

The effects of T helper 17 and regulatory T cells on patients with carotid atherosclerosis

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Abstract: This study was aimed to observe the level of T helper 17 (Th17) cells and CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells (Tregs) and their related factors in carotid atherosclerosis (AS) patients and AS model rats to explore the influence of Th17 on the pathological process of AS and its specific mechanism. 60 cases with AS in our hospital from July 2012 to July 2014 were recruited for this study as the observation group, and 40 healthy people who came to the hospital for a physical examination were the control group. Flow cytometry (FCM) was used to detect the Th17 and Treg cells in the peripheral blood of the two groups, ELISA was used to detect IL-17 and transforming growth factor- β (TGF- β), and RT-PCR was used to test the ROR γ T mRNA and Foxp3 mRNA expression levels. An AS model was created in rats using high-fat+ VD₃ to explore the mechanism of Th17 on AS. The Th17 count, serum level of IL-17, and ROR γ T mRNA level of the observation group were significantly higher than those of the control group ($P < 0.001$). The Tregs count, serum TGF- β level, and Foxp3 mRNA level were significantly lower in the observation group than in the control group ($P < 0.001$). In addition, the findings of the AS model in rats showed that the Th17 cell count and serum level of IL-17 in high-fat rats were significantly higher than in the normal rats ($P < 0.05$). The Treg count and TGF- β levels of the observation rats were significantly lower than in the normal rats ($P < 0.05$). The IL-17 level, serum total cholesterol, and triglyceride in the high-fat-feed rats decreased after being injected with the IL-17 neutralizing antibody, but TGF- β levels increased, and the difference was significant ($P < 0.05$). Th17 cells and their related factors can be involved in promoting the pathological process of AS, while Tregs and its related factors can be involved in the inhibition of AS. Blocking IL-17 can be one potential method of treating AS.

Keywords: Th17, Regulatory T cells, IL-17, TGF- β , Carotid artery atherosclerosis.

INTRODUCTION

Atherosclerosis (AS) is a complex pathological process of lipid accumulation in the arterial wall, and can cause critical diseases which results in damages similar to coronary heart disease (CHD) and cerebral infarction. AS is usually induced by the adsorption, involvement and activation of various cells (e.g. monocytes, macrophages, T cells, endothelial cells and smooth muscle cells), which can trigger local inflammatory responses (Manduteanu *et al.*, 2012). However, the atherosclerotic cellular mechanism is still not known. Cluster of differentiation 4-Positive T-Lymphocytes (CD4⁺T) play an important role in the human immune system, and can be differentiated into different cell subsets (e.g. Th1, Th2, Th17 and regulatory cells (Tregs)) (2-5). The functional imbalance of Th17 and Tregs can play an important role in atherosclerotic formation and development. Th17 is a kind of subset under the context of the stimulation of cytokines (e.g. IL-6 and TGF- β) in the TCR pathway (S Taleb *et al.*, 2015), and this subset participates in the chronic pathological process of atherosclerotic inflammation (Mallat *et al.*, 2004). Ammirati *et al.* (2015) discussed the findings supporting a pro-atherogenic role of T cell subsets, such as effector memory T cells, or the potential protective function of regulatory T cells (E Ammirati *et al.*, 2015). Tregs is a kind of cell group

regulating immune function. It can also maintain immune tolerance and immunity homeostasis. The findings in some studies show that Tregs in the Apoe^{-/-} mice can produce IL-10 to alleviate the pathological immune response and reduce the formation of atherosclerotic plaque (Chen *et al.*, 2014). Del Porto *et al.* showed that Th17 counts were related to the late stages of CAS, but not to plaque instability. Moreover, Treg expansion seems to represent a specific cellular pattern displayed by patients with symptomatic CAS and is associated with brain injury (Del Porto *et al.*, 2016). However, the specific function of Th17/Treg on the atherosclerotic pathological process is not clear.

