Regulatory T cells ameliorate hyperhomocysteinaemia-accelerated atherosclerosis in apoE−/− mice

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Aims Atherosclerosis is an inflammatory disease with T cell-driven immunoinflammatory responses contributing to disease initiation and progression. We investigated the potential role of regulatory T cells (Tregs) in hyperhomocysteinaemia (HHcy)-accelerated atherosclerosis in apoE−/− mice.

Methods and results apoE−/− mice were fed normal mouse chow supplemented with or without a high level of homocysteine (Hcy) (1.8 g/L) in drinking water for 2, 4, and 6 weeks. Atherosclerotic lesion area was slightly increased at 2 weeks and substantially elevated at 4 and 6 weeks in HHcy apoE−/− mice. Cotransfer of normal Tregs significantly attenuated atherosclerotic lesion size and infiltration of T cells and macrophages into plaque. Furthermore, Treg cotransfer reversed HHcy-accelerated proliferation of T cells, increased pro-inflammatory, and decreased anti-inflammatory cytokine secretion from activated splenic T cells. With a clinically relevant level of plasma Hcy, the proportion of Tregs and suppressive activity in splenic T cells were reduced in HHcy apoE−/− mice, which was associated with reduced mRNA and protein expression of Foxp3, a factor governing mouse Treg development and function. In addition, Hcy significantly attenuated the proportion and suppressive effects of Tregs in vitro.

Conclusion HHcy suppresses the function of Tregs, which may be responsible for HHcy-accelerated atherosclerosis in apoE−/− mice.

1. Introduction

Atherosclerosis is known as a chronic inflammatory disease in which T lymphocytes play an important role.1–3 Atherosclerotic lesions contain T cells, macrophages, and smooth muscle cells, which produce cytokines, growth factors, and other pro-inflammatory mediators in response to hypercholesterolaemia. T cells, including Th1, Th2, or natural killer T cells, may contribute to the development and/or progression of atherosclerosis. Most CD4+ T cells of murine and human atherosclerotic plaques are the Th1-cell type, producing interleukin 2 (IL-2) and interferon γ (IFN-γ).4 Several experimental studies have clearly shown a critical pathogenic role for Th1-mediated responses in atherosclerosis.5–8 However, a subtype of T cells, regulatory T cells (Tregs, CD4+CD25+), have recently been shown to play a critical role in maintaining immunological tolerance against self and non-self antigens and preventing the development of various immunoinflammatory diseases. Transcription factor Foxp3 governs mouse CD4+CD25+ Treg development and function. Emerging data have revealed that Tregs can exert their suppressive role on atherosclerosis in mouse9,10 and human.11 During this process, an imbalance between number of pathogenic T cells and Tregs in response to ‘altered’ self antigens may lead to reciprocal and mutual amplification of the innate and adaptive immune responses responsible for plaque development.4,12–15

Homocysteine (Hcy) is a sulfur-containing amino acid formed during the metabolism of methionine. Hyperhomocysteinaemia (HHcy) has been implicated as an independent risk factor for atherosclerosis.16,17 HHcy as a potent pro-inflammatory factor might accelerate the development of atherosclerosis.18,19 We previously demonstrated that pathophysiological levels of Hcy could interfere with human monocyte function by up-regulating monocyte chemotactic protein 1 and IL-8 expression and secretion via oxidative stress.20 Hcy potentiates mitogen-induced proliferation of mouse T lymphocytes, in which reactive oxygen species (ROS) generated by the thiol (-SH) of Hcy auto-oxidation are involved.21 HHcy accelerates atherosclerosis development in the immunoinflammatory mechanism of apoE−/− mice.21,22 However, whether Tregs participate in HHcy-accelerated atherogenesis is unknown. In the present study, we aimed to investigate the contribution of Tregs to atherosclerotic lesion development and Th1-mediated response in an HHcy model.

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apoE−/− mice significantly attenuated T-cell hyperresponsiveness, T-cell and macrophage infiltration, and lesion formation in aortas.

2. Methods

2.1 Animals and treatments

Female apoE−/− and C57BL/6J mice, 6 weeks old, were provided by the Animal Center of Peking University Health Science Center (Beijing, China). apoE−/− mice were fed normal mouse chow supplemented with or without 1.8 g/L DL-Hcy (Sigma Chemical Co., St Louis, MO, USA) in drinking water for 2, 4, and 6 weeks, as we previously reported with some modifications. The treatment of the laboratory animals and experimental protocol followed the guidelines of Peking University and were approved by the Institutional Authority for Laboratory Animal Care.

2.2 Cell isolation and flow cytometry

Splenocytes were collected from mouse spleens, and cell suspensions were prepared by passing cells through a nylon mesh after removal of erythrocytes by ACK lysing buffer (155 mM NH4Cl, 0.1 mM EDTA-Na2, and 10 mM KHCO3, pH 7.5). T cells were purified by positive immunomagnetic cell sorting with use of standard protocols and CD90 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The sorted cell populations were >98% pure on FACS reanalysis. For FACS assay, T cells were co-stained with the following antibodies: fluorescein-isothiocyanate-labelled anti-CD4 (GK1.5), phycoerythrin-labelled anti-CD25 (7D4, both Miltenyi Biotech), fluorescein-isothiocyanate-labelled rat IgG2b isotypic control (A95-1), and phycoerythrin-labelled rat IgM isotypic control (R4-22, both BD Pharmingen, USA). Stained cells were analysed by FACSScan flow cytometry with Cell QuestPro software (BD Biosciences, USA).

CD4+CD25+ cells from murine spleen were isolated by use of a CD4+CD25+ regulatory T-cell isolation kit (Miltenyi Biotech) according to the manufacturer’s instructions. The CD4+CD25+ cells, which did not bind to the beads, were harvested from the flow-through. The purity of all cell subsets processed by this method was determined by FACS analysis (data not shown).

