observed in an animal model[12]. However, the effects and mechanism of action of ADAM10 in humans are still unclear. The pathogenesis of Buerger's disease is poorly understood; most hypotheses are controversial, and the above-mentioned modern immunology concepts have not yet been applied to the study of Buerger's disease. Therefore, this investigation was carried out to evaluate the levels of the relevant cytokines in the plasma of patients with Buerger's disease.

Specifically, this study aimed to investigate the pathogenic role of IL-17 and HMGB1 in patients with Buerger's disease. We demonstrated that ADAM10 inhibits the interaction between IL-17 and HMGB1. Thus, we investigated the applicability of ADAM10 as a candidate immunological therapeutic drug for the treatment of Buerger's disease.

Patients and Methods

Study Population

From January 2009 to July 2020, 15 patients with Buerger's disease were enrolled in this study at the Korea University Guro Hospital (Seoul, Korea). The clinical diagnostic inclusion criteria for Buerger's disease were as follows: (1) onset before the age of 55 years; (2) a history of smoking; (3) presence of distal-extremity ischemia indicated by claudication, pain at rest, ischemic ulcers or gangrenes documented by non-invasive vascular testing. The exclusion criteria were as follows: (1) autoimmune diseases, hypercoagulable states, and diabetes mellitus; (2) a proximal source of emboli detected by echocardiography or arteriography; (3) consistent arteriographic findings in the clinically involved and non-involved limbs. At least two doctors reviewed the clinical and imaging findings of the patients. Patients treated with sufficient and optimal medication, including high-intensity statin therapy, with a clinically stable condition, were enrolled in the study. Percutaneous transluminal angioplasty (PTA) for critical limb ischemia was performed at least one year before enrollment. Ten healthy donors aged over 19 years without a previous adverse medical history were enrolled as controls. All participants provided written informed consent, and the study was approved by the Institutional Review Board of Korea University Guro Hospital (2019GR0087).

Clinical Data and Data Collection

The baseline characteristics of each patient with Buerger's disease are shown in Table I. Participants were interviewed to collect demographic data. All clinical indicators were collected from medical records and result sheets. Clinical data of the patients extracted from electronic medical records (EMRs) were as follows: sex, age at onset, history of smoking, history of particular diseases, laboratory findings, and history of pharmaceutical treatments with peripheral vascular disease agents.

Collection of Peripheral Blood Mononuclear Cells (PBMCs)

Buerger's disease patients and healthy donors (as controls) were enrolled in this study. Blood samples were collected in BD Vacutainer® tubes containing heparin (Becton-Dickinson, Franklin Lakes, NJ, USA) to prevent coagulation. PBMCs were prepared within 2 h of blood collection and were isolated by standard density gradient

Table I. Baseline characteristics of patients with Buerger's disease and controls.

Variables	Patients (n = 15)	Controls (n = 10)
Sex, male	15 (100%)	6 (60%)
Age at onset, year	43.27 [35-51]	36.4 [26-45]
BMI* (kg/m^2)	23.31 [20.7-25.1]	23.5 [20.4-28.1]
Height (cm)	172 [165-180]	165 [160-183]
Weight (Kg)	69.07 [62-72]	60.4[55-102]
Blood pressure, mmHg		
Systolic	127.13 [115-136]	126.2 [110-130]
Diastolic	76.8 [64-86]	75.5[65-82]
Heart rate (bpm)	86.07 [75-106]	83.2 [75-95]
Smoking information		
History of smoking	15 (100%)	4 (40%)
Currently smoking	8 (53.3%)	2(20%)
Period of smoking (month)	277.6 [180-360]	202 [150-300]
Amount of smoking (pack/day)	1.03 [1-1]	1
Quit smoking	7 (46.7%)	2 (20%)
Clinical presentation		
Claudication	10 (66.7)	
Resting Ischemic Pain	6 (40)	
Wound (Major+Minor)	9 (60)	
Gangrene	8 (53.3)	
Patient history		
Hypertension	3 (20%)	
Diabetes mellitus	3 (20%)	
Insulin	1 (6.7%)	
Oral medication	1 (6.7%)	
Dietary intervention	1 (6.7%)	
Dyslipidemia	2 (13.3%)	
Atrial fibrillation	2 (13.3%)	
Congestive heart failure	1 (6.7%)	
Superficial vein thrombosis	3 (20%)	

*BMI, body mass index

centrifugation using Ficoll-Hypaque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The cells were then washed and re-suspended at a density of 1×10^6 cells/mL in RPMI-1640 (Welgene Inc., Gyeongsan-si, Gyeongsangbuk-do, Korea) containing 10% fetal bovine serum (FBS; Access Biological Inc., Vista, CA, USA).

PBMC Culture and Stimulation

Isolated PBMCs (5×10^5 cells/500 µL) were pretreated with 125 ng/mL of ADAM10 for 2 h, and then stimulated with or without 100 or 500 ng/mL recombinant human HMGB1 and 10 ng/mL of IL-17 (R&D systems, Minneapolis, MN, USA) for 24 or 72 h.

