Original Research

Changes in $CD4^+CD25^+$ Tregs in the pathogenesis of atherosclerosis in $ApoE^{-/-}$ mice

Li Xue-Mei*, Chen Jie*, Dai Xuan, Liao Xiao-Xing, Hu Chun-Lin and Li Yu-Jie

Department of Emergency, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, China
*These authors contributed equally to this paper.
Corresponding authors: Hu Chun-Lin. Email: hu-chunlin@163.com; Li Yu-Jie. Email: Yujieligd@163.com

Impact statement

In this article, we conclude that Tregs decreased with atherosclerosis (AS) as determined in ApoE knockout mice fed a high fat diet. It is an important matter for understanding the AS pathology.

Abstract

The goal of this study was to observe the pathological characteristics of atherosclerotic plaques in the aortic walls of $ApoE^{-/-}$ and C57BL/6J mice and the changes of $CD4^+CD25^+$ regulatory T cells (Tregs) in atherosclerotic mice. Twenty $ApoE^{-/-}$ mice were split into high-fat diet (AH) and normal diet (AN) groups and 10 C57BL/6J male mice were designated as the control group (BN). The serum concentrations of IL-10 and TGF-β1 were detected by enzyme-linked immunosorbent assay; paraffin sections of the aorta were stained with hematoxylin & eosin, and morphometric parameters were measured using the Image Pro Plus 6.0 system. Verhoeff stain was used to observe the distribution of elastic fibers, and immunohistochemical staining was performed to verify the phenotype of the forkhead box protein 3 ($Foxp3^+$) $CD25^+$ cells in the atherosclerotic tissue. The proportion of $CD4^+CD25^+$ Tregs in the spleen was calculated by flow cytometry. The thickness of the intima, the intima/media ratio, the plaque area, and the plaque/lumen ratio of mice in AN group were significantly larger than those of mice in BN group. The thickness of the intima, the plaque area, and the plaque/lumen ratio of the mice in AH group were significantly increased compared with those of the AN group mice. The serum concentrations of IL-10 and TGF-β1 and the percentage of splenic $CD4^+CD25^+$ Tregs in AN group mice were significantly decreased compared with the control group. The serum concentrations of IL-10 and TGF-β1 and the percentage of splenic $CD4^+CD25^+$ Tregs in the mice in AH group were significantly decreased compared with those in AN group. The proportions of $Foxp3^+$ and $CD25^+$ cells within the total lymphocyte population were significantly decreased in AH group mice compared with those in AN group mice. Atherosclerosis in an experimental mouse model was correlated with Treg depletion in the lymphoid tissues and plaques, indicating the important antiatherosclerotic role of $CD4^+CD25^+$ Tregs.

Keywords: Atherosclerosis, $CD4^+CD25^+$ Tregs, forkhead box protein 3, ApoE-KO mouse ($ApoE^{-/-}$ mice)

***Experimental Biology and Medicine* 2017; 242: 918–925. DOI: 10.1177/1535370216689826**

Introduction

Atherosclerosis (AS) is a complex disease characterized by smooth muscle cell proliferation, cholesterol deposition, and the infiltration of mononuclear cells. The formation and progression of atherosclerotic plaques results in the disruption of organ perfusion, causing cardiovascular and cerebrovascular diseases. The pathogenesis of AS is still unclear, but increasing evidence has demonstrated that the immunoinflammatory response plays a critical role in the development and progression of AS. Recent studies have suggested an important role for regulatory T cells (Tregs) in the inhibition of disease-related vascular inflammation. Accumulating evidence has shown that $CD4^+$ effector T cells may accelerate the development of AS.[1–3] In contrast, $CD4^+$ Treg cells play a protective role in AS.[4–7] Tregs, a special T cell subtype, were discovered in recent years. Tregs exhibit a weak immune response, have immune-suppressive characteristics, and play an important role in immune tolerance and immune regulation. In recent years, several studies have confirmed the role of Tregs in the development of atherosclerotic lesions.[4,8–12] The transcription factor forkhead box protein 3 (Foxp3) plays an important role in regulating the maturity and function of Tregs and has been shown to promote resistance to AS.[13] Tregs inhibit AS via the production of IL-10 and TGF-β1. At present, research on the role of Tregs in AS has focused on Tregs in the peripheral blood or lymphatic tissues, and few studies have evaluated the distribution of Tregs in local plaques. Thus, the possible antiatherosclerotic mechanisms of Tregs in a local tissue environment and their relationship with the distribution of peripheral Tregs remain unclear.

The aim of this study was to explore the relationship between $CD4^+CD25^+$ Tregs and local AS in arteries by establishing different degrees of atherosclerotic lesions in a mouse model and observing the distribution of Tregs in the spleens and local plaques as well as the concentrations of cytokines in the peripheral blood.

Materials and methods

Animals

Thirty 8-week-old male mice provided by the Laboratory Animal Center of Peking University Health Science Center (Certification Number: SCXK()2006-0008) were used for this experiment. Twenty $ApoE^{-/-}$ mice (on a C57BL/6J background, originating from Jackson Laboratories) were randomly divided into two groups by feeding different diets: 10 mice were placed in the $ApoE^{-/-}$ mice + high-fat diet (AH) group, and the other 10 mice were placed in the $ApoE^{-/-}$ mice + normal diet (AN) group. Another 10 male C57BL/6J mice fed a normal diet were designated as the control group (BN). The high-fat diet (a mixture containing 2% cholesterol, 0.2% propylthiouracil, 15% fat, 5% yolk and ordinary feed) and the normal diet were supplied by the Guangdong Medical Laboratory Animal Center (Certification Number: SCXK(Yue)2008-0002, 2008D007). All animals were maintained in an SPF environment at the Sun Yat-sen University School of Public Health Animal Center for 12 weeks.

