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Original Article

Characterization and functional elucidation of CD8+AT2+ lymphocytes in human thoracic aortic aneurysm

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Abstract: Human thoracic aortic aneurysm (TAA) is one of the most fatal cardiovascular diseases. However, the underlying molecular mechanism behind this disease remains uncertain. Previous studies have proven the importance of angiotensin II receptor type 2 (AT2) and endothelial cells in aneurysm pathology. This study aimed to elucidate the effect of CD8+AT2+ lymphocytes in TAA pathogenesis. The expression levels of CD8, AT2, IL-2, and IFN-r were determined by real-time quantitative PCR. CD8+AT2+ lymphocytes were counted by semi-quantitative immunofluorescence and flow cytometric analysis. The wound-healing assay was used to detect endothelial cell migration. The expression levels of CD8 and AT2 in human nonsyndromic TAA tissues were significantly higher than those of the control. TAA tissues have more CD8+AT2+ lymphocytes than the controls. Furthermore, circulating CD8+AT2+ lymphocytes were significantly elevated in TAA patients. The expression levels of IL-2 and IFN-r in CD8+AT2+ lymphocytes were significantly increased compared with those in CD8+AT2- lymphocytes. The CD8+AT2+ lymphocytes had a greater inhibitory effect on endothelial migration compared with CD8+AT2- cells. Our findings showed that the increment of CD8+AT2+ lymphocytes in human TAA exhibited a protective effect by downregulating the release of pro-inflammation cytokines and the inhibition of endothelial cell migration.

Keywords: Angiotensin II type 2 receptor, thoracic aortic aneurysm, endothelial cell, cytokine, inflammation

Introduction

Thoracic aortic aneurysm (TAA) is a life-threatening disease characterized by progressive dilatation of the thoracic aorta. Usually, the disease continues to develop without any symptoms until the terminal stage, which is detected by computed tomographic scans. Patients with TAA die because of aorta rupture [1]. Unfortunately, the current standard therapeutic options are limited and include open surgical replacement or endovascular aortic repair [2]. Pharmaceutical treatment is only applied preoperationally for surgical preparation [3]. Therefore, molecular pathogenesis is necessary for pharmacotherapy.

The pathogenesis of aneurysms is well known to involve tissue remodeling and chronic inflammation. Aneurysmal arteries are characterized by leukocyte infiltrates, particularly lymphocytes that interact with each other to elicit immune responses and vascular disease [4]. Lymphocytes facilitate pathological changes through release of cytokines and endothelial activation [5]. Evidence shows that endothelial cells (ECs) may trigger macrophage infiltration, as well as inflammation in the adventitia and media by upregulating the expression of adhesion molecules [6]. Naturally, endothelial cells are regularly arranged only in inner layer and secret little cytokines. However, the behavior and function of endothelial such as migration and neoangiogenesis, are proved abnormal in aortic aneurysm [7, 8]. Angiogenesis contributes to the dilation of the aneurysm wall and plays a key role in the development and rupture of aneurysms [9]. Our previous study demonstrated that ECs in aneurysm walls abnormally secreted more Matrix metalloproteinase 2 (MMP2), which is the main cytokine in extracellular matrix degradation [10, 11]. Therefore,

ECs indirectly interfere in the pathological progress of aortic aneurysms.

The chronic infusion of angiotensin II (Ang II) into hypercholesterolemic mice promotes the formation of thoracic aortic aneurysms and augments atherosclerotic lesions [12-15]. Ang II exerts its bioactive effects mainly through binding to AT1 and AT2 receptors [16]. AT2 is sparsely expressed in normal healthy adult tissues but strongly upregulated damage such as myocardial infarction [17]. AT2 exhibits tissue-protective properties by inhibiting inflammation, fibrosis, and apoptosis [16]. We previously identified a new cell population, the CD4+ AT2+ T lymphocytes, in TAA and defined its functions in the inhibition of cell growth and MMP2 expression, and promotion of apoptosis in ECs. In the present study, we found another cell population, the CD8+AT2+ lymphocytes, in TAA tissues. Although CD8+AT2+ lymphocytes have been observed in ischemic heart injury and show cardioprotective functions [18], the role of this cell subpopulation in TAA has not been explored.

