Angiotensin II Promotes the Development of Carotid Atherosclerosis in Type 2 Diabetes Patients via Regulating the T Cells Activities: A Cohort Study

Background: Specific T cell phenotype has been reported to potentially contribute to the development of angiotensin II (Ang II)-induced several vascular disorders. Type 2 diabetes mellitus (T2DM) is intimately associated with cardiovascular disease. The present study aimed to investigate the relationship between T cell phenotypes and Ang II in T2DM patients combined with carotid atherosclerosis (CA).

Material/Methods: This study was performed on 50 patients with T2DM in our hospital. Based on the presence of CA, they were divided into CA group (presence of CA, n=30) or T2DM group (absence of CA, n=20). Additionally, 10 healthy participants were selected as controls. Basic characteristics of all participants were collected and recorded. Peripheral blood mononuclear cells (PBMCs) isolated from patients and controls with or without Ang II and Ang II receptor blocker (ARB) treatment were used to detect Th1, Th2, and Th17 cell proportions, mRNA levels of T-bet, GATA3, and ROR$\gamma$ as well as the expression of IFN-$\gamma$, IL-4, and IL-17 by flow cytometry, ELISA, and Real-Time PCR.

Results: Ang II levels were notably higher in patients in the CA group than those in the T2DM and control group ($p<0.05$). Th1 and Th17 positive cells, mRNA levels of T-bet and ROR$\gamma$ as well as the expression of IFN-$\gamma$ and IL-17 were significantly increased in the CA group compared with the T2DM group and control group ($p<0.05$). Moreover, the activities of T cells and related cytokines were significantly increased of healthy controls after Ang II treatment ($p<0.05$), while these changes were notably weakened by ARB treatment ($p<0.05$).

Conclusions: Ang II promotes the development of CA in T2DM patients by regulating T cells activities.

MeSH Keywords: Angiotensin II • Carotid Artery Diseases • Diabetes Mellitus, Type 2

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Background

Type 2 diabetes mellitus (T2DM) refers to inadequate insulin secretion and impaired insulin sensitivity of several tissues [1]. Nowadays, the prevalence and incidence of T2DM continue to increase worldwide and the economic burden for health care in treating diabetes as well as its related complications is quite large [2]. T2DM is intimately associated with peripheral vascular disease, cerebrovascular disease, coronary artery disease [3], and sclerosing mesenteritis [4]. Cardiovascular disease has been reported to account for 70% to 75% of total diabetes-related mortality [5], and the increased cardiovascular risk in T2DM is reflected by the presence of atherosclerosis [6]. Glycemic management for T2DM patients has become complex and controversial due to mounting concerns about the potential adverse effects and new uncertainties of the pharmacological agents on macrovascular complications [7]. Therefore, investigation for the mechanism of arteriosclerosis in T2DM is a key challenge and necessary to be conducted.