Experimental methods

After 12 weeks, the mice were anesthetized with 4% chloral hydrate before sacrifice. Blood extracted from the left ventricle was centrifuged for 20 min to obtain the serum. The serum concentrations of IL-10 and TGF-β1 were detected by enzyme-linked immunosorbent assay (ELISA); total cholesterol, LDL, and triglycerides levels were assayed by using commercially available kits. After perfusing the tissue with 4% paraformaldehyde and saline, 600 μm of tissue from the root of the aorta was isolated. Then, 5 μm paraffin sections of the aorta, arch, and bifurcations were stained with hematoxylin & eosin (H&E), and morphometric parameters, such as the plaque area, the lumen area, the plaque/lumen ratio, the thickness of the intima and tunica media, and the intima/media ratio, were measured using the Image Pro Plus 6.0 system. Verhoeff stain was used to observe the distribution of elastic fibers, and immunohistochemical staining was performed to verify the phenotype of the $Foxp3^+$ and $CD25^+$ cells in the atherosclerotic tissue. The spleens were isolated and ground to obtain a splenocyte suspension. The proportion of $CD4^+CD25^+$ Tregs in the spleen was calculated by flow cytometry.

Tissue pathological morphology

H&E-stained sections were imaged at 400 × using an optical microscope, and each blood vessel was randomly selected from six fields. The arterial intima media thickness (I) and medium thickness (M) were measured using the Pro Plus Image 6 image analysis system, and the vessel lumen area was calculated. The elastic fibers in each specimen were stained, and the arrangement of elastic fibers was observed using an optical microscope.

Peripheral blood TGF-β1, IL-10, total cholesterol, LDL, and triglycerides concentration determination

The serum concentrations of the cytokines TGF-β1 and IL-10 in the mice were detected using the double antibody sandwich ELISA method, in strict accordance with the manufacturer's instructions for the relevant kits. Cytokine standard curves were constructed using a dilution series of the standard, and the corresponding OD450 values were measured for the standard curves using CurveExpert1.3 analysis software. The absorbance of the samples at a detection wavelength of 450 nm was obtained, and then the concentrations of the cytokines (pg/mL) in each sample were calculated based on the standard curve. Total cholesterol, LDL, and triglycerides levels were assayed by using commercially available kits (Roche Molecular Biochemicals, Indianapolis, IN, USA), respectively.

The proportion of $CD4^+CD25^+$ Tregs in splenic cell suspensions

Splenocytes or lymph node cells were labeled with fluorescein isothiocyanate-conjugated anti-CD4 (clone RM4-5, eBioscience) and allophycocyanin-conjugated anti-CD25 (clone PC61.5 eBioscience) antibodies and then analyzed by flow cytometry on a Canto II flow cytometer (Becton Dickinson). Intracellular Foxp3 staining was performed using phycoerythrin-conjugated anti-mouse/rat Foxp3, and the results were expressed as the proportion of $CD4^+CD25^+$ Tregs within all $CD4^+$ cells.

Foxp3 and CD25 immunohistochemical staining of the vascular intima

Immunohistochemical staining for Foxp3 and CD25 was performed on paraffinized sections of the aortic root, arch, and bifurcations. Elivision's two-step method was used for immunohistochemistry and 3,3′-diaminobenzidine chromogenic assays, with phosphate-buffered saline instead of a primary antibody as a negative control. Brown granules were defined as positive. Foxp3 was mainly found to be nuclear, while CD25 was located in the membrane. The counting and size tools in the IPP image analysis system were used to count the $Foxp3^+CD25^+$ cell numbers as well as the number of lymphocytes in the plaques. The measured values were expressed as the ratio proportion of $Foxp3^+CD25^+$ cells out of all lymphocytes.

Statistics

SPSS version 13.0 was used to analyze the data and make bar graphs. All values were expressed as the mean ± standard deviation. Differences between the groups were compared by analysis of variance after testing the homogeneity of the variance. $P < 0.05$ was considered statistically significant.

Results

Body weight

There were no obvious differences among the three groups in behavior mobility, fur brightness, and feeding throughout the course of the experiments. Before the experiment, there was no significant difference in the weights of the mice between the three groups. After feeding for 12 weeks, the weights of the mice were not significantly different between the AH and AN groups; compared with the mean body weight for group BN, the mice in AN group increased in weight significantly ($P = 2.490$) (Table 1).

Aortic pathological morphology

AS plaques were significantly increased in the aortas of the mice in AH group. Additionally, extensive foam cell formation and accumulation was observed; the majority of these cells were formed from dendritic cells (DCs) in the vascular lumen. The intima was thickened at the root of the aorta, the gap between the endothelial cells and the membrane was widened significantly, and the smooth muscle in the membrane was atrophic and arranged in a disordered fashion (Figure 1(a)). Early plaque formation was observed in the aortic walls of the mice in AN group, and mild pathological changes were observed in the mice in AH group (Figure 1(a)). The aortic walls of the mice in BN group were normal, clear, and had no AS plaque formation (Figure 1(a)). The elastic fibers in AH group were arranged loosely, disordered, and partially ruptured (Figure 1, Masson B, red arrow), which was more severe than the pathology observed in AN group (Figure 1(b)). In contrast, the elastic fibers in BN group were arranged neatly and closely together without rupture (Figure 1(b)).