Materials and methods

Human aorta specimen

A total of 20 TAA patients participated in the present study. Patients with Marfan syndrome or aortic dissection were excluded. In addition, 20 non-aneurysmal thoracic aortic samples were collected from patients with coronary heart disease undergoing coronary artery bypass surgery. The protocol was approved by the Ethical Committee of Renji Hospital. Informed consent was obtained from all included participants in the study. All the procedures performed in studies involving human participants agreed with the ethical standards of the institutional and/or national research committee, as well as the 1964 Helsinki declaration and its later amendments, or comparable ethical standards.

Immunofluorescence (IF) staining

Aorta tissues for IF staining were fetched from the operation room and immediately delivered to the laboratory. Tissues around the dilated region were cut and frozen for examination. Cryo-tissue section preparation and examination was performed as previously described [10].

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated with TRIzol Reagent (Invitrogen, USA), according to the manufacturer's instructions. Briefly, 1 μg of total RNA was used to synthesize complementary DNA (cDNA) with PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Dalian, China), according to the manufacturer's instructions. RT-qPCR was performed in triplicate with the SYBR Premix Ex TaqII kit (Takara, Dalian, China) on an ABI 7900 system (Applied Biosystems, CA, USA), according to the manufacturer's instructions. The following primer sequences were used: β-actin, 3'-AGAAAATCTGGCACCACACC-5' (forward) and 3'-AGAGGCGTACAGGGATAG-CA-5' (reverse); AT2, 3'-ACTGGCTCTTTGGACC-TGTG-5' (forward) and 3'-GCCATACACCAAACA-AGGGG-5' (reverse); CD8, 3'-GCCAGTGACCAT-TCCGGTA-5' (forward) and 3'-GACCTAGCCTG-GACCTTGGA-5' (reverse); IL-2, 3'-GAATCACGT-TACGTTCTGTCCT-5' (forward) and 3'-CTTAGG-GTTTGAGTGGTCCTAC-5' (reverse); IFN-r 3'-AC-CTGTAAGTTCAGTCAATGGCT-5' (forward) and 3'-CGTCGATTTTGTCCCTTCGCT-5' (reverse). The relative expression levels of CD8, AT2, IL-2, and IFN-r were calculated and normalized by the $2^{-\Delta\Delta Ct}$ method relative to β-actin.

Flow cytometry analysis and sorting

Peripheral blood was collected prior to surgery. Mononuclear cell extraction from blood and its analysis was previously described [10]. After sorting, the cell subpopulations was kept in a mixed liquid for 30 min at 25°C and resuspended for incubation.

Wound-healing assay

To measure endothelial cell migration, the wound-healing assay was performed. ECs were plated on 24-well culture plates. The culture medium contained PromoCell Cell Growth medium (PromoCell) and RPMI 1640 (3:1). After treatment for 3 days, the transwells were removed and the endothelial cell plates were wounded with pipette tips of the same seize. Photographs of the wounded area were taken every 6 h with a microscope (Nikon, Tokyo, Japan). Three different fields from each sample were considered for quantitative estimation of the distance between the borderlines. In each image, three different equidistant points were measured to better estimate the real width of

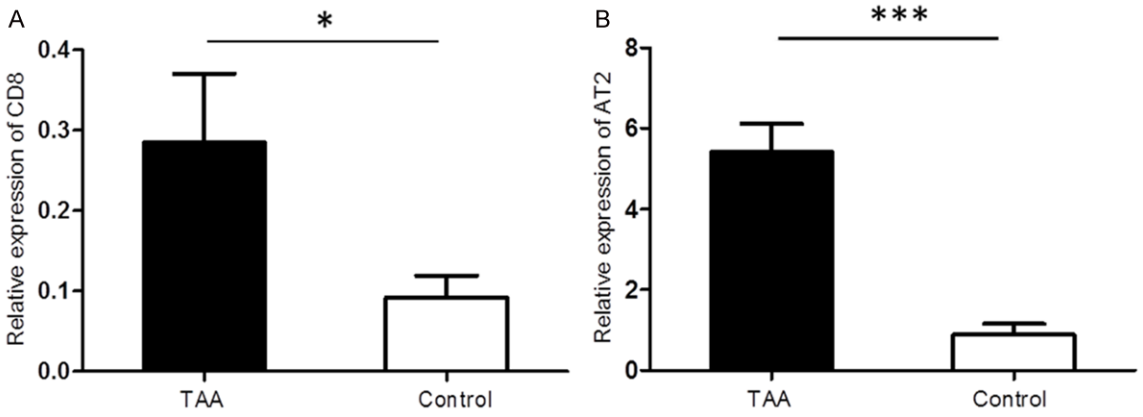

Figure 1. The expression levels of CD8 and AT2 in human TAA tissues. Both CD8 (A) and AT2 (B) were significantly upregulated in TAA tissues. *$P < 0.05$, ***$P < 0.001$.