Intracellular Staining and Flow Cytometry

For flow cytometry staining, PBMCs were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma Aldrich, St. Louis, MO, USA), 500 ng/mL ionomycin (Sigma-Aldrich, St. Louis, MO, USA), and 0.34 µL/well Golgi Stop (BD Biosciences, San Jose, CA, USA) for 4 h in an incubator with 5% CO_2 at 37°C. The cells were then harvested and washed with fluorescence-activated cell sorting buffer. Then, PBMCs were stained with phycoerythrin (PE)/cyanine (Cy) 7-conjugated anti-CD4 antibody (BioLegend, San Diego, CA, USA), fixed, and permeabilized according to the manufacturer's instructions. The cells were then stained with antibodies specific to intracellular markers for 30 min at 4 °C in the dark. For intracellular staining, PE-conjugated anti-IL-17 (BD Biosciences, San Jose, CA, USA), FITC-conjugated anti-IFN-γ (eBioscience, San Diego, CA, USA), and allophycocyanin (APC)-conjugated anti-IL-4 (BD Biosciences, Xi San Jose, CA, USA) antibodies were used. For the analysis of Tregs, PBMCs were stained with PE/Cy7-conjugated anti-CD4 (BioLegend) and APC-conjugated anti-CD25 (eBioscience, San Diego, CA, USA) antibodies, followed by fixation and permeabilization using a Foxp3 staining

kit (eBioscience), according to the manufacturer's instructions. For intracellular staining of Tregs, PBMCs were stained with PE-conjugated anti-Foxp3 antibody (eBioscience) for 30 min at 4 °C in the dark. All samples were assessed using an LSRForessa™ cell analyzer (BD Bioscience).

Measurement of Cytokine Levels

Human plasma and cell culture supernatants were obtained and assessed using Milliplex™ MAP (Millipore, Billerica, MA, USA) Multiplex Magnetic Bead-Based Antibody Detection kits, according to the manufacturer's instructions. Plasma and cell culture supernatant samples were analyzed using an eight-analyte (8-plex) kit. A custom Milliplex™ kit was used to measure the levels of interferon (IFN)-γ, IL-4, IL-6, IL-10, IL-17, IL-23, and tumor necrosis factor (TNF)-α, resulting in eight protein targets to be analyzed. Concentrations of soluble receptors for advanced glycation end product (sRAGE) cytokines were measured using a human soluble cytokine receptor magnetic bead panel using Milliplex™ MAP (Millipore). HMGB1 levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (Arigo Biolaboratories, Hsinchu City, Taiwan). Microtiter plates pre-coated with antibodies specific for HMGB1 were used. The absorbance was measured at 450 nm using an ELISA microplate reader. All procedures were performed according to the manufacturer's instructions.

RNA Isolation and Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted using TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from the extracted total RNA (2 μg) using a Dyne 1st-Strand cDNA Synthesis Kit (Dynbio Inc., Seongnam-si, Gyeonggi-do, Korea). mRNA expression was estimated using quantitative real-time PCR using SFCgreen qPCR Master Mix (SFCprobes, Yongin-si, Gyeonggi-do, Korea) fluorescent dye, according to the manufacturer's instructions. An ABI QuantStudio™ 6 (Applied Biosystems, Foster City, CA, USA) was used for quantitative PCR. Primer sequences are shown in **Supplementary Table I**. All primers were designed using Primer Express (Applied Biosystems). mRNA levels were calculated using the 2^{-ddCp} method, and mRNA expression of the genes of interest was normalized to that of β-actin.

Western Blotting

PBMCs were lysed in Halt protein lysis buffer containing the Halt phosphatase inhibitor (Thermo Pierce, Waltham, MA, USA). The cells were then harvested and lysed with lysis buffer (Roche Applied Science, Vienna, Austria). Protein concentration was determined using the Bradford dye-binding method (Bio-Rad, Hercules, CA, USA). For this purpose, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto Hybond ECL membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for Western Blotting using SNAP i.d. Protein Detection System (Millipore). For Western blot hybridization, the membrane was pre-incubated with a blocking buffer for 2 h, and then incubated with primary antibodies against total nuclear factor kappa B (NFκB), phospho-NF-κB, total extracellular-signal-regulated kinase (ERK), phospho-ERK, total p38 mitogen-activated protein kinase (MAPK), phospho-p38 MAPK, and β-actin, for 1 h (Cell Signaling, Danvers, MA, USA). After washing, primary antibodies were detected using goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies, and the membranes were incubated for 1 h at room temperature. After washing, the hybridized bands were detected using enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA), a detection kit (Pierce, Rockford, IL, USA), and Hyperfilm (Agfa, Antwerp, Mortsel, Belgium).

Statistical Analysis

For continuous variables, differences between the two groups were evaluated using the unpaired t-test or Mann-Whitney rank test. Data are expressed as the mean ± standard deviation or median ± interquartile range (IQR). For discrete variables, differences were expressed as counts and percentages, and were analyzed using the $\chi 2$ or Fisher's exact test between the two groups. For all analyses, a two-tailed $p < 0.05$ was considered to indicate significance. All data were processed using SPSS software (version 25.0, SPSS-PC, Inc., IBM Corp., Armonk, NY, USA).

Results

Clinical Characteristics of Patients with Buerger's Disease

Fifteen patients diagnosed with Buerger's disease based on clinical features from their EMRs,

Table II. Laboratory findings of patients with Buerger's disease.