More thickening of the aortic intima was observed in AH group than in AN group ($P = 0.041$ in aortic root, 0.023 in aortic arch, and 0.003 in aortic bifurcations), but no difference in the I/M ratio was observed between the two groups. Similarly, more thickening of the aortic intima was observed in AN group than in BN group (all $P < 0.001$), but the I/M ratio was also increased in AN group compared with BN group (all $P < 0.01$). The media thicknesses were no different between the three groups (Table 2).

The plaque area and the ratio of the plaque area to the lumen cross-sectional area were higher in AH group than in AN group (plaque area: $P = 0.002$; ratio: $P = 0.01$ in aortic root; both the plaque area and ratio in aortic arch and bifurcations, $P < 0.001$). These two parameters were also higher in AN group than in BN group (plaque area: $P < 0.001$; ratio: $P < 0.001$ all in aortic root, arch, and bifurcations), and there were no plaques formed in BN group (Table 3).

Table 1 The body weights of mice in each group before experiment and after feeding for 12 weeks (g)

Group	n	Before	After
AH	10	20.48 ± 1.73	28.73 ± 4.54
AN	10	20.74 ± 1.06	28.53 ± 2.72*
BN (Control)	10	20.87 ± 1.40	25.60 ± 2.54

AH: $ApoE^{-/-}$ mice + high-fat diet; AN: $ApoE^{-/-}$ mice + normal diet; BN: control group.
*$P < 0.05$, AN versus BN.

Concentrations of TGF-β1, IL-10 total cholesterol, LDL, and triglycerides in the peripheral blood and $CD4^+CD25^+$ Tregs in a splenic cell suspension from mice in each group

The concentrations of TGF-β1 and IL-10 (pg/mL) in the peripheral blood and the proportion of $CD4^+CD25^+$ Tregs within a splenic cell suspension from the mice in AH group were 83.97 ± 33.45, 27.50 ± 11.54, and 5.8 ± 1.51%, respectively, which was significantly lower than those observed for AN group, which were 116.05 ± 32.27, 41.83 ± 16.15, and 9.4 ± 4.00% ($P = 0.035$, 0.043, and 0.017, respectively). The same parameters were also significantly lower in AN group than in BN group (191.27 ± 95.27, 61.84 ± 23.05,

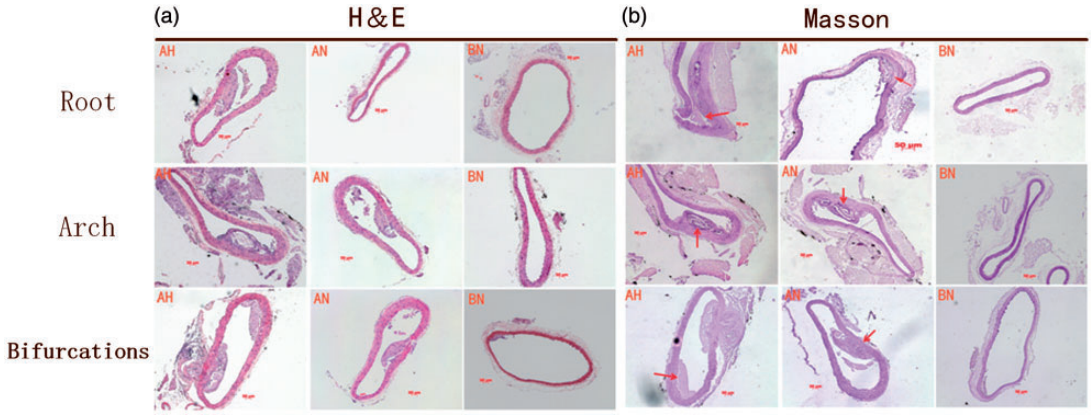

Figure 1 The pathological morphology of the aortic artery in different groups revealed by H&E and Masson staining. AS plaques were significantly increased in the aortas of mice in AH group (H&E (a)). Early plaque formation was observed in the aortic walls of mice in AN group (H&E (a)); the aortic walls of mice in BN group were normal, with no AS plaque formation (H&E (a)). The elastic fibers in AH group were arranged loosely, disordered, and partially ruptured (Masson b, red arrow), which was a more severe phenotype than AN group (Masson b), while the elastic fibers in BN group were arranged neatly (Masson b), bar represented 50 μm. AH: $ApoE^{-/-}$ mice + high-fat diet; AN: $ApoE^{-/-}$ mice + normal diet; AS: atherosclerosis; BN: control group; H&E: hematoxylin & eosin. (A color version of this figure is available in the online journal.)

13.8 ± 3.97%) ($P = 0.039$, 0.037, and 0.023, respectively) (Figure 2; Table 4).The concentrations of total cholesterol, LDL, and triglycerides in the peripheral blood in AH group were all significantly higher than those in AN and BN groups; the concentrations of total cholesterol, LDL, and triglycerides in group AN were also significantly higher than those in group BN (Table 5).

Immunohistochemistry of the aortic vascular intima

$Foxp3^+$ and $CD4^+CD25^+$ cells were present in atherosclerotic plaques. However, none of these cells were observed in the intima of the mice in BN group. When comparing the severe lesions in group AH with the mild lesions in group AN, we observed that the proportion of $Foxp3^+$, $CD4^+CD25^+$ cells within the total lymphocyte population was significantly decreased (1.28 ± 1.20 versus 3.04 ± 1.92, $P = 0.024$; 2.00 ± 1.39 versus 3.98 ± 1.67, $P = 0.010$ in aortic root; 1.55 ± 0.98 versus 3.20 ± 1.46, $P = 0.032$; 2.13 ± 0.99 versus 3.86 ± 1.24, $P = 0.022$ in aortic arch; and 1.08 ± 0.76 versus 3.00 ± 1.31, $P = 0.012$; 1.45 ± 0.78 versus 3.45 ± 1.26, $P = 0.008$ in aortic, respectively) (Figure 3(a) and (b)).