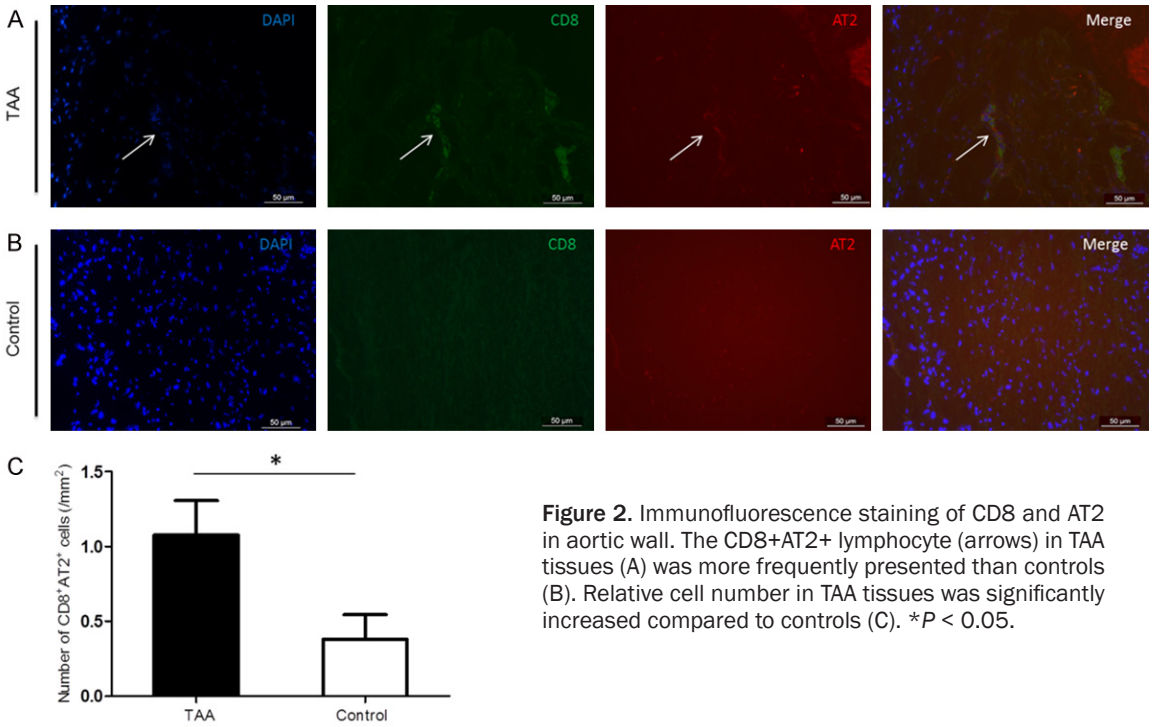

Figure 2. Immunofluorescence staining of CD8 and AT2 in aortic wall. The CD8+AT2+ lymphocyte (arrows) in TAA tissues (A) was more frequently presented than controls (B). Relative cell number in TAA tissues was significantly increased compared to controls (C). *$P < 0.05$.

the wounded area. Three independent series of experiments were performed. The distance of cell migration from the initial wound was measured with the NIS-Elements D software (Nikon, Tokyo, Japan).

Statistical analyses

Data were expressed as mean ± SEM. Comparison between two groups was performed by the two-tailed Student's t-test. Multiple comparisons were analyzed with one-way ANOVA followed by the Bonferroni post hoc test. Differences were considered significant at $P < 0.05$.