It is well accepted that the development of atherosclerosis is affected by genetic, environmental, and epigenetic factors. Cardiovascular genetics has led to numerous clinical studies focusing on candidate genes. A review conducted by Starčević et al. discussed the cardiovascular disease risk genes involved in lipid metabolism and statin therapy [8]. By genetic and epigenetic fine mapping, Turner et al. identified a causal coronary artery disease-associated single nucleotide polymorphism rs17293632C>T, which is associated with protection from coronary artery disease [9]. But gene polymorphisms such as the interleukin-1 receptor antagonist gene and the inhibitor of xB protein gene polymorphisms have been demonstrated to be not associated with myocardial infarction in patients with T2DM [10]. In addition, Reynolds et al. focused on the associations of epigenetic modification-DNA methylation with cigarette smoking and subclinical atherosclerosis and they indicated that the aryl hydrocarbon receptor repressor (AHRR) methylation may be functionally related to AHRR expression in monocytes and represents a potential biomarker of subclinical atherosclerosis in smokers [11]. Furthermore, Declerck et al. give an overview of epigenetic alterations in atherosclerosis, mainly focusing on DNA and histone modifications [12]. Immune disorders are reported to be associated with the development of T2DM and its complications [13]. T lymphocytes can be divided into several subsets and subtypes, which produce various inflammation-related factors responding to the infection and immune reaction [14]. It has been reported that B cells promote inflammation in T2DM through regulating all helper CD4+ T-cell subsets including Th1, Th2, Th17, and Tregs activities and a number of related inflammatory cytokine secretion [15]. Adaptive immune cytokines including IFN-γ, IL-4, and IL-17 were secreted by polarized Th subsets (Th1, Th2, and Th17, respectively) mounting an immune response involved in reciprocal activation of eosinophils and macrophages [16]. Recently, the occurrence and development of atherosclerosis has been identified to be related to the predominance of Th1 cells and the imbalance of Th1/Th2 [17]. Angiotensin II (Ang II) is one of the most potent mitogens that induce monocytes and macrophages entering into vessel walls to cause inflammatory responses in vascular smooth muscle cells [18]. Meanwhile, Ang II has been reported to have several important effects on the pathogenesis of atherosclerosis [19]. Furthermore, increasing evidences have identified that specific T cell phenotype potentially contribute to the development of Ang II-induced hypertension and several other vascular disorders [14,20]. Accordingly, it was hypothesized that Ang II might be associated with the development of atherosclerosis in T2DM patients via regulating specific T cell phenotypes.

To confirm whether Ang II level contributes to the development of carotid atherosclerosis (CA) in T2DM patients via regulation of specific T cell phenotypes, we investigated the relationship between T cell phenotypes and Ang II levels in T2DM patients combined with or without CA. Meanwhile, the effects of Ang II on T cell activities were further confirmed using peripheral blood mononuclear cells (PBMCs) cells obtained from health controls after Ang II only or Ang II plus Ang II receptor blocker (ARB) treatment.

Material and Methods

Patients

Between September 2014 and June 2015, 57 patients with T2DM presented to the affiliated hospital of Inner Mongolia Medical University. T2DM was diagnosed in patients as blood glucose levels >200 mg/dl, glycated hemoglobin (HbA1c) >6.5% as recommended by the American Diabetes Association criteria [21] or use of diabetic medication. If patients had one of the following: 1) presence of hypertension; 2) with cardiovascular disease like coronary heart disease and congestive heart failure; 3) presence of acute or chronic inflammation, or having inflammation-suppressing drugs such as nonsteroidal anti-inflammatory drugs and corticosteroids before; 4) with history of stroke; 5) with hepatic and/or renal dysfunction; 6) with cancer and/or rheumatic diseases, they would be not included in the present study. After excluding seven patients for presence of hypertension (three cases), congestive heart failure (one case), chronic inflammation (two cases) and with a history of stroke (one case), a total of 50 patients diagnosed with T2DM were enrolled in the present study. The average duration of T2DM was 6.4 years of these included patients. According to the presence of CA evaluated by the carotid ultrasound, patients were assigned to CA group (patients with...
T2DM combined with CA, n=30) or T2DM group (patients with T2DM, n=20). Meanwhile, 10 healthy participants were selected as a control group. Basic demographic and clinical data of all participants in terms of age, gender, systolic blood pressure (SBP), diastolic blood pressure (DBP), history of hypertension, total cholesterol (TC) level, triglycerides (TG) level, low-density lipoprotein-cholesterol (LDL-C) level, high-density lipoprotein-cholesterol (HDL-C) level, and smoking were collected and recorded after a standard interview and clinical examination. Informed consent was obtained from all of the participants and the Research Ethics Committee of the affiliated hospital of Inner Mongolia Medical University approved the present study.