Therefore, in this study, the Th17, Tregs and their cytokine levels of the peripheral blood in AS patients, normal people and AS mice models were respectively compared and analyzed to explore the relationship of Th17/ Tregs and AS.

MATERIALS AND METHODS

General information

60 patients diagnosed with AS via carotid ultrasonography in our hospital from July 2012 to July 2014 were recruited for this study and were the observation group. 21 cases had a medical history of hypertension, 15 cases had a medical history of diabetes, 12 cases had a medical history of coronary heart disease.

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Correspondingly, we selected 40 healthy patients who came to the hospital for a physical examination as the control group. In the observation group, there were 25 male cases and 35 female cases, ranging from 42-68 years old (an average of 55.26 ± 8.32 years). In the control group, there were 14 male cases and 26 female cases, ranging from 44-67 years old (an average of 54.51 ± 9.17 years). Gender, age, and other differences in general information had no statistically significant difference between the two groups ($P > 0.05$). Therefore, the two groups were comparable. All patients signed a written informed consent and the study was approved by the Ethical Committee of the Ethics committee of The First Affiliated Hospital of Zhengzhou University, China.

Experimental animal models

12 healthy Wistar rats were selected and divided into a high fat + VD₃ group (with 6 rats) and a control group (with 6 rats). The Wistar rats in the high fat + VD₃ group were injected with 7×10^5 u/kg of VD₃ for three consecutive days, and then given a 20g high-fat diet per day for 21 continuous days. Each rat in the control group was injected with an equal volume of normal saline and given a 20g standard diet for the same number of days.

Methods

Flow cytometry dying of Th17 and Tregs

5ml of PB was collected separately from the normal and observation groups. Then, Ethylenediaminetetraacetic acid (EDTA) anticoagulant and red cell lysis solution was added to the samples. After, the samples were put into the complete RPMI-1640 at a concentration of 1×10^6 cells/ml. Th17 staining was completed by the centrifugation and suspension of the sample into 100 μ l of phosphate buffer saline (PBS). Then, CD3-APC (eBioscience, 0.2mg/ml) and CD4 - FITC (eBioscience, 0.25mg/ml) were added. The samples were then blended and kept away from light in an incubation room at room temperature for 40 min. Then, 1ml of fixation liquid was added at 4°C in darkness for 30 min. Then, the samples were centrifuged and suspended into a 100 μ l PBS. IL-17-PE (BioLegend, 0.25 mg/ml) was then added, and the samples were kept at 4°C and away from light for 30 min, washed with PBS twice, and then suspend into 500 μ l of PBS and tested with FCM (FACS Calibur, BD company, USA). Tregs staining was carried out as follows: the samples were centrifuged and suspended into 100 μ l of PBS, then CD25-APC (eBioscience, 0.25mg/ml) and CD4-FITC (eBioscience, 0.25mg/ml) was added, mixed up and kept away from light for 40min at room temperature. The samples were then washed with PBS once, had 1ml of fixation liquid added, and were kept for 40 min at 4°C in darkness. Then, the samples were centrifuged and suspended in 100 μ l of PBS, had 2 μ l of Fc receptor blockers added to them (eBioscience), were kept at 4°C in darkness for 20min. After this, the anti-FoxP3-PE antibody (eBioscience, 0.15 mg/ml) was added, the samples kept at 4°C in darkness for 40 min, washed with PBS, then suspend into 500 μ l of PBS and then detected with FCM.

Detection of IL-17 and TGF- β by ELISA

All the detection processes in this study were strictly in accordance with the specifications of the kits (Dakewe Biotech Co., Ltd.).