Variables	Patients (n = 15)
Hemoglobin, g/dL	13.71 [12.7-15.1]
Hematocrit, %	41.25 [38.7-45.4]
Red blood cell count, $\times 100^3/\mu L$	4.5 [4.1-4.9]
Mean corpuscular volume, fL	91.79 [89.9-94.7]
Mean corpuscular hemoglobin, pg	30.48 [30-31.7]
Mean corpuscular hemoglobin concentration, g/dL	33.17 [32.7-33.6]
Red cell distribution width, %	13.79 [13.2-14.15]
White blood cell count, $\times 10^3/\mu L$	7.25 [5.7-8.7]
Neutrophil, %	58.16 [54-66.1]
Lymphocyte, %	30.24 [26.2-33.6]
Monocyte, %	7.15 [6.1-8]
Eosinophil, %	3.79 [1.3-5]
Basophil, %	0.67 [0.5-0.9]
Platelet count, $\times 10^3/\mu L$	280.67 [183-325]
Platelet distribution width, %	16.69 [16.1-17.025]
Mean platelet volume, fL	8.03 [7.4-8.2]
Aspartate aminotransferase, IU/L	24.07 [19-26]
Alanine aminotransferase, IU/L	32.13 [20-35]
Blood urea nitrogen, mg/dL	13.51 [10-17.2]
Creatinine, mg/dL	0.78 [0.65-0.84]
Total cholesterol, mg/dL	121.27 [107-135]
Triglyceride, mg/dL	108.67 [76-150]
High-density lipoprotein cholesterol, mg/dL	47.73 [35-50]
Low-density lipoprotein cholesterol, mg/dL	66.87 [42-94]
Glucose, mg/dL	93.07 [82.25-102.25]
Glycated hemoglobin, % of hemoglobin	5.94 [5.4-5.9]
C-reactive protein, mg/L	2.85 [0.56-4.67]
N-terminal pro b-type natriuretic peptide, pg/mL	110.64 [29.47-95.12]
Creatine kinase-MB, ng/mL	1.46 [0.745-2.145]
Troponin T, ng/mL	0.01 [0.004-0.008]

and angiography findings, were enrolled for this study. In addition, ten healthy donors who did not have any medical conditions were enrolled as controls for blood samples. The median age of the healthy donors was 36.4 (IQR, 26-45) years. Six healthy donors were male, and four healthy donors had a history of smoking in the control group.

The baseline characteristics of the patients are shown in Table I. The median age of the patients was 43.27 (IQR, 35-51) years, and all patients had a history of smoking. Patients commonly presented with wounds (9 [60%]), gangrene (8 [53.3%]), claudication (10 [66.7%]), and resting pain (6 [40%]). Three patients [20%] had superficial vein thrombosis. In addition, some patients had hypertension, diabetes, dyslipidemia, atrial fibrillation, and congestive heart failure after disease onset (3 [20%], 3 [20%], 2 [13.3%], 2 [13.3%], and 1 [6.7%], respectively). Laboratory findings, including white blood cell counts, lymphocyte percentages, C-reactive protein levels, and other inflammation-related parameters, were within the reference range (Table II). The angiographic findings and procedural characteristics are shown in Table III. Thirteen patients underwent PTA for critical limb ischemia, and two patients were treated with medications. Most patients had lesions in both limbs (n = 8, 53.3%). All patients who underwent PTA had lesions below the knee. Patients were prescribed cilostazol (n =

Table III. Angiographic characteristics of patients with Buerger's disease.

Variables	Patients (n = 15)
Previous history of PTA*	13 (86.7%)
Lesion Site	
Both limbs	8 (53.3%)
Iliac artery	2 (13.3%)
Femoral artery	5 (33.3%)
Popliteal artery	5 (33.3%)
Below the knee	13 (86.7%)
Tibioperoneal trunk	2 (13.3%)
Anterior tibial artery	10 (66.7%)
Posterior tibial artery	11 (73.3%)
Peroneal artery	3 (20%)

*PTA: Percutaneous Transluminal Angioplasty.

11, 73.3%); statins (n = 13, 86.7%); and peripheral vascular disease agents, such as sulodexide (n = 13, 86.7%), sarpogrelate (n = 11, 73.3%), ginkgo (n = 10, 66.7%), kallidinogenase (n = 7, 46.7%), and beraprost (n = 4, 26.7%) (Table IV).

Cytokine Profiles of Patients with Buerger's Disease

First, we measured the plasma levels of cytokines IL-17, IL-23, IL-6, TNF-α, IFN-γ, IL-4, IL-10, HMGB1, and sRAGE in patients with Buerger's disease. The plasma levels of pro-inflammatory cytokines (TNF-α, IL-6, and HMGB1) were higher in patients than in controls. The levels of Th1 (IFN-γ), Th1 (IL-4), and Th17 (IL-17 and IL-23) cytokines were higher, whereas those of Treg cytokine IL-10 were lower in patients than in controls. The plasma levels of sRAGE, a competitive inhibitor of ligand-RAGE interactions, were lower in patients with Buerger's disease than in controls (Figure 1A).

Next, we compared the mRNA expression of IL-17, RORγt, HMGB1, and RAGE in the PBMCs of patients with Buerger's disease and controls. The mRNA expression of Th17-associated molecules, such as IL-17 and RORγt, was significantly higher in patients than in controls. Furthermore, the mRNA expression of pro-inflammatory cytokine-associated molecules, including HMGB1 and RAGE, was significantly higher in patients than in controls. Therefore, the mRNA expression of IL-17, RORγt, HMGB1, and RAGE was positively correlated with symptom severity in patients with Buerger's disease (Figure 1B). Increased levels of IL-17 and IL-23 also suggest that autoimmune mechanisms are involved in Buerger's disease development. Additionally, increased production of HMGB1 cytokines in patients could be reflected in the vascular inflammatory response.

Table IV. Angiographic characteristics of patients with Buerger's disease.