Before this study, a preliminary experiment was performed on Th1 proportion between T2DM patients combined with CA and those without CA to estimate the sample size. In the preliminary experiments, the Th1 proportion in T2DM patients combined with CA and patients without CA was 20% and 15%, and the standard deviation values were both 0.05. To detect the difference in Th1 proportion between T2DM patients with and without CA with a two-sided 5% significance and a power of 80%, a sample size of 21 patients per group was necessary. Considering the incidence of CA in T2DM patients of about 65% in our hospital and total of 53 patients with T2DM should be recruited. However, due to recruitment difficulties and the restriction of inclusion criteria, the sample size was reduced to 50, with 30 patients in the CA group and 20 patients in the T2DM group.

**Blood sampling**

After 12-hour fasting, venous blood samples were collected from median cubital vein from each participant. A portion of the blood samples was sent to assess plasma glucose concentration and the levels of TC, TG, LDL-C, and HDL-C. The remaining blood samples were divided into two aliquots and placed into anticoagulant tube containing heparin sodium. The blood in the upper layer was used for isolation of peripheral blood mononuclear cells (PBMCs). Meanwhile, the PBMCs obtained from the healthy controls were divided into eight aliquots and these cells were treated with 0, 0.1, 1, and 10 μM Ang II combined with or without 10 μg/mL losartan potassium tablets (one kind of ARB; Merck Sharp & Dohme, UK) for 48 hours.

**Flow cytometry for determination of Th1, Th2, and Th17 cell proportions**

The PBMCs were seeded into 24-well plate at a density of 2×10^5 cells/well. After culture for 24 hours, cells were treated with 25 ng/mL polymethyl acrylate (PMA), 50 ng/mL ionomycin and 1 μL monensin was added into each wells and placed at 37°C in 5% CO_2 atmosphere for another four hours. Cells were harvested by centrifugation at the speed of 300 rpm/minutes for 10 minutes. After blocking with rabbit serum for 30 minutes, the cells were incubated with mouse anti-human CD4-PE antibody for 20 minutes in the dark. After washed with PBS, cells were fixed with 200 μL fixative solution for 10 minutes and permeated using 1 mL rupture solution for 10 minutes. Then cells were incubated with mouse anti-human FITC-conjugated IFN-γ, IL-4, and IL-17 antibodies (eBioscience, San Diego, CA, USA), respectively, in the dark for 20 minutes. Finally, proportions of Th1 (positive with FITC-IFN-γ), Th2 (positive with FITC-IL-4), and Th17 (positive with FITC-IL-17) cells were determined by flow cytometry.

**Real-time reverse transcription polymerase chain reaction (RT-PCR)**

Total RNA was extracted from PBMCs using TRIzol reagent and reverse transcription into cDNA by using cDNA synthesis kit (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. Real-time PCR (RT-PCR) was performed utilizing a SYBR Green Mix (TaKaRa, Shiga, Japan) on iQ5 Detection System (Bio-Rad, Hercules, CA, USA). The procedure was as follows: 95°C for four minutes; 45 cycles of 95°C for five seconds and 62°C for 30 seconds; 1 μL monensin was added into each wells and placed at 37°C in 5% CO_2 atmosphere for another four hours. Cells were harvested by centrifugation at the speed of 300 rpm/minutes for 10 minutes. After blocking with rabbit serum for 30 minutes, the cells were incubated with mouse anti-human CD4-PE antibody for 20 minutes in the dark. After washed with PBS, cells were fixed with 200 μL fixative solution for 10 minutes and permeated using 1 mL rupture solution for 10 minutes. Then cells were incubated with mouse anti-human FITC-conjugated IFN-γ, IL-4, and IL-17 antibodies (eBioscience, San Diego, CA, USA), respectively, in the dark for 20 minutes. Finally, proportions of Th1 (positive with FITC-IFN-γ), Th2 (positive with FITC-IL-4), and Th17 (positive with FITC-IL-17) cells were determined by flow cytometry.