Detection of ROR γ T mRNA and Foxp3 mRNA in the PB by RT-PCR

150 μ l of super blood plasma was extracted after centrifugation. 1ml of Trizol was added and mixed up, and the total RNA was extracted according to the kit specifications. The total RNA was put into a 25 μ l DEPC. The RNA were analyzed by ultraviolet spectrometry. Then, the RNA was reverse transcribed by Femantes and stored at -20°C. The PCR reaction conditions were as follows: 95°C, 20 s; 60°C, 20 s; 70°C, 1 s (40 circles). The ROR γ T primer sequence was as follows: F: 5'-GCAGCGCTCCAACATCTTCT-3', R: 5'-ACGTACTGATGGCCTCGGT-3'. Foxp3 primer sequence: F: 5'-CACCTGGCTGGGAAAATGG-3', R: 5'-GGAGCCCTGTTCGGATGA-3'; Internal GAPDH primer sequence: F: 5'-GCATGGGTCAGAAGGATTCC-3', R: 5'-TCGTCCCAGTTGGTGACGAT-3'. The ABI 7900 Real-Time PCR instrument was used for amplification, and the results were quantitatively analyzed by $2^{-\Delta\Delta C_t}$ (Livak et al., 2001).

Experiment of blocking IL-17 in rat model

Each of the rat model was injected with the neutralizing antibody of IL-17 (Shanghai laboratory reagent Co., Ltd.) by 100 μ g.

Detection of serum total cholesterol and triglyceride

The serum total cholesterol (TC) and triglyceride levels were tested by the automatic biochemical analyzer (TNA-40FR, Toshiba, Japan). The regents were purchased from Beijing Kangda Taike Medicine Technology Co. Ltd.

STATISTICAL ANALYSIS

SPSS18.0 software was used to analyze all the data in this study. The observational data (mainly the measurement data) was tested by a normality test, expressed by Standard Mean Deviation ($\bar{x} \pm S$), and checked by a T test. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of Th17 and Tregs of PB in the patients with carotid AS

The results of FCM showed that the Th17 count in PB of the control and observation group were (0.57 ± 0.41) % and (1.87 ± 0.31) %, respectively. The difference was significant ($t = 18.03$, $P < 0.001$). The Treg counts were (1.52 ± 0.47)% and (0.81 ± 0.38)%, respectively, and the difference was significant ($t = 8.32$, $P < 0.001$) (fig. 1).

Table 1: the population of Th17 and Tregs in the AS model in rats

Groups	Cases	Th17 (%)	Treg (%)	IL-17 (pg/ml)	TGF- β (pg/ml)
Control group	6	1.58 \pm 0.34	5.34 \pm 0.78	51.12 \pm 7.98	31.67 \pm 9.16
High fat +VD ₃ group	6	2.14 \pm 0.45	3.41 \pm 0.77	87.17 \pm 11.25	20.12 \pm 8.17
<i>t</i> value	/	2.432	4.313	6.402	2.305
<i>P</i> value	/	, 0.035	, 0.002	, 0.000	, 0.044

Table 2: The serum total cholesterol and triglyceride after injecting with IL-17 neutralizing antibodies