Table 2 The thickness of intima (I), media (M) (μm) and ratio of I/M (%) in three groups

Group	n	Intima	Media	I/M
Aortic root				
AH	10	14.80 ± 3.42$^{\Delta}$	23.13 ± 3.65	0.663 ± 0.222
AN	10	12.24 ± 1.34$^{\#}$	19.45 ± 4.22	0.658 ± 0.167$^{\#}$
BN	10	7.33 ± 2.23	17.51 ± 2.08	0.429 ± 0.145
Aortic arch				
AH	10	16.54 ± 2.48$^{\Delta}$	25.11 ± 3.57	0.659 ± 0.235$^{\#}$
AN	10	13.25 ± 1.43$^{\#}$	18.51 ± 4.65	0.687 ± 0.178$^{\#}$
BN	10	7.29 ± 2.33	18.12 ± 2.21	0.413 ± 0.126
Aortic bifurcations				
AH	10	17.79 ± 3.32$^{\Delta}$	27.10 ± 3.22	0.656 ± 0.213$^{\#}$
AN	10	14.11 ± 1.24$^{\#}$	21.41 ± 4.39	0.659 ± 0.207$^{\#}$
BN	10	8.31 ± 2.44	18.54 ± 2.28	0.448 ± 0.158

AH: $ApoE^{-/-}$ mice + high-fat diet; AN: $ApoE^{-/-}$ mice + normal diet; BN: control group.
$^{\Delta}P < 0.05$, compared with AN; $^{\#}P < 0.01$, compared with BN.

Discussion

This study developed an AS model using $ApoE^{-/-}$ knockout mice fed a high-fat diet daily to induce different degrees of pathological changes in AS plaque formation, with wild-type C57BL mice as a negative control group. The aim of this study was to systematically observe changes in $CD4^+CD25^+$ Tregs in the pathogenesis of AS. Analyses included comparing AS lesion severity by evaluating the pathological characteristics of AS plaques, measuring the number of vascular intima $CD4^+CD25^+$Tregs and the Foxp3 distribution using immunohistochemistry, measuring cytokine (IL-10, TGF-β1) concentrations in the peripheral blood, and measuring the number of Treg cells in the spleen. The results of the present study confirmed that $ApoE^{-/-}$ mice are an ideal animal model to study the pathological changes in AS because supplementation with different diets resulted in different degrees of plaque-related pathological changes compared with wild-type C57BL/6J mice. In addition to the pathological changes of AS, the proportion of $CD4^+CD25^+$ Tregs in the atherosclerotic lesions and the spleen as well as the concentrations of the cytokines IL-10 and TGF-β1 in the peripheral blood was further decreased.

Tregs are important members of regulatory networks in the human body, with broad inhibitory effects on the immune response. Tregs inhibit the activation of other lymphocytes via the direct secretion of cytokines or inducing other cells to secrete cytokines. During the occurrence and development of AS, diverse types of interactions between immune cells, cytokines, and antibodies form a very complex network of cellular and humoral immune mechanisms.[14] Once Tregs are activated, they can secrete IL-10 and TGF-β1 to suppress several cell types, including antigen-specific T cells, thereby limiting the occurrence and development of AS.

A study by Ait-Oufell *et al.*[4] discovered that in contrast to $CD45.1^+CD4^+CD25^-$ cells, $CD45.1^+CD4^+CD25^+$ Tregs

Table 3 The aortic plaques area, lumen cross-sectional area (μm^2), and the ratio of them in each groups

Group	n	Plaques area	Cross-sectional area	Ratio
Aortic root				
AH	10	905,265.9 ± 259,356.16$^{\Delta\#}$	3,648,599 ± 571,428.72	0.251 ± 0.073$^{\Delta\#}$
AN	10	600,265.4 ± 263,876.25$^{\#}$	3,696,993 ± 722,599.43	0.159 ± 0.055$^{\#}$
BN	10	0	3,460,847 ± 677,018.27	0
Aortic arch				
AH	10	967,918.2 ± 259,356.16$^{\Delta\#}$	3,217,157 ± 525,621.36	0.301 ± 0.078$^{\Delta\#}$
AN	10	641,178.2 ± 265,967.10$^{\#}$	3,511,663 ± 522,609.21	0.183 ± 0.052$^{\#}$
BN	10	0	3,360,766 ± 577,117.14	0
Aortic bifurcations				
AH	10	1,381,869.2 ± 325,693.11$^{\Delta\#}$	3,623,245 ± 461,346.13	0.381 ± 0.054$^{\Delta\#}$
AN	10	865,665.2 ± 366,043.15$^{\#}$	3,934,783 ± 445,774.24	0.221 ± 0.039$^{\#}$
BN	10	0	4,390,674 ± 368,243.31	0

AH: $ApoE^{-/-}$ mice + high-fat diet; AN: $ApoE^{-/-}$ mice + normal diet; BN: control group.
$^{\Delta}P < 0.05$, compared with AN; $^{\#}P < 0.01$, compared with BN.