**Isolation and culture of PBMCs**

Mononuclear cells were isolated from whole blood cells via Ficoll-Hypaque density-gradient centrifugation at 2,500 rpm/minutes for 10 minutes of Ficoll-Hypaque. Then the monocytes were isolated by magnetic cell sorting using the Monocyte Isolation Kit II (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Flow cytometry was conducted to detect the purity of the PBMCs. Then isolated PBMCs were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Gibco, Grand Island, USA) and supplemented with 2 mM L-glutamine, 100 U/mL penicillin, and 100 g/mL streptomycin in a 37°C, 5% CO_2 incubator.
GATA-3 reverse: 5’-TGCACGCTGGTAGCTCATAC-3’
RORγt forward: 5’-GGGAAAGTCCCAATCCTGA-3’
RORγt reverse: 5’-TCTGATCTTGCCTTCCGACT-3’
Actin forward: 5’- ACTTAGTTGCGTTACACCCTT-3’
Actin reverse: 5’- GTCACCTTCACCGTTCCA-3’

The fold-change of gene expression was normalized to β-actin and calculated using the 2\(^{-ΔΔCt}\) method. Each sample was repeated in triplicates and all the experiments were conducted for three times.

Enzyme-linked immunosorbent assay (ELISA)

The levels of Ang II, IFN-γ, IL-4, and IL-17 in the plasma were detected using ELISA kits (Bender Medsystems, Vienna, Austria) in accordance with the manufacturer’s instructions. After reacted with substrate solution for 30 minutes, the absorbance was measured using a Microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

Statistical analysis

All data were presented as mean ± standard deviation (SD) and analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Differences among different groups were compared using one-way analysis of variance (ANOVA), followed by least significant difference (LSD) test. A value of p<0.05 was considered as significant difference.

### Results

#### Comparison of basic characteristics and Ang II levels among different groups

Basic demographic and clinical data were presented in Table 1. Significant difference was found in terms of age, blood glucose concentration, TC level, and LDL-C level among three groups (p<0.05). Patients in the CA group were obviously older than those in T2DM and control group. There was an obviously higher rate of presence of hypertension in the CA group compared to that in T2DM and control groups (p<0.05). The rate of presence of smoking was also obviously higher in both CA and T2DM groups than the control group (p<0.05). However, no significant difference was found in terms of gender, TG level, HDL-C level, SBP, and DBP among these different groups (p>0.05). Furthermore, Ang II levels were notably higher in T2DM combined with CA patients (80.66±17.20 pg/mL) than those in T2DM patients (35.49±15.01 pg/mL) and controls (35.14±0.43 pg/mL) (p<0.05).

### Changes of T cell activities among different groups

The cell proportions of Th1, Th2, and Th17 cells in PBMCs from different groups were detected using flow cytometry (Figure 1). There was an obvious increase in Th1 and Th17 positive cells proportion in CA group compared with T2DM group and control group (p<0.05) and no significant difference was found.

| Characteristics | CA group (n=30) | T2DM group (n=20) | Control (n=10) | P-value |
|-----------------|----------------|------------------|----------------|---------|
| Age (years)     | 67.03±8.14     | 38.4±10.70       | 33.20±4.83     | <0.001  |
| Gender(males)   | 8 (26.67%)     | 11 (55%)         | 4 (40%)        | 0.129   |
| Plasma glucose concentration (mmol/L) | 5.79±1.41 | 10.10±1.63 | 4.69±0.75 | <0.001 |
| TC (mmol/L)     | 4.21±0.88      | 2.17±0.75        | 2.99±0.49      | <0.001  |
| TG (mmol/L)     | 1.56±0.80      | 1.25±0.29        | 1.40±0.63      | 0.129   |
| LDL-C (mmol/L)  | 2.62±0.78      | 1.40±0.36        | 2.11±0.84      | <0.001  |
| HDL-C (mmol/L)  | 3.22±0.70      | 2.33±0.78        | 2.05±0.52      | 0.532   |
| Presence of hypertension (n) | 14 (46.67%) | 0 | 0 | <0.001 |
| Presence of smoking (N) | 16 (53.33%) | 9 (45%) | 1 (10%) | <0.001 |
| SBP (mmHg)      | 120.73±10.44   | 117.20±6.34      | 112.2±12.12    | 0.052   |
| DBP (mmHg)      | 77.73±10.53    | 77.55±5.24       | 79.5±4.37      | 0.812   |
| Ang II (pg/ml)  | 80.66±17.20    | 35.49±15.01      | 35.14±0.43     | <0.001  |