Variables	Patients (n = 15)
Aspirin	7 (46.7%)
Clopidogrel	5 (33.3%)
Cilostazol	11 (73.3%)
Sarpogrelate	11 (73.3%)
Angiotensin II receptor blockers	1 (6.7%)
Calcium channel blockers	2 (13.3%)
Nitrates	2 (13.3%)
β-Blockers	1 (6.7%)
Statins	13 (86.7%)
Vasodilators	1 (6.7%)
Sulodexide	13 (86.7%)
Ginkgo	10 (66.7%)
Kallidinogenase	7 (46.7%)
Beraprost	4 (26.7%)

Helper T Cell Subtypes Involved in Buerger's Disease

To investigate the helper T cell subtypes involved in Buerger's disease, IFN-γ-expressing (mainly Th1), IL-4-expressing (Th2), IL-17-expressing (Th17), and $CD25^{high}$ $Foxp3^{+}$ (Treg) $CD4^{+}$ T cells were isolated from the PBMCs of patients with Buerger's disease and controls. Th1, Th2, and Th17 cell populations were higher and Treg cell populations were lower in the patients, than in controls (Figure 2). These results indicate that the number of pathogenic T cells had increased markedly, but that of immune-regulatory T cells had decreased, in patients with Buerger's disease.

Interaction Between IL-17 and HMGB1 in Buerger's Disease

We investigated whether HMGB1 and IL-17 interact with each other in patients with Buerger's disease. To study the induction of HMGB1 and RAGE expression by IL-17, PBMCs were treated with IL-17 (10 ng/mL for 72 h). Interestingly, the mRNA expression of HMGB1 and RAGE was strongly induced by IL-17 in the PBMCs of patients compared to the controls (Figure. 3A).

To determine whether HMGB1 treatment can promote IL-17 and RORγt production in the PBMCs of patients, PBMCs were cultured with 100 or 500 ng/mL HMGB1 for 72 h. We noted that IL-17 production was significantly increased by HMGB1 treatment in a dose-dependent manner (Figure 3B). Moreover, the mRNA expression of Th17-associated molecules, including IL-17 and RORγt, also increased with HMGB1 treatment in a dose-dependent manner (Figure 3C). These results indicate that HMGB1 treatment promotes the expression of IL-17 and Th17-associated transcription molecules in patients with Buerger's disease. This suggests that the suppression of HMGB1 by IL-17 regulation may modulate the progression of Buerger's disease.

Role of ADAM10 in Buerger's Disease

We examined whether ADAM10 inhibits the interaction between HMGB1 and IL-17. To this end, we investigated how HMGB1-associated molecules change depending on the presence or absence of ADAM10 in patients. HMGB1 and sRAGE levels decreased with ADAM10 treat-