Results

Increased CD8+AT2+ lymphocytes in TAA tissues

To investigate the presence of CD8+AT2+ lymphocytes in TAA tissues, we first examined the expression levels of CD8 and AT2 in the aneu-

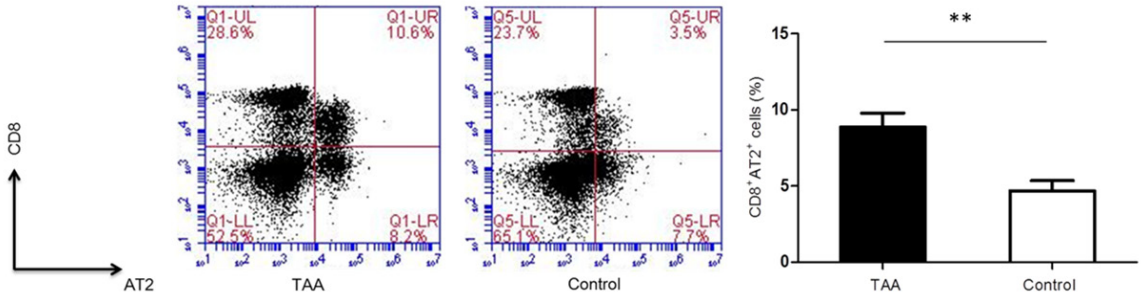

Figure 3. Circulating CD8+AT2+ lymphocyte. The percentage of CD8+AT2+ lymphocyte in TAA patients was significantly increased than those in controls. $**P < 0.01$.

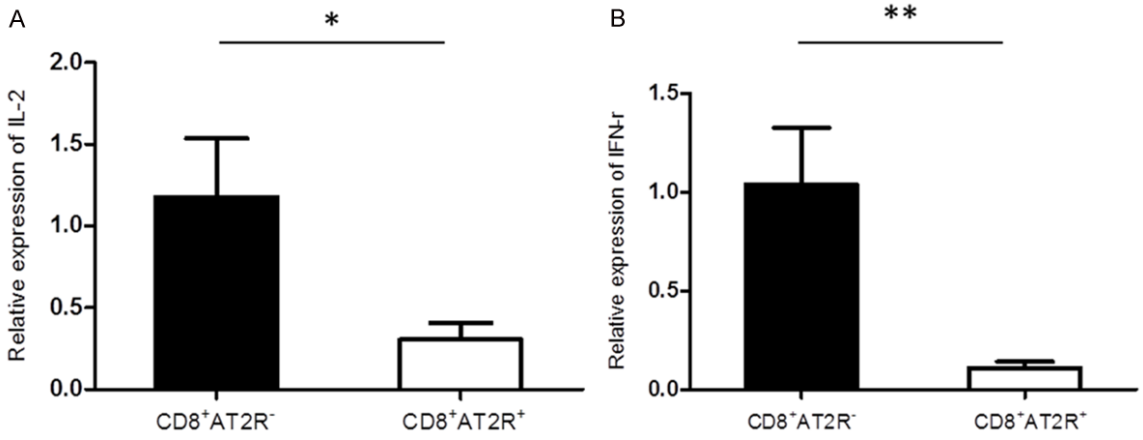

Figure 4. The expression levels of IL-2 (A) and IFN-r (B) in CD8+AT2+ lymphocytes were increased than those in CD8+AT2- lymphocytes. $*P < 0.05$, $**P < 0.01$.

rysmal walls and controls. Both CD8 ($P < 0.05$) and AT2 ($P < 0.001$) were significantly upregulated in TAA tissues compared with the control (**Figure 1**). Furthermore, we examined the protein levels of CD8 and AT2 in the aortic wall by immunofluorescence. CD8+AT2+ lymphocytes were rarely detected in the control. However, more cellular distribution was observed and the CD8+AT2+ lymphocytes had the tendency to cluster in TAA tissues (**Figure 2**). The density of CD8+AT2+ lymphocytes in TAA tissues (1.08 ± 0.23 cells/mm^2, n = 16) was notably increased relative to the control (0.38 ± 0.16 cells/mm^2, $P < 0.05$, n = 16; **Figure 2**).

Increased circulation of CD8+AT2+ lymphocytes in TAA patients

Alteration of peripheral lymphocyte subsets implies the local response of the immune system. The percentage of CD8+AT2+ lymphocytes from PBMCs was 8.87 ± 0.91% (n = 13) in TAA patients, which was significantly higher than that in the control (4.70 ± 0.65%, $P = 0.001$, n = 13; **Figure 3**).