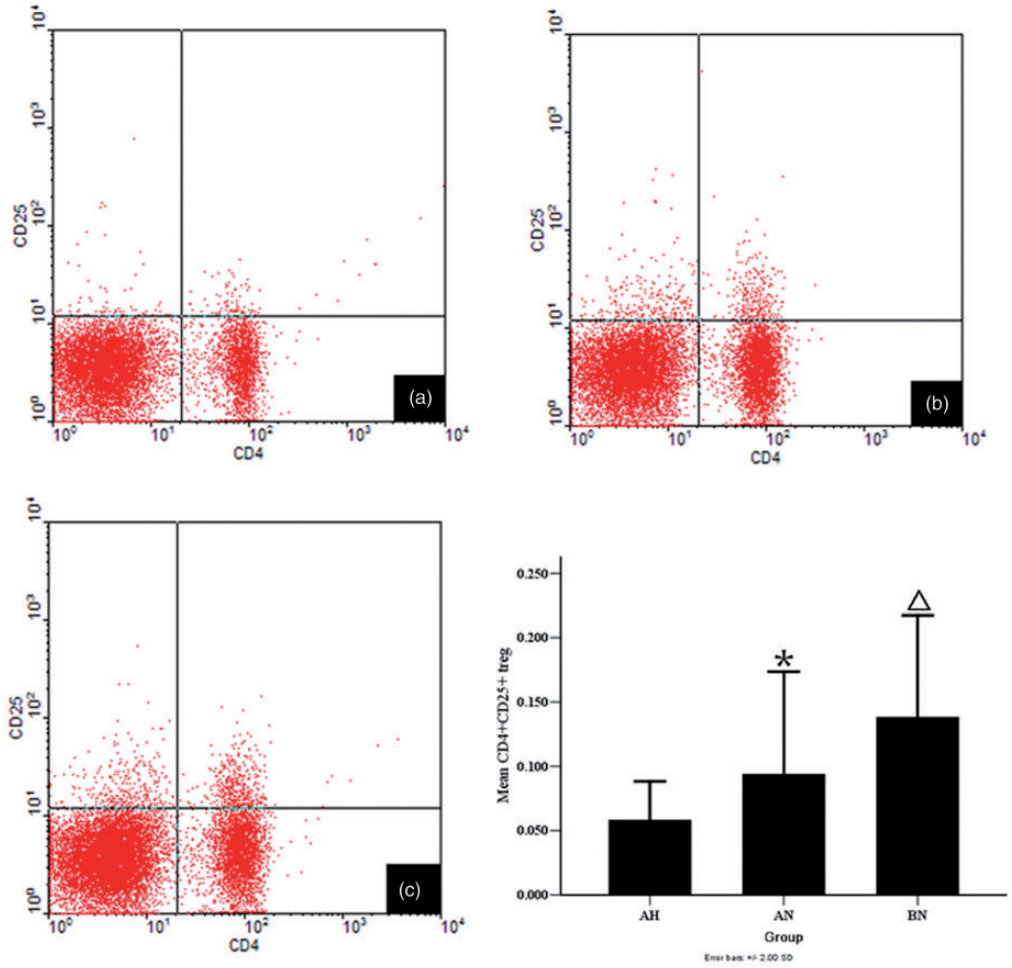

Figure 2 The proportion of $CD4^+CD25^+$ Tregs in a splenic cell suspension in different treatment groups. The proportion of $CD4^+CD25^+$ Tregs within splenic cell suspensions from the mice in AH group was lower than that in AN group ($P = 0.017$), and the proportion of $CD4^+CD25^+$ Tregs was lower in AN group than in BN group ($P = 0.023$). AH: $ApoE^{-/-}$ mice + high-fat diet; AN: $ApoE^{-/-}$ mice + normal diet; BN: control group; Treg: regulatory T cells. (A color version of this figure is available in the online journal.)

Table 4 The concentrations of TGF-β1, IL-10 (pg/mL) in peripheral blood and the ratio of $CD4^+CD25^+$ Treg cells (%) in spleen cells suspension in each group

Group	n	TGF-β1	IL-10	Ratio of $CD4^+CD25^+$ Treg
AH	10	$83.97 \pm 33.45^{\Delta}$	$27.50 \pm 11.54^{\Delta}$	$5.8 \pm 1.51^{\Delta}$
AN	10	$116.05 \pm 32.27^{*}$	$41.83 \pm 16.15^{*}$	$9.4 \pm 4.00^{*}$
BN	10	191.27 ± 95.27	61.84 ± 23.05	13.8 ± 3.97

AH: $ApoE^{-/-}$ mice + high-fat diet; AN: $ApoE^{-/-}$ mice + normal diet; BN: control group; Treg: regulatory T cells.
$^{\Delta}P < 0.05$, compared with AN; $^{*}P < 0.05$, compared with BN.

Table 5 The concentrations of total cholesterol, LDL, and triglycerides (mmol/L) in the peripheral blood in each group

Group	n	Total cholesterol	LDL	Triglycerides
AH	10	$21.82 \pm 3.6^{\Delta *}$	$11.89 \pm 4.51^{\Delta *}$	$2.89 \pm 1.01^{\Delta *}$
AN	10	$11.24 \pm 2.33^{*}$	$9.09 \pm 3.12^{*}$	$1.62 \pm 0.55^{*}$
BN	10	2.53 ± 1.02	0.80 ± 0.51	0.94 ± 0.22

AH: $ApoE^{-/-}$ mice + high-fat diet; AN: $ApoE^{-/-}$ mice + normal diet; BN: control group.
$^{\Delta}P < 0.05$, compared with AN; $^{*}P < 0.05$, compared with BN.

prevented the proliferation stimulated by CD3 cells and also reduced the induction of IL-10 production in T cells by threefold and TGF-β1 mRNA by fourfold, demonstrating that Tregs could induce the secretion of inhibitory cytokines and thus play a role in resistance to AS. Klein *et al.*[15] have established that $CD4^+CD25^+$ Tregs can produce IL-10 in *in vivo* experiments. The role of IL-10 in AS has been established previously; IL-10 inhibits antigen presentation, Th1- and Th2-mediated inflammatory responses, and inflammation of the blood vessel walls.