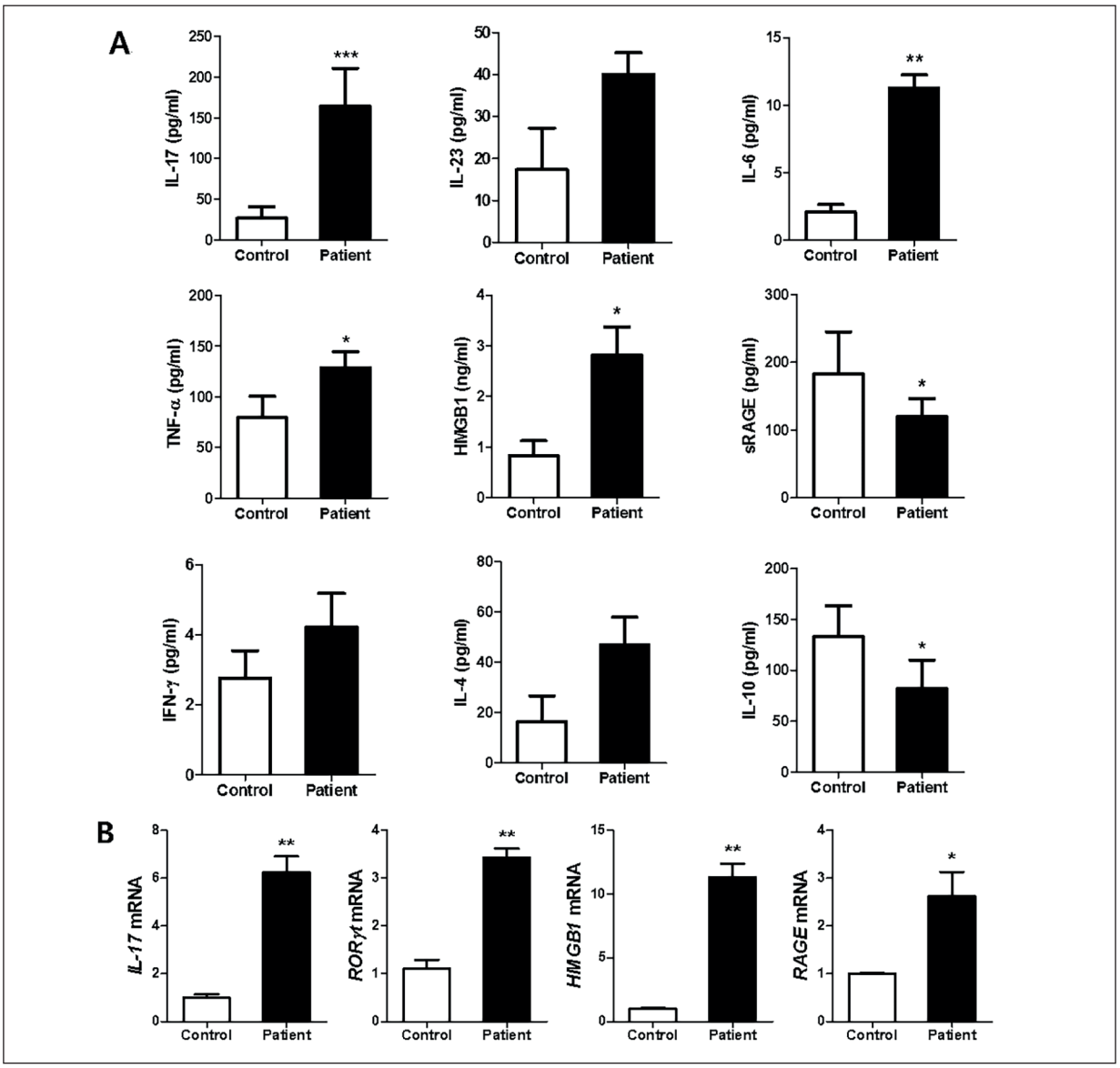

Figure 1. Cytokine profiles in patients with Buerger's disease. **A,** Plasma levels of IL-17, IL-23, IL-6, TNF-α, IFN-γ, IL-4, IL-10, HMGB1 and sRAGE cytokines measured in each group. **B,** Expression of IL-17, RORγt, HMGB1 and RAGE was determined using quantitative real-time PCR in PBMCs obtained from each group and normalized to β-actin expression. Data are presented as the mean ± SD of two independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to healthy control.

ment compared with no treatment (Figure 4A). Additionally, treatment with ADAM10 decreased HMGB1, RAGE, and TBXA2R mRNA levels (Figure 4B).

To confirm whether the presence of ADAM10 inhibits IL-17 promoted by HMGB1, isolated PBMCs from patients were pretreated with 125 ng/mL of ADAM10 for 2 h, and then treated with HMGB1 (500 ng/mL). HMGB1 treatment significantly increased IL-17 and IL-23 levels in patient PBMCs, which were normalized by ADAM10 treatment. Likewise, IL-17 and RORγt mRNA levels were increased by HMGB1 treatment and normalized by ADAM10 treatment (Figure 4C).

To investigate the inhibitory effect of ADAM10 on NF-κB, p38, and ERK phosphorylation, western blot analysis was performed using PBMCs from patients. Treatment with ADAM10 significantly suppressed the phosphorylation of NF-κB, while the total amount of NF-κB remained unchanged. Phosphorylation of P38 and ERK was also significantly reduced by ADAM10

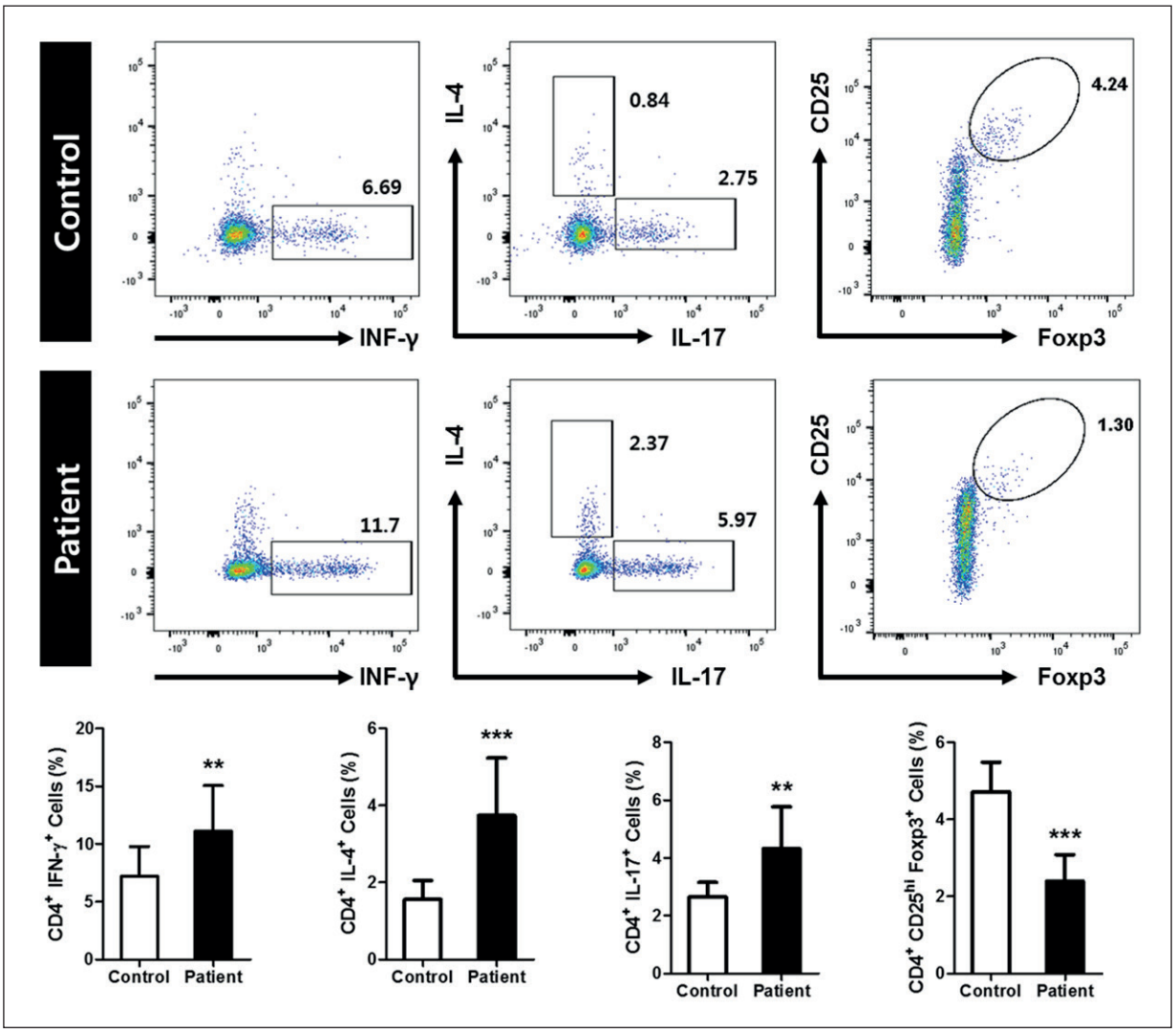

Figure 2. Comparison of helper T cell subtypes in Buerger's disease. A, PBMCs were collected from each group, and CD4+ cell populations were analyzed for IFN-γ (Th1), IL-4 (Th2), IL-17 (Th17), and CD25 high, Foxp3+ (Treg) expression. The FACS plots show representative data. **B,** Graphs depict the percentage of T cell subsets remaining in the CD4+ gated population. Data are presented as the mean ± SD of three independent experiments *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to healthy control.