Downregulated IFN-r and IL-2 levels in CD8+AT2+ lymphocytes

Lymphocytes participate in local immune response by releasing pro-inflammation cytokines. We examined the expression levels of IFN-r and IL-2 in CD8+AT2+ and CD8+AT2- lymphocytes. RT-qPCR revealed that the expression levels of IFN-r (n = 6, $P < 0.01$) and IL-2 (n = 6, $P < 0.05$) in CD8+AT2- lymphocytes were significantly higher than those in CD8+AT2+ lymphocytes (**Figure 4**).

Effect of CD8+AT2+ lymphocytes on the migration of aortic ECs

To investigate the effect of CD8+AT2+ lymphocytes on aortic aneurysms, we examined the different migration abilities of aortic ECs co-cultured with CD8+AT2+ lymphocytes, CD8+

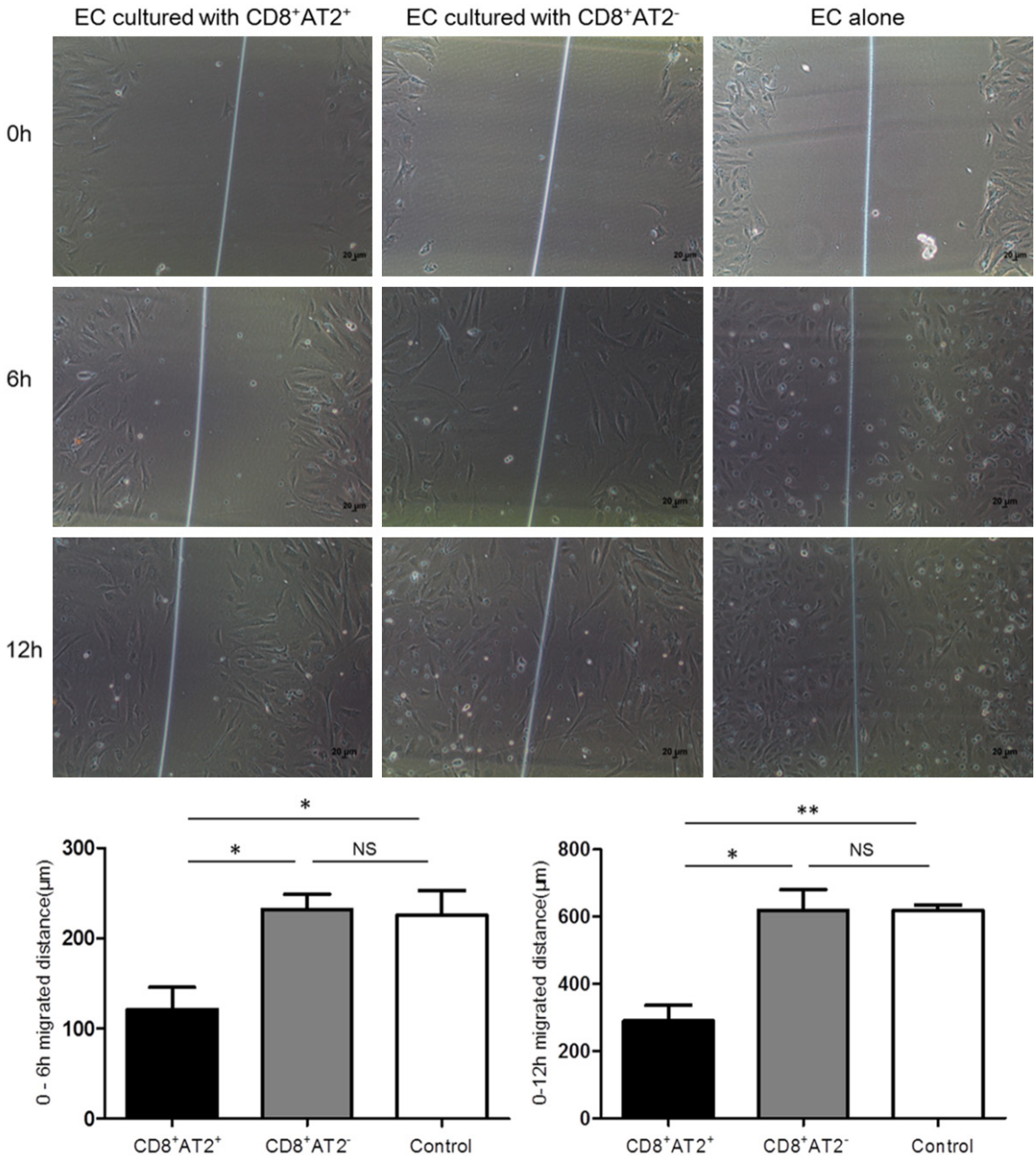

Figure 5. CD8+AT2+ lymphocytes inhibited ECs migration. There was significant difference in migration distance between ECs cocultured with CD8+AT2+ lymphocytes and CD8+AT2- lymphocytes or ECs alone for 6 h and 12 h. *P < 0.05, **P < 0.01.