CA – carotid atherosclerosis; T2DM – type 2 diabetes mellitus; TG – triglycerides; TC – total cholesterol; SBP – systolic blood pressure; DBP – diastolic blood pressure; Ang II – angiotensin II.

CA – carotid atherosclerosis; T2DM – type 2 diabetes mellitus; TG – triglycerides; TC – total cholesterol; SBP – systolic blood pressure; DBP – diastolic blood pressure; Ang II – angiotensin II.
between T2DM group and control group (p>0.05). Besides, the cell proportions of Th2 differed insignificantly among three groups (p>0.05).

T-bet, GATA3, and RORγt are specific transcription factors for Th1, Th2, and Th17 cells and were also detected for changes in PBMCs by RT-PCR assay (Figure 2A). Consistently, the levels of T-bet and RORγt were significantly higher in the CA group than those in T2DM and control groups (p<0.05), whereas the level of GATA3 was similar among these three groups (p>0.05).

Furthermore, the IFN-γ, IL-4, and IL-17 levels in plasma were also examined in participants from different groups and the results were displayed in Figure 2B. Compared with T2DM and control groups, IFN-γ and IL-17 levels were elevated remarkably in the CA group (p<0.05), whereas IL-4 levels remained unchanged (p>0.05).

**T cell activities is closely associated with the levels of Ang II**

In order to investigate the correction between T cell activities and Ang II levels, the activities of T cell activities in PBMCs obtained from healthy controls were detected after Ang II with or without ABR treatment. According to our results, an increase of the cell proportions of Th1 and Th17 (Figure 3), upregulated mRNA levels of T-bet and RORγt (Figure 4A), as well as IFN-γ and IL-17 levels in plasma (Figure 4B) were observed with the increasing concentrations of Ang II (p<0.05). As expected, all these changes were remarkably alleviated after the treatment of ARB (p<0.05). At the same time, Ang II and ARB had no obvious effects on the cell proportions of Th2, the mRNA levels of GATA3, as well as the IL-4 levels in plasma (Figures 3, 4, p>0.05).
In addition, we also found that Th1 cells, T-bet, and IFN-γ levels were consistent with previous report that verified the role of inflammation disorder and occurrence of atherosclerosis [23]. Our results clarify the inflammatory response, as a result of immune functions, leading to formation of a positive feedback loop to amplify the inflammatory response and induces Th2 differentiation [31]. GATA3 selectively induces initial CD4+ T cells into Th2 cells [32]. It is still controversial about the role of Th2-type immune response in the process of atherosclerosis susceptibility in several animal model such as ApoE−/− mice and LDLr−/− mice and plays an important role in the early stage of atherosclerosis development, owing to the significantly delayed fatty streak formation in CD4-deficient C57BL/6 mice [24,25]. Th1-related cytokines including IFN-γ, IL-2, TNF-α, and TGF-β were largely expressed in plaques of AC, activating macrophages, and endothelial cells, amplifying inflammation and promoting the development of atherosclerosis and the plaque instability [26]. As the most important cytokine and polar reaction indictor of Th1, IFN-γ is also abundantly expressed in lesions of human and CA susceptibility models [27,28]. IFN-γ regulates a series of progresses during AC, including formation of macrophage foam cell, recruitment of leukocytes to the activated endothelium, and stability and remodeling of plaque [29]. Meanwhile, the activity of transcription factor of T-bet was considered to be essential for Th1 cell differentiation and CA plaque progression was significantly delayed in T-bet-deleted mice [30]. Additionally, Th2 cells exert their effect by secreting a variety of immunological cytokines, including IL-4 which is mainly secreted by Th2 cells exert their effect by secreting a variety of immunological cytokines, including IL-4 which is mainly secreted by Th2 cells, as well as the IFN-γ and IL-17 levels in plasma were significantly elevated in patients with both T2DM and CA, compared with those in T2DM patients. Moreover, the increase of the sclerosis degree could amplify these changes (data not shown). Previous research indicated that inflammation and the immune cell infiltration (a symbol of vascular inflammation) are associated with many of the cardiovascular diseases and also play an important role in the development of atherosclerosis [22,23]. Inflammatory response is capable of activating a large number of immune T lymphocytes, leading to formation of a positive feedback loop to amplify the inflammatory response, as a result of immune function disorder and occurrence of atherosclerosis [23]. Our results were consistent with previous report that verified the role of T cells activation in the development of CA in T2DM patients.