In experimental models of AS, IL-10 negatively regulated the oxidation of low-density lipoprotein (LDL) to stimulate monocytes to produce IL-12, thus blocking AS. Some studies have shown that feeding C57BL/6J mice defective in IL-10 expression a high-fat diet can promote early atherosclerotic plaque formation, characterized by increases in inflammatory cell infiltration (especially that

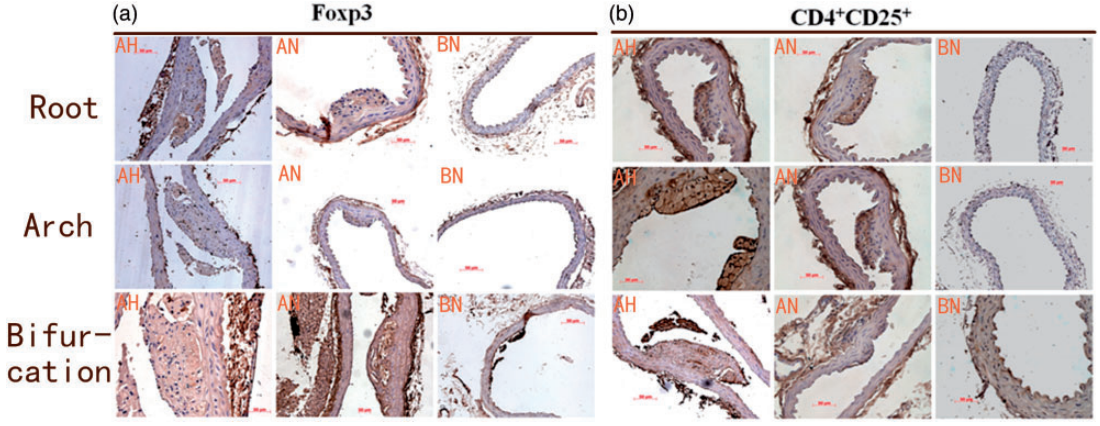

Figure 3 The numbers of Foxp3$^+$ and CD4$^+$CD25$^+$ cells in different treatment groups. Foxp3$^+$ and CD4$^+$CD25$^+$ cells were present in atherosclerotic plaques. However, none of these cells were observed in the intima of the mice in BN group. The proportion of Foxp3$^+$, CD4$^+$CD25$^+$ cells within the total lymphocyte population was significantly decreased (1.28 ± 1.20 versus 3.04 ± 1.92, $P = 0.024$; 2.00 ± 1.39 versus 3.98 ± 1.67, $P = 0.010$ in aortic root; 1.55 ± 0.98 versus 3.20 ± 1.46, $P = 0.032$; 2.13 ± 0.99 versus 3.86 ± 1.24, $P = 0.022$ in aortic arch; and 1.08 ± 0.76 versus 3.00 ± 1.31, $P = 0.012$; 1.45 ± 0.78 versus 3.45 ± 1.26, $P = 0.008$ in aortic, respectively) ((a) and (b)), bar represented 50 μm. BN: control group; Foxp3$^+$: forkhead box protein 3. (A color version of this figure is available in the online journal.)

of active T cells) and in the expression of proinflammatory factors.[16,17] Similar results were obtained in IL-10$^{-/-}$/ApoE$^{-/-}$ mice fed a high-fat diet.[18] In LDLr$^{-/-}$ mouse models, defective expression of IL-10 by bone marrow cells led to increased Th1-mediated inflammatory reactions, with the accumulation of T cells and macrophages in local plaques.[19] These studies clearly demonstrated that IL-10 could prevent AS by regulating both the systemic immune response and the proportion of cells and elastic fiber components in local plaques.[20] The role of IL-10 secretion by other cells and cell subtypes, such as macrophages, DCs, or T cells, in local plaques is unclear. Similar to previous studies, the results of the current study indicated that the serum levels of TGF-β1 were obviously decreased in AS mice.[18,21–23] TGF-β1 antibodies and soluble TGF-β1 receptor could accelerate AS plaque formation, increase the accumulation of inflammatory cells, and decrease the amount of elastic fiber components in local AS plaques in TGF-β1-deficient mice; this indicates that TGF-β1 plays a role in preventing AS.

The present experimental results indicate that the lack of Tregs in the spleens of AS mice accompanied by the decreased serum levels of IL-10 and TGF-β1 in the peripheral blood is correlated with the severity of AS lesions. A correlation between Treg cell numbers and the expression of IL-10 and TGF-β1 was not found in AS mice, but the proportion of CD4$^+$CD25$^+$ Tregs tended toward a decrease with the decreased expression of IL-10 and TGF-β1 in the spleens of AS mice. IL-10 and TGF-β1 are necessary for Treg cell function; both mediate the regulatory function of Tregs in AS and potentially regulate inflammatory factors in local plaques. Both of these proteins were decreased in the present study, which indicates that the anti-AS effect of Tregs was decreased in AS mice.