treatment, whereas the total amount of these proteins remained unchanged compared to that in the untreated cells (Figure 4D). These results indicate that ADAM10 inhibits the interaction of HMGB1 and IL-17 through the NFκB, P38, and ERK pathways in Buerger's disease.

Discussion

The present study reported three main findings. Firstly, we observed that the increased levels of inflammatory cytokines, and increased population of Th cells, are important markers of Buerger's disease in Korean patients. The levels of pro-inflammatory cytokines (IL-17, IL-23, IL-6, TNF-α, IFN-γ, and HMGB1) were higher in patients with Buerger's disease than in controls. The levels of Th1 (IFN-γ), Th1 (IL-4), and Th17 (IL-17 and IL-23) cytokines were also higher in patients with Buerger's disease, than in healthy controls.

Secondly, we measured the mRNA levels of HMGB1, RORγt, RAGE, and IL-17 in PBMCs from patients with Buerger's disease using quantitative real-time PCR. We found that the mRNA levels of HMGB1, RORγt, RAGE, and IL-17 were higher in patients than in controls.

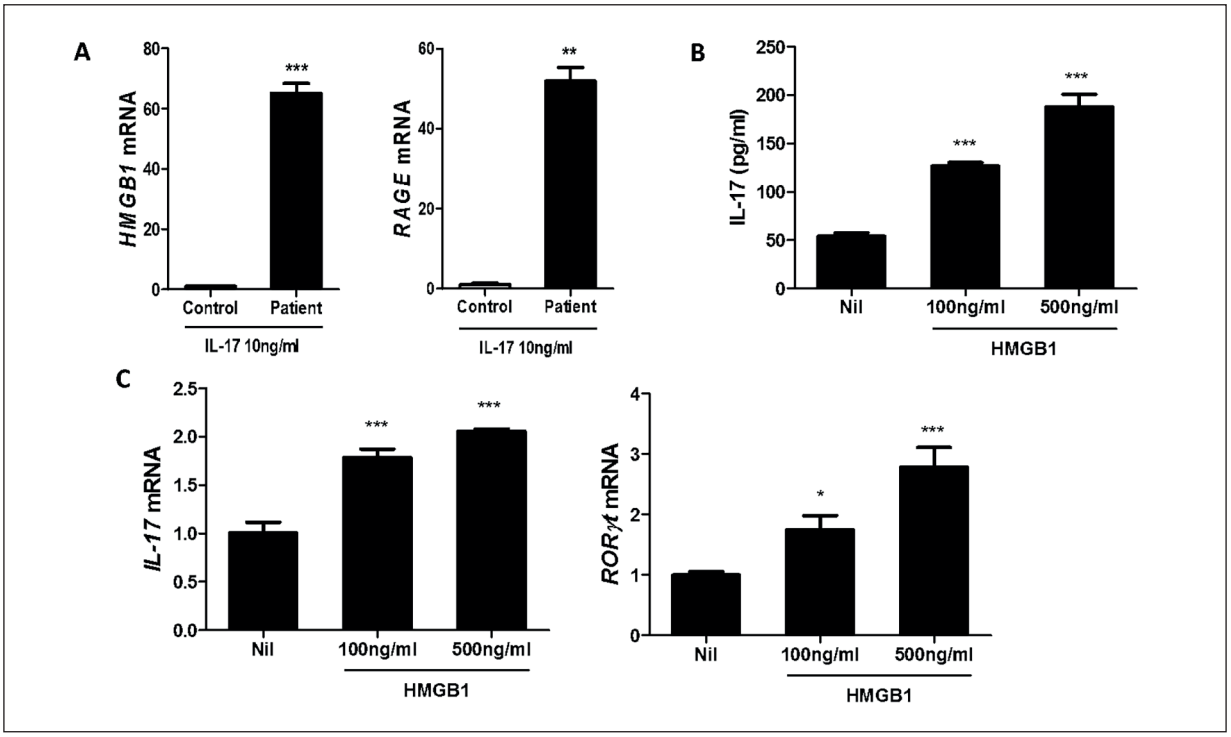

Figure 3. The mutual induction of HMGB1 and IL-17 in patients with Buerger's disease. **A,** The mRNA expression of HMGB1 and RAGE in PBMCs was measured by quantitative real-time PCR. **B,** The protein levels of IL-17 in PBMCs of Buerger's disease patients were measured by ELISA (n = 3). **C,** The mRNA expression of IL-17 and RORγt in PBMCs of patients was measured by quantitative real-time PCR (n = 3). Data are presented as the mean ± SD of three independent experiments *$p <$ 0.05, ** $p < 0.01$, *** $p < 0.001$ compared to control.

The mRNA expression of IL-17 and RORγt produced by CD4(+) T cells (Th17) increased with 100 or 500 ng/mL HMGB1 treatment for 72 h in a dose-dependent manner, which indicated that the increased levels of HMGB1 might contribute to Th 17 cell activation in Buerger's disease. HMGB1/RAGE signaling may promote the differentiation and maturation of Th 17 cells, increase the number of Th 17 cells, and increase the secretion of IL-17 in patients. This study suggests that T cell-mediated immunity plays a role in the pathogenesis of Buerger's disease. It is likely that endothelial cell injury is related to early arterial occlusion through an immunological mechanism.