First, we found that the cell proportions of Th1 and Th17, the mRNA levels of T-bet and RORγt, and the IFN-γ and IL-17 levels in plasma.

In addition, we also found that Th1 cells, T-bet, and IFN-γ levels were all significantly increased in T2DM patients combined with CA than in T2DM patients and controls. As previously described, activation of CD4 + T cells contributes to atherosclerosis susceptibility in several animal model such as ApoE−/− mice and LDLr−/− mice and plays an important role in the early stage of atherosclerosis development, owing to the significantly delayed fatty streak formation in CD4-deficient C57BL/6 mice [24,25]. Th1-related cytokines including IFN-γ, IL-2, TNF-α, and TGF-β were largely expressed in plaques of AC, activating macrophages, and endothelial cells, amplifying inflammation and promoting the development of atherosclerosis and the plaque instability [26]. As the most important cytokine and polar reaction indictor of Th1, IFN-γ is also abundantly expressed in lesions of human and CA susceptibility models [27,28]. IFN-γ regulates a series of progresses during AC, including formation of macrophage foam cell, recruitment of leukocytes to the activated endothelium, and stability and remodeling of plaque [29]. Meanwhile, the activity of transcription factor of T-bet was considered to be essential for Th1 cell differentiation and CA plaque progression was significantly delayed in T-bet-deleted mice [30]. Additionally, Th2 cells exert their effect by secreting a variety of immunological cytokines, including IL-4 which is mainly secreted by Th2 cells and Th2-related cytokine and polar reaction indictor of Th1, IFN-γ is also abundantly expressed in lesions of human and CA susceptibility models [27,28]. IFN-γ regulates a series of progresses during AC, including formation of macrophage foam cell, recruitment of leukocytes to the activated endothelium, and stability and remodeling of plaque [29].

**Discussion**

Following the results in our study, Ang II is involved in the process of CA in T2DM patients via regulating specific T cell activities such as increasing the cell proportions of Th1 and Th17, the mRNA levels of T-bet and RORγt, and the IFN-γ and IL-17 levels in plasma.

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cytokines were found in human atherosclerotic plaques [26]. However, several studies reported that IL-4 promoted the progress of atherosclerosis and the plaques was smaller after silencing IL-4 [24,33]. In this study, no obvious changes of Th2, GATA3, and IL-4 were found among different groups. Consistent with the increase of IFN-γ level, proportion of Th17 cells, levels of RORγt and IL-17 were all obviously elevated in T2DM combined with CA patients than those in T2DM patients and controls in our study, suggesting the role of Th17 and related cytokines in the development of CA in T2DM patients. Th17, a new separated T cell subset, secrets IL-17 and other chronic inflammatory diseases and is closely related to autoimmune diseases [34]. RORγt is one of the key transcription factors of Th17 cell differentiation. The roles of Th17 cells and IL-17 in atherosclerosis are not fully understood. Smith et al. reported that atherosclerosis was suppressed by blocking interleukin-17 in Apoe (−/−) mice [35]. Eid et al. also confirmed that IL-17 was associated with human coronary atherosclerosis and induced the production of pro-inflammatory cytokines and chemokines in VSMCs to promote pro-arteriosclerotic vascular changes [36]. Moreover, IL-17 is reported to induce the production of IFN-γ and neutralizing IFN-γ activity is expected to induce an increase of IL-17, indicating an additional interaction between these two cytokines [36]. Furthermore, we also identified that Th1 and Th17 cells, as well as related cytokines were notably increased by addition of Ang II in a dose-dependent manner and the promoted effects were significantly attenuated after treatment with ABR, which demonstrated the relationship between Ang II and T cell activity in the progress of CA in T2DM patients. Ang II has been widely suggested to be involved in the development and progression of atherosclerosis and coronary heart disease [37].