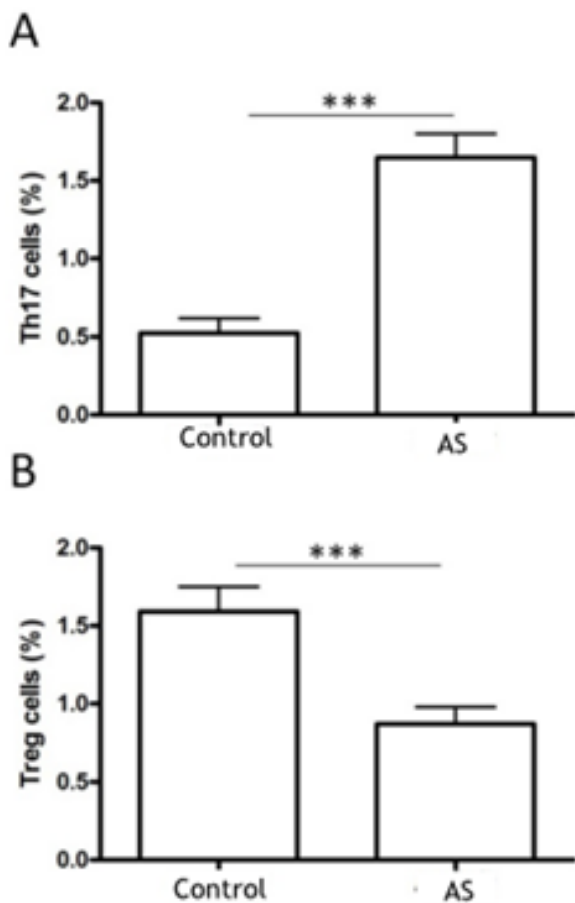
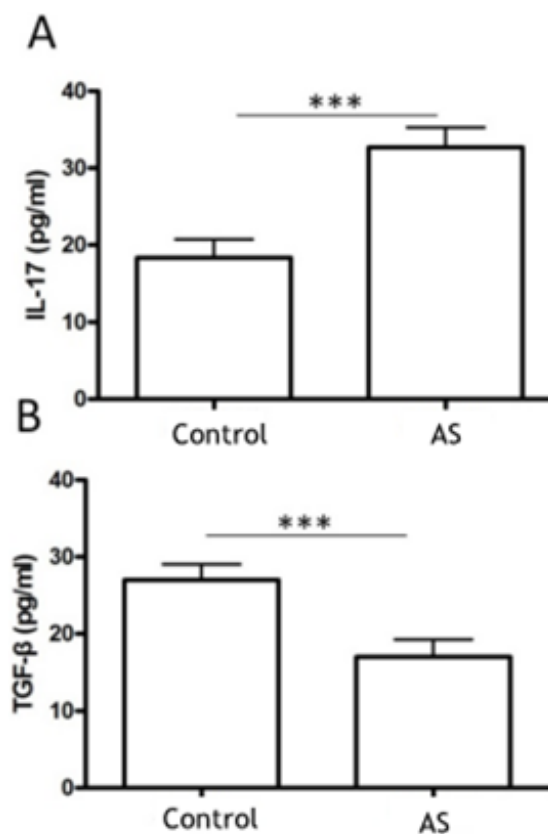
Groups	Cases	IL-17 (pg/ml)	TGF- β (pg/ml)	Total cholesterol (mmol/L)	Triglyceride (mmol/L)	Area of AS plaque (%)
Control group	6	91.26 \pm 8.16	22.51 \pm 9.82	2.31 \pm 0.41	242.47 \pm 37.48	24.06 \pm 7.32
IL-17 Neutralizing antibody group	6	46.26 \pm 8.16	36.12 \pm 8.61	1.56 \pm 0.43	150.67 \pm 47.58	17.49 \pm 4.26
<i>t</i> value	/	9.552	2.553	3.092	3.713	3.024
<i>P</i> value	/	0.000	0.029	0.011	0.004	0.025

Comparison of IL-17 and TGF- β level between the two groups

The findings of ELISA showed that serum level of IL-17 in the observation group (33.21 \pm 8.41 pg/ml) was much higher than in the control group (17.12 \pm 8.80 pg/ml) ($t=9.20$, $P<0.001$). In addition, the TGF- β (17.21 \pm 8.73 pg/ml) level was much lower in the observation group than in the control group ($t=5.47$, $P<0.001$) (fig. 2).

Comparison of ROR γ T mRNA and Foxp3 mRNA between the two groups

ROR γ T and Foxp3 are two important transcription factors that determine the Th17 and Treg functions (Barbi et al., 2013). Therefore, we used RT-PCR to detect the level of ROR γ T mRNA and Foxp3 mRNA. The findings showed that the ROR γ T mRNA level in the observation group was much higher than in the control group ($P<0.001$), while the Foxp3 mRNA level was much lower in the observation group than in the control group ($P<0.001$) (fig. 3).