This study demonstrated that Foxp3$^+$CD4$^+$CD25$^+$ cells are present in atherosclerotic plaques. However, no Foxp3$^+$CD4$^+$CD25$^+$ cells were observed in the intima of the mice in BN group. When we compared the severe lesions in AH group with the mild lesions in AN group, we observed that the proportion of Foxp3$^+$CD4$^+$CD25$^+$ cells within all lymphocytes was significantly decreased in AH group. Foxp3 is a molecular marker that plays an important role in Tregs and maintains Tregs functions. Thus, the decreased expression of Foxp3 could influence the immunosuppressive function of Tregs.[24,25]

The present study shows that a small number of Foxp3-positive cells is present in local AS plaques, and the numbers of these cells decreased with increased AS lesion severity. Van Es *et al.*[26] have conducted a meaningful attempt to study whether the regulation of Foxp3 expression can help to treat AS; they found that Foxp3 vaccination may lead to a reduction in the number of Foxp3-positive cells in multiple organs, exacerbate early atherosclerotic plaques, and increase the number of inflammatory cells in local plaques, indicating that the expression of Foxp3 by Tregs plays an important role in the formation of AS plaques. Many studies[27–29] have shown that Foxp3-positive T cells are present in early human atherosclerotic plaques; these cells coexist with IL-10, TGF-β1, and the chemokine receptor CCR4, all of which are related with Tregs and regulated by the ligand CCL17.[30]

AS is considered an immune inflammatory disease, and the T cell-mediated immune inflammatory response plays an important role in the pathogenesis of AS. Tregs can inhibit T cell activation to limit or reduce the severity of the local inflammatory response. The numbers of Tregs migrating to local plaques increased in conjunction with the exacerbation of the T cell-mediated immune inflammatory response in local plaques, which could explain why Tregs were present in lesion-associated blood vessels but not in normal blood vessel walls. The present results indicate that the proportion of Tregs is very low (0.4–6%) in local plaques compared with normal or inflammatory skin tissue (24–28%),[31] but the mechanism underlying the decrease in Treg cell numbers in local AS plaques is unknown. It has been shown that ox-LDL inhibits Foxp3 expression and the

function of Tregs *in vitro*; therefore, the early presence of ox-LDL in AS plaques could suppress the expression of Foxp3, which may lead to a reduction in the numbers of Tregs in the local plaque environment.[8] In addition, this study confirmed that the numbers of Tregs in the spleens of AS mice are decreased significantly, and thus the numbers of Tregs directionally migrating to AS plaques are decreased. This is one of the reasons for the reduction in the number of Tregs in local plaques, but the exact mechanism remains to be elucidated in a future study.

Limitations

This study was a simple observational study with small samples. Due to lack of present methods, the evaluation of $CD4^+CD25^+$ Tregs in atherosclerotic plaques was performed by observational count on microscope images and expressed as a proportional data of $Foxp3^+CD4^+CD25^+$ cells within the total lymphocyte population, which inevitably carries certain measuring errors. This measuring error was equalized by consolidated calculation in every single sample, even though these data could not serve as an accurate measurement of $CD4^+CD25^+$ Tregs in the end. The results indicated that $CD4^+CD25^+$ Tregs were decreased both in the spleen and in atherosclerotic plaques, and this decrease was accompanied by a decrease in the concentration of IL-10 and TGF-β1 in the peripheral blood. However, further studies are needed to validate the relationship between these changes observed during AS. In the future, we intend to design a larger sampled intervention study with more accurate detection and calculation of $Foxp3^+CD4^+CD25^+$ cells in AS plaques.

Conclusions

The numbers of Tregs were obviously decreased in the spleen and in local plaques, and the concentrations of IL-10 and TGF-β1 were decreased in the peripheral blood. Furthermore, the expression of Foxp3 was decreased in local plaques in AS mice, indicating that the numbers of $CD4^+CD25^+$Tregs were decreased and that their immunosuppressive function was weakened in AS lesions. Overall, the data demonstrate that Tregs play an important role in the formation of atherosclerotic plaques.

Authors' contributions: LX-M and HC-L contributed equally to this work; LY-J designed the research; LX-M, HC-L, DX, and CJ performed the research; LY-J and LX-X analyzed the data; LX-M and HC-L wrote the paper.