Thirdly, this study demonstrated that ADAM10 attenuates the mutual induction of HMGB1 and IL-17 via the NFκB, P38, and ERK pathways at both the mRNA and protein levels. These results suggest that ADAM10 alleviates endothelial inflammation in Buerger's disease via the HMGB1 and RAGE/NF-κB signaling pathways. Thus, our study provides insights into the molecular basis of Buerger's disease, as well as a potential therapeutic strategy to treat it.

The precise cause and course of development of Buerger's disease have not yet been elucidated, and different hypotheses have been put forward. A reaction to the constituents of cigarettes is recognized as a factor in the initiation, progression, and prognosis of this disease. Genetic modifications or autoimmune disorders may also be implicated. The direct action of inflammatory mediators has been observed in the vascular endothelium of patients [13-15]. In this study, we evaluated cytokine profiles and populations of T helper cell subtypes in the plasma of Korean patients with Buerger's disease. Our study revealed that the levels of most pro-inflammatory cytokines were higher than normal in patients with Buerger's disease. Immune pathway-specific therapies targeting systemic inflammation may be used alongside topical treatments, such as PTA and bypass surgery.

IL-17-producing T cells have been classified as a new effector T cell subset called Th17, which is distinct from Th1, Th2, and Treg subsets. There has been much progress in the past year, in the identification of the molecular mechanisms that drive the differentiation of Th17 T cells. This

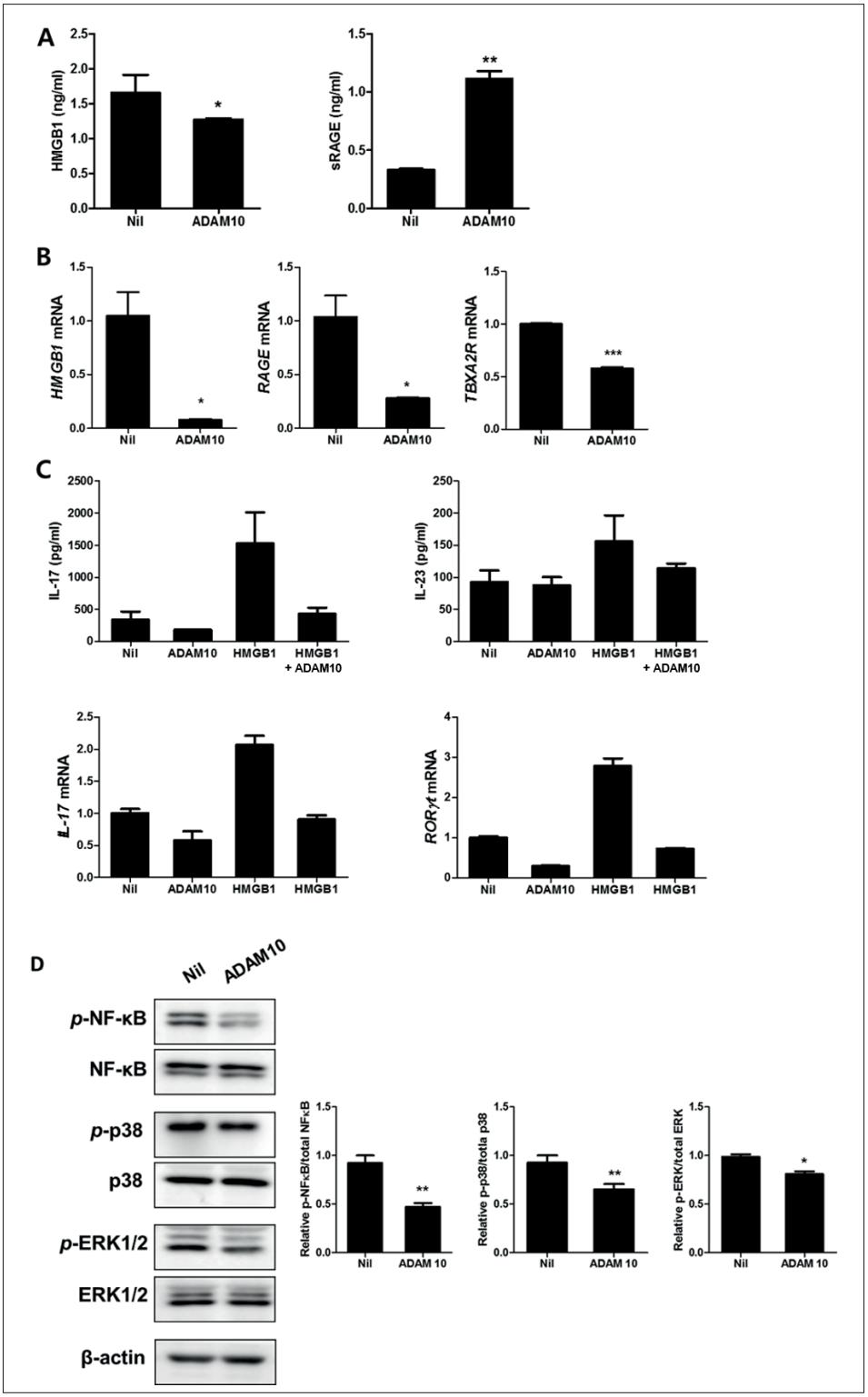

Figure 4. Effect of ADAM10 on patients with Buerger's disease *in vitro*. **A,** The levels of HMGB1 and sRAGE in cell culture supernatant of patients with Buerger's disease were evaluated by ELISA. **B,** The mRNA expression of HMGB1, RAGE, and TBXA2R in PBMCs of patients with Buerger's disease was analyzed by quantitative real-time PCR. **C,** IL-17 and IL-23 levels were measured by ELISA (Top). The mRNA levels of IL-17 and RORγt were evaluated by quantitative real-time PCR (bottom). **D,** Isolated PBMCs were cultured in the absence or presence of ADAM10. The expression of NFκB, P38 and ERK signaling molecules was determined by Western blotting. Graphs on the right depict the relative protein levels. Data are presented as the mean ± SD of three independent experiments $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to control.