AT2- lymphocytes, and ECs alone. As shown in **Figure 5**, the distance migrated by ECs with CD8+AT2+ lymphocytes (121 ± 25 μm, n = 3, 6 h; 289 ± 47, n = 3, 12 h) was shorter than either CD8+AT2- lymphocytes (232 ± 16 μm, 6 h, P < 0.05; 619 ± 60 μm, 12 h, P < 0.05) or ECs alone (225 ± 27 μm, 6 h, P < 0.05; 618 ± 17 μm, 12 h, P < 0.01) within 6 or 12 h. However, no statistically significant differences were observed in the migration distance of ECs between the CD8+AT2- lymphocyte and EC groups (n = 3 for each group).

Discussion

Previous studies have clarified the protective role of AT2 in aortic aneurysms of mice and humans [10, 19]. Similarly, our previous studies

identified the role of CD4+AT2+ lymphocytes to slow the dilation of aneurysm rupture. However, the underlying pathogenesis is complicated and has not been fully explored. In this study, we proved that the cell population of CD8+ AT2+ lymphocytes is abundant in TAA aortic wall and the peripheral blood circulation of TAA patients. Similar with CD4+AT2+ lymphocytes, the pro-inflammation cytokines, such as IL-2 and IFN-r, were downregulated in CD8+ AT2+ lymphocytes. Naturally, IL-2 and IFN-r are both released by CD8+ lymphocytes and further stimulate lymphocyte proliferation, thereby leading to inflammation cascades, cell infiltration, and MMP production [20, 21]. IFN-r may activate NK cells and upregulate their killing ability to cause direct tissue damage. Compared with CD8+AT2- lymphocytes, CD8+AT2+ lymphocytes secreted low levels of IL-2 and IFN-r, thereby indicating that the anti-inflammation effect of CD8+AT2+ lymphocytes may be mediated by the AT2 pathway. Although the level of CD8+AT2+ lymphocytes in both peripheral blood circulation and aortic wall is increased, it is not clear where the cell subpopulation originate from. The underlying mechanism of elevated level of CD8+AT2+ lymphocytes in TAA requires further investigation.

Another important result of our study is the inhibition of endothelial migration by CD8+AT2+ lymphocytes. Aortic ECs play an important role in the dilation of aneurysmal wall [10]. ECs can secrete MMP2 and inflammation cytokines to hydrolyze the aortic media and cause invasive migration [10, 11]. Endothelial cell migration within the vessels is marked by cell rearrangement, which was clearly demonstrated in our previous study [10, 22]. Therefore, EC-related neoangiogenesis could contribute to the final breakdown of the aortic wall [23]. The wound-healing assay reflects the motility and proliferation of ECs [22]. Our study indicated that CD8+ AT2+ lymphocytes can dramatically inhibit EC migration. We concluded that the EC invasion into the aortic media could be significantly prevented by increasing the number of CD8+ AT2+ lymphocytes. EC disorder is one of the main causes of immune cell chemotaxis and aggregation. Therefore, the inflammatory reaction may be notably reduced by EC inhibition. Although the underlying mechanism of inhibition remains unclear, the effect of CD8+AT2+ lymphocytes on ECs may prevent media breakdown and protect the structure of the aortic wall, thereby slowing the TAA progression.

In conclusion, the findings of the present study provide the first evidence that CD8+AT2+ lymphocytes are present in the TAA wall and the blood circulation of TAA patients. CD8+AT2+ lymphocytes play a protective role in TAA through inhibition of EC migration and release of inflammation cytokines. However, further studies are required to elucidate the exact mechanism of the effect of CD8+AT2+ lymphocytes on ECs.

Disclosure of conflict of interest

None.

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