Figure 3. Cell proportions of Th1, Th2, and Th17 in PBMCs from different groups. Cell proportions were detected using flow cytometry. The PBMCs were isolated from whole blood cells of health participants and then divided into eight groups. Then cells were treated with 0, 0.1, 1, and 10 μM Ang II combined with or without 0.1 μM valsartan (one kind of ARB) for 48 hours. Data were presented as mean ± standard deviation (S.D.). ** p<0.01, *** p<0.001 vs. 0 μM Ang II; * p<0.05, ** p<0.01, *** p<0.001 vs. 0 μM ARB.
Increasing evidences revealed the interplay between inflammatory events and renin-angiotensin system [38]. Ang II induces inflammation and improves the activation of the immune system, and T cell activation is prevented in Ang II receptor-deficient mice [39]. Meanwhile, activated T cells may generate Ang II locally by themselves to influence multiple cell functions in an autocrine manner [40]. Recently, several studies proved that Ang II contributes to atherosclerosis by increasing the levels of vascular inflammatory genes [41]. Furthermore, modulation of Th1/Th2 phenotype is identified to be a major downstream mechanism of Ang II in atherosclerosis [42]. Based on the results from our study, we supposed that Ang II could promote the process of CS through regulating activities of specific T cell phenotypes.

There were still several limitations in this study. First, significant differences in age were found among different groups which was attributed to the frequent occurrence of CA in elder patients. Age-matched controls might be more appropriate and persuasive. Meanwhile, the underlying mechanism of the relationship between the Ang II and T cell activation was not explored, which should be investigated in the further studies.

**Conclusions**

The proportions of Th1 and Th17 cells were significantly higher in the CA group than those in the T2DM group and control group. Consistently, a remarkable increase of T-bet and RORγt, as well as IFN-γ and IL-17 was found in the CA group compared with the T2DM group and control group. Additionally, Th1 and Th17 cells, as well as related cytokines, were notably elevated by Ang II treatment in a dose-depended manner; and the promoted effects were significantly attenuated by treated with

![Figure 4. T cells related cytokines were detected before and after Ang II and Ang II receptor blocker (ARB) treatment. The PBMCs were isolated from whole blood cells of health participants and then divided into eight groups, and the cells were treated with 0, 0.1, 1, and 10 μM Ang II combined with or without 0.1 μM valsartan (one kind of ARB) for 48 hours. (A) The mRNA levels of T-bet, GATA3, and RORγt were detected in PBMCs by RT-PCR assay. (B) The IFN-γ, IL-4 and IL-17 levels in plasma were assessed using ELISA for different patients and controls. Data were presented as mean ± standard deviation (S.D.). ** p<0.01, *** p<0.001 vs. 0 μM Ang II; * p<0.05, ** p<0.01, *** p<0.001 vs. 0 μM ARB.](https://example.com/figure4.png)
ABR. Therefore, we concluded that Ang II was closely associated with the development of CA in T2DM patients through regulating Th1 and Th17 cells as well as the related pro-inflammatory cytokines. Our study might provide new insights for investigating the mechanism and novel therapeutic targets for cardiovascular disease in T2DM patients.

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Conflict interests

The authors declared that there is no conflict of interest.