**Fig. 1:** Comparison of Th17 and Tregs of PB in the patients with carotid AS.**Fig. 2:** Comparison of IL-17 and TGF- β level between the two groups.

The level of Th17 and Tregs in the mice AS model

A recent study conducted by Zhao *et al.* demonstrated that a high fat diet + VD3 is a good method for creating an AS model in mice (13). We found that Th17 and serum IL-17 levels in the high-fat diet + VD3 rats were much higher than those of the normal rats ($P < 0.05$). The Tregs and serum TGF- β levels were much lower in the high-fat diet + VD3 rats than in the normal rats ($P < 0.05$) (table 1).

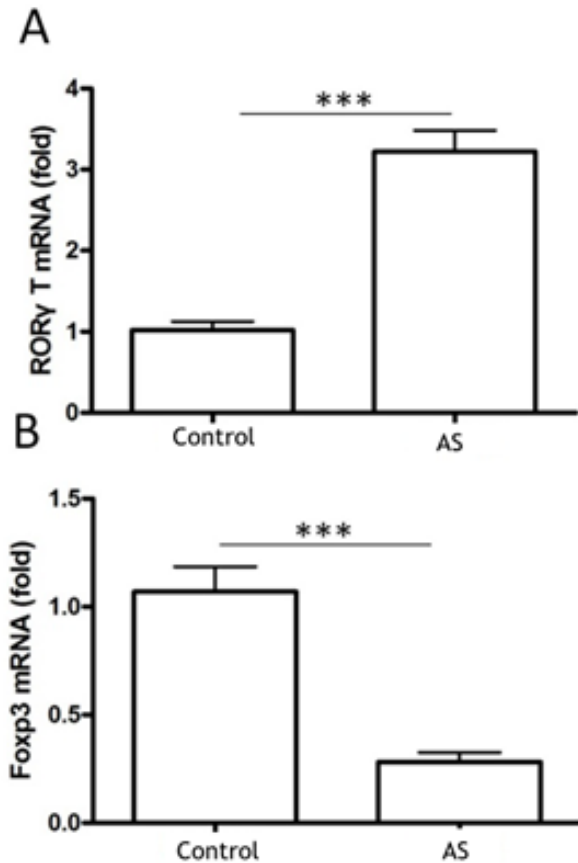


Fig. 3: Comparison of ROR γ TmRN and Foxp3mRN between the two groups

The serum total cholesterol and triglyceride after injecting with IL-17 neutralizing antibodies in the the AS model in rats

In order to discuss the accurate effect of IL-17 (generated by Th17) on the AS patients, we injected IL-17 neutralizing antibodies into high-fat diet + VD3 rats. The findings showed that the IL-17 level decreased and the TGF- β level increased, while the serum total cholesterol and triglyceride levels were significantly lowered. This is shown in table 2.

DISCUSSION

AS refers to the vessel stenosis or total vessel occlusion involving the aorta, coronary artery, cerebral artery, renal artery, and large and medium muscle elastic-type arteries, leading to ischemia hypoxia, functional disorders, or even

necrosis of the surrounding vital organs. The major clinical manifestation of AS is a heightened serum lipid level, accompanied by inflammation and autoimmune system diseases (Chen *et al.*, 2014). An inflammatory response is the most important initiator during the pathological process of AS. With the development of inflammation, the activated white blood cells and endothelial cells in the patients with AS will release a variety of cytokines, such as fiber growth factor (FGF), transforming growth factor- β (TGF- β), and epidermal growth factor (EGF), which will stimulate the proliferation and migration of the fibroblast and smooth muscle cells. This eventually results in endomembrane fibro muscular proliferative disease, which will make AS ingravescent (An *et al.*, 2014). Therefore, it is of clinical significance to explore the function and onset mechanisms of inflammatory cells during the pathological process of AS.