ACKNOWLEDGEMENTS

This study was supported by the Science and Technology Program of Guangdong Province, China (2012B031800290), NSFC (81372021, 81571867), and the Science and Technology Foundation of Guangdong Province, China (S2013010015524). The funders had no role in the study design, data collection, research analysis, publishing decision, or manuscript preparation.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Frostegård J, Ulfgren AK, Nyberg P, Hedin U, Swedenborg J, Andersson U, Hansson GK. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* 1999;**145**:33–43
2. Methe H, Brunner S, Wiegand D, Nabauer M, Koglin J, Edelman ER. Enhanced T-helper-1 lymphocyte activation patterns in acute coronary syndromes. *J Am Coll Cardiol* 2005;**45**:1939–45
3. Eid RE, Rao DA, Zhou J, Lo SF, Ranjbaran H, Gallo A, Sokol SI, Pfau S, Pober JS, Tellides G. Interleukin-17 and interferon-gamma are produced concomitantly by human coronary artery-infiltrating T cells and act synergistically on vascular smooth muscle cells. *Circulation* 2009;**119**:1424–32
4. Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, Merval R, Esposito B, Cohen JL, Fisson S, Flavell RA, Hansson GK, Klatzmann D, Tedgui A, Mallat Z. Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med* 2006;**12**:178–80
5. Mallat Z, Gojova A, Brun V, Esposito B, Fournier N, Cottrez F, Tedgui A, Groux H. Induction of a regulatory T cell type 1 response reduces the development of atherosclerosis in apolipoprotein E-knockout mice. *Circulation* 2003;**108**:1232–7
6. Zhou X, Johnston TP, Johansson D, Parini P, Funa K, Svensson J, Hansson GK. Hypercholesterolemia leads to elevated TGF-beta1 activity and T helper 3-dependent autoimmune responses in atherosclerotic mice. *Atherosclerosis* 2009;**204**:381–7
7. Klingenberg R, Lebens M, Hermansson A, Fredrikson GN, Strodthoff D, Rudling M, Ketelhuth DF, Gerdes N, Holmgren J, Nilsson J, Hansson GK. Intranasal immunization with an apolipoprotein B-100 fusion protein induces antigen-specific regulatory T cells and reduces atherosclerosis. *Arterioscler Thromb Vasc Biol* 2010;**30**:946–52
8. Mor A, Planer D, Luboshits G, Afek A, Metzger S, Chajek-Shaul T, Keren G, George J. Role of naturally occurring CD4+ CD25+ regulatory T cells in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol* 2007;**27**:893–900
9. Mallat Z, Taleb S, Ait-Oufella H, Tedgui A. The role of adaptive T cell immunity in atherosclerosis. *J Lipid Res* 2009;**50**:S364–9
10. Taleb S, Tedgui A, Mallat Z. Regulatory T-cell immunity and its relevance to atherosclerosis. *J Intern Med* 2008;**263**:489–99
11. Mallat Z, Ait-Oufella H, Tedgui A. Regulatory T-cell immunity in atherosclerosis. *Trends Cardiovasc Med* 2007;**17**:113–8
12. Mallat Z, Ait-Oufella H, Tedgui A. Regulatory T cell responses: potential role in the control of atherosclerosis. *Curr Opin Lipidol* 2005;**16**:518–24
13. Nik TN, Hambly BD, Sullivan DR, Bao S. Forkhead box protein 3: essential immune regulatory role. *Int J Biochem Cell Biol* 2008;**40**:2369–73
14. Ait-Oufella H, Taleb S, Mallat Z, Tedgui A. Cytokine network and T cell immunity in atherosclerosis. *Semin Immunopathol* 2009;**31**:23–33
15. Klein L, Khazaie K, von Boehmer H. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc Natl Acad Sci USA* 2003;**100**:8886–91
16. Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, Soubrier F, Esposito B, Duez H, Fievet C, Staels B, Duverger N, Scherman D, Tedgui A. Protective role of interleukin-10 in atherosclerosis. *Circ Res* 1999;**85**:e17–24
17. Pinderski LJ, Hedrick CC, Olvera T, Hagenbaugh A, Territo M, Berliner JA, Fyfe AI. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 1999;**19**:2847–53
18. Caligiuri G, Rudling M, Ollivier V, Jacob MP, Michel JB, Hansson GK, Nicoletti A. Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Mol Med* 2003;**9**:10–7

19. Potteaux S, Esposito B, van Oostrom O, Brun V, Ardouin P, Groux H, Tedgui A, Mallat Z. Leukocyte-derived interleukin 10 is required for protection against atherosclerosis in low-density lipoprotein receptor knockout mice. *Arterioscler Thromb Vasc Biol* 2004;**24**:1474–8
20. Pinderski LJ, Fischbein MP, Subbanagounder G, Fishbein MC, Kubo N, Cheroutre H, Curtiss LK, Berliner JA, Boisvert WA. Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient mice by altering lymphocyte and macrophage phenotypes. *Circ Res* 2002;**90**:1064–71
21. Mallat Z, Gojova A, Marchiol-Fournigault C, Esposito B, Kamaté C, Merval R, Fradelizi D, Tedgui A. Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. *Circ Res* 2001;**89**:930–4
22. Lutgens E, Gijbels M, Smook M, Heeringa P, Gotwals P, Koteliansky VE, Daemen MJ. Transforming growth factor-beta mediates balance between inflammation and fibrosis during plaque progression. *Arterioscler Thromb Vasc Biol* 2002;**22**:975–82
23. Grainger DJ, Mosedale DE, Metcalfe JC, Böttinger EP. Dietary fat and reduced levels of TGFbeta1 act synergistically to promote activation of the vascular endothelium and formation of lipid lesions. *J Cell Sci* 2000;**113**:2355–61
24. Entin-Meer M, Afek A, George J. Regulatory T-cells, FoxP3 and atherosclerosis. *Adv Exp Med Biol* 2009;**665**:106–14
25. Ziegler SF. FOXP3: of mice and men. *Annu Rev Immunol* 2006;**24**:209–26
26. van Es T, van Puijvelde GH, Foks AC, Habets KL, Bot I, Gilboa E, Van Berkel TJ, Kuiper J. Vaccination against Foxp3(+) regulatory T cells aggravates atherosclerosis. *Atherosclerosis* 2010;**209**:74–80
27. Tomura M, Honda T, Tanizaki H, Otsuka A, Egawa G, Tokura Y, Waldmann H, Hori S, Cyster JG, Watanabe T, Miyachi Y, Kanagawa O, Kabashima K. Activated regulatory T cells are the major T cell type emigrating from the skin during a cutaneous immune response in mice. *J Clin Invest* 2010;**120**:883–93
28. Zhang N, Schröppel B, Lal G, Jakubzick C, Mao X, Chen D, Yin N, Jessberger R, Ochando JC, Ding Y, Bromberg JS. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. *Immunity* 2009;**30**:458–69
29. Belkaid Y, Tarbell KV. Arming Treg cells at the inflammatory site. *Immunity* 2009;**30**:322–3
30. Heller EA, Liu E, Tager AM, Yuan Q, Lin AY, Ahluwalia N, Jones K, Koehn SL, Lok VM, Aikawa E, Moore KJ, Luster AD, Gerszten RE. Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells. *Circulation* 2006;**113**:2301–12
31. de Boer OJ, van der Loos CM, Teeling P, van der Wal AC, Teunissen MB. Immunohistochemical analysis of regulatory T cell markers FOXP3 and GITR on $CD4^+CD25^+$ T cells in normal skin and inflammatory dermatoses. *J Histochem Cytochem* 2007;**55**:891–8

(Received August 10, 2016, Accepted December 18, 2016)