has helped clarify many aspects of their role in host defense as well as autoimmunity[16]. Th17 cells are a subset of T helper cells, that play a key role in inflammation by activating neutrophils and enhancing the production of inducible nitric oxide synthase by neutrophils[17]. It has been demonstrated that Th17 cells can play a role in autoimmune diseases[18]. The most significant finding in this study was the reciprocal activity of HMGB1 and IL-17 in patients with Buerger's disease. The pro-inflammatory effects of HMGB1 are believed to be mediated by RAGE. Previous studies have shown that increased HMGB1 levels play a key role in vascular lesions in patients with Buerger's disease[9]. IL-17 induces the secretion of proinflammatory cytokines, such as IL-6 and TNF-α from macrophages. These pro-inflammatory cytokines in turn form a positive feedback loop with IL-17, thereby amplifying its effects[19-21]. The increased levels of IL-17 and IL-23 suggest that the alterations noted in Buerger's disease are mediated via autoimmunity[22]. Shi et al[23] demonstrated a strong association between Th17 cell activity and HMGB1 in patients with rheumatoid arthritis, an autoimmune disease. However, to the best of our knowledge, studies probing the interaction between HMGB1 and IL-17 in Buerger's disease have not yet been conducted. In this study, we discovered the inflammatory function of HMGB1, which induces elevation of IL-17 levels in the PBMCs of patients with Buerger's disease.

Our findings demonstrate that ADAM10 inhibits the expression of HMGB1 and RAGE in PBMCs of patients with Buerger's disease. ADAM10 is a sheddase of RAGE and is a membrane protease responsible for RAGE cleavage[24]. Two RAGE isoforms have been reported: fl-RAGE, which is located on the cell membrane and plays a role in signal transduction, and s-RAGE, which is derived from cleaved RAGE and does not participate in cellular signaling[25,26]. Although RAGE shedding is enhanced by binding of the ligand HMGB1, it has been speculated that ADAM10 increases s-RAGE levels, inhibiting the HMGB1/RAGE signaling pathway. Additionally, TBXA2R is a potent stimulator of platelet aggregation[27]. Thus, reduced TBXA2R levels following ADAM10 treatment may be a key factor leading to the anticoagulation of vascular occlusion in patients with Buerger's disease.

Activation of the NF-κB, p38 MAPK, and ERK pathways increases expression of inflammatory cytokines and is associated with several inflammatory diseases, including Buerger's disease[28-30]. ADAM10 treatment suppressed NF-κB, p38, and ERK phosphorylation in patients with Buerger's disease. This indicates that ADAM10 treatment inhibits the interaction between HMGB1 and IL-17 by acting on the NF-κB, p38 MAPK, and ERK pathways. Although inflammatory cytokine levels are elevated in patients with Buerger's disease, further studies are needed to correlate cytokine levels with disease severity. Although HMGB1 and IL-17 facilitate inflammation in this disease, further studies are needed to determine whether they play a role in IL-17-producing CD4+ T helper cell (Th17) differentiation.

We validated the results of a previous study that demonstrated the efficacy of ADAM10 in the treatment of Buerger's disease, using sodium laurate-induced thromboangiitis obliterans rat models[12]. In our study, the therapeutic effect and the mechanism of action of ADAM10 were demonstrated in patients *in vitro*. Furthermore, we used a relatively small number of samples to perform the *in vitro* assay, to determine the effect of inhibiting the reciprocal activity of HMGB1 and IL-17 via ADAM10 treatment. To our knowledge, this is the first study to report the pathogenic mechanism of Buerger's disease. However, to further validate our hypothesis, further studies involving a larger number of participants, as well as *in vivo* experiments, are necessary.

This study had several limitations. Firstly, the inflammatory profile status is not exclusive to Burger's disease. In other diseases, where damage to the vascular wall is recognized, proinflammatory and anti-inflammatory cytokines have also been described for other types of vasculitis. Secondly, Buerger's disease is rare; therefore, the sample size in this study was relatively small. Thirdly, although HMGB1 has the potential to induce IL-17 expression and elevate its serum levels in patients with Buerger's disease, *in vivo* animal studies are needed to confirm the inflammatory effects of HMGB1 treatment. Some animal models are required to further demonstrate that HMGB1 results in an increase in IL-17 expression. Moreover, *in vitro* assays probing the upregulation of proinflammatory cytokines through HMGB1 treatment were performed using a relatively small number of samples. However, this investigation is the first to report the possible pathogenic role of HMGB1 and IL-17 in Buerger's disease. Future studies with a larger sample size and *in vivo* animal experiments are required to verify our hypothesis.

Conclusions

Our results indicate that IL-17 and HMGB1 cytokines contribute to the pathogenesis of Buerger's disease, and ADAM10 alleviates inflammation in Buerger's disease via the HMGB1 and RAGE/NF-κB signaling pathways. This study provides insights into the molecular basis and potential therapeutic strategies for this condition.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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