Th17 and Tregs are two important subgroups of CD4⁺ T. Th17 is a kind of subgroup induced by IL-6 and TGF- β factors, and can generate IL-17, which can participate in the body’s defense against (Kleinewietfeld *et al.*, 2013; Liu *et al.*, 2012). Liu *et al.* reported that TGF- β 1 can regulate the cell differentiation processes of Th17and Treg cells (Liu *et al.*, 2012). Treg cells are another kind of subgroup that participates in immune suppression, which can be generated by the induction of IL-10 and TGF- β . Tregs can generate TGF- β to take part in the onset and development of various autoimmune diseases, like systemic lupus erythematosus (SLE), aplastic anemia, and diabetes mellitus type 1 (Kleinewietfeld *et al.*, 2013; Wang *et al.*, 2013; Hou *et al.*, 2013).

Today, people are paying more and more attention to Th17 and Tregs levels in AS patients. Zhu *et al.* demonstrated the idea that the imbalance of Th17/Tregs can significantly increase the incidence rate of AS (Zhu *et al.*, 2013). Liu *et al.* found that in an AS mouse model induced by angiotensin II, Th17 can promote the occurrence of AS (Liu *et al.*, 2011). Rosario *et al.* also found that Th17 cells increased significantly in patients with cardiogenic shock, which was taken as one risk factor (Del Rosario Espinoza Mora *et al.*, 2014). The imbalance of Th17/Tregs, prone to Th17 differentiation, can be seen in a mouse liver injury model induced by tripterygium wilfordii, (Wang *et al.*, 2014). This study aimed to explore the clinical significance of Th17 and Treg cells in the treatment of AS through analyzing and comparing the Th17 and Treg levels and their related factors in the AS cases and AS mice models. The results showed that the Th17 level in AS patients was much higher than in normal people, while the Tregs level was much lower, indicating that the imbalance of Th17/Treg can be involved in the immune response in AS patients. This corresponded with the results of previous studies (Mallat *et al.*, 2004; Wang *et al.*, 2014). In addition, we

tested the functional factors of Th17 and Treg cells (e.g. IL-17 and TGF- β). The results showed that the serum IL-17 level in AS patients was much higher than in the normal people, while the serum TGF- β level was decreased, indicating that the imbalance of Th17/Treg can affect their functional factors in the involvement of AS development. Then, we further detected the ROR γ T mRNAs and Foxp3 mRNAs and found that the ROR γ T mRNA levels were much higher in AS patients than in normal people, while the Foxp3 mRNA levels were lower in AS patients than in normal people. These results supported our study. In addition, we also created an AS rat model to confirm the findings.

Subramanian *et al.* thought that the Tregs-mediated inhibition process of AS was mainly dependent on the Myd88 signaling pathway (Subramanian *et al.*, 2013). Wasserman *et al.* found that IL-33 can suppress AS development by increasing the Tregs level (Wasserman *et al.*, 2012). However, Ammirati *et al.* (2010) proposed that no correlation was observed between Treg-cell levels and intima-media thickness in a carotid study (Ammirati *et al.*, 2010). Also, Wigren *et al.* found that there were no associations between Treg-cell levels and the development of an acute coronary event or stroke, reflecting the more heterogeneous cause of this disease (Wigren *et al.*, 2015). Therefore, the influence of IL-17 on AS patients was still not clear. In this study, we injected an IL-17 neutralizing antibody into AS rats, and the results showed that after IL-17 was neutralized, the TGF- β level increased, while the total cholesterol and triglyceride levels significantly decreased. This indicated that IL-17 can participate in the pathological process of AS.

CONCLUSION

The imbalance of Th17/Tregs and their related factors play an active role in the pathological process of AS. The increase of Th17 levels and its related factors can be an important cause of AS. It is of great importance to decrease the IL-17 level for the treatment and prognosis of AS patients.

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