2.3 Western blot analysis of Foxp3

A total of 3 × 105 purified T cells were lysed. Proteins underwent SDS-PAGE with a 10% running gel and were electrophoretically transferred onto a nitrocellulose membrane. The membrane was incubated with 5% skim milk in Tris Tween-buffered saline at room temperature for 1 h, with polyclonal anti-Foxp3 antibody (eBioscience, San Diego, CA, USA) at a dilution of 1:500 at 4°C for 12 h and then with IRDye™-conjugated secondary antibody for 1 h successively. Immunofluorescence bands were detected by the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

2.4 RNA extraction and RT–PCR for Foxp3

Total RNA from primary mouse T cells from spleens was isolated by use of TRIzol reagent (Promega, Madison, WI, USA) and reverse transcribed by the reverse transcription system (Promega), then the reaction mixture underwent real-time PCR. The amount of PCR products formed in each cycle was evaluated by SYBR Green I fluorescence. Primers for mouse Foxp3 were as follows: sense, CACTCAGCCACAACACTCTGG; antisense, GTATCCGCTTTCTCCGCTG. All amplification reactions involved use of the Mx3000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA). Results were analysed with use of Stratagene Mx3000 software.

2.5 Cell culture and cytokine assays

For co-culture experiments, 96-well plates (Nunc) were coated with anti-CD3 monoclonal antibody (145-2C11, BD Pharmingen) 0.5 μg/mL overnight at 4°C and washed with phosphate-buffered saline three times. CD4+CD25+ (effector) and CD4+CD25− (regulatory) T cells (5 × 105 cells/well) were cultured in RPMI1640 medium supplemented with 10% foetal bovine serum at different ratios of regulatory to effector cells (1:1, 1:2, and 1:4). All cells were cultured in a final volume of 200 μL at 37°C in a humidified atmosphere of 5% CO2 for 72 h. [3H]-thymidine (TDR) (1 μCi/well) was added for 16 h before proliferation was assayed. The cells were then harvested onto glass filter paper, and radioactivity was assayed with the use of a liquid scintillation counter and presented as counts per minute (CPM).

For lymphocyte proliferation assays, we cultured purified T cells at 3 × 105 cells/well with or without Hcy for 48 h in anti-CD3-antibody-coated (0.5 μg/mL) microplates. All cultures were performed in triplicate. [3H]-Tdr (1 μCi/well) was added to each culture 6 h prior to analysis by liquid scintillation counter and presented as CPM. ELISA assays (JingMei Biotech, Shenzhen) were used to measure the secretion of IL-2, IFN-γ, IL-17, IL-10, and transforming growth factor β (TGF-β) in supernatant.

2.6 In vivo adoptive transfer study

CD4+CD25− Tregs (1 × 106) were purified from C57BL/6J mice, suspended in saline, and successively transferred into 6-week-old female apoE−/− mice by injection through the tail vein. A total volume of 200 μL saline with or without cells was injected per animal. Mice were euthanized 2, 4, and 6 weeks after the initial injection. Splenocytes were obtained for further experiments. Plasma was obtained for assessment of lipid profiles, and aortic roots were frozen for morphology analysis by Oil red O staining. For quantification of atherosclerotic lesion area, the aortic root was cross-sectioned serially at 7 μm intervals, with every other section collected on glass slides. Six sections, spaced 70 μm apart and thus spanning 350 μm of the aortic root starting at the level where the aortic valve leaflets were joined and upwards, were stained with Oil red O to visualize lesion areas. Atherosclerotic lesion areas were measured blindly by one observer using Quantity One software, and the mean lesion areas from six sections for each mouse were calculated. In addition, we studied the presence of macrophages and T lymphocytes in plaque using specific anti-Mac-3 (Santa Cruz Biotechnology, USA) and anti-CD4 (BD Pharmingen) antibodies, respectively. Collagen content in lesion was determined on Sirius red staining.

2.7 Lipid profile

Total plasma Hcy level was quantified by means of gas chromatography–mass spectrometry. Plasma total triglyceride and cholesterol levels were assayed by the use of kits from Zhong Sheng Bio-technology (Beijing, China).

2.8 Statistical analysis

All data are reported as mean ± SEM unless otherwise stated. Data were analysed by the use of GraphPad Prism software. Statistical analysis involved one-way ANOVA for multiple comparisons and the Student’s unpaired t-test for comparisons between two groups. A value of P < 0.05 was considered statistically significant.
3. Results

3.1 Adoptive transfer of Tregs reduced atherosclerotic plaque size and infiltration of macrophages and T cells into plaque

We have previously shown that HHcy accelerates atherosclerosis development during the early stages in apoE<sup>−/−</sup> mice, and T lymphocytes and macrophages may be involved. Oil red O staining was used to examine the lipid composition in root areas of aortas with adoptive Treg cell transfer to explore the role of Tregs during this process. apoE<sup>−/−</sup> mice with a clinically relevant level of plasma Hcy (see Supplementary material online, Table S1), showed slight development of atherosclerotic lesions at 2 weeks and substantial elevated at 4 and 6 weeks (Figure 1A and Supplementary material online, Figure S1A–C). These alterations were associated with increased infiltration of T cells (Figure 1B) and macrophages (Figure 1C) into the plaque. The groups did not differ in body weight (data not shown) or plasma triglyceride or total cholesterol levels (see Supplementary material online, Table S1). Cotransfer of normal Tregs from C57BL/6J mice significantly attenuated atherosclerotic lesion size (Figure 1A) and infiltration of T cells (Figure 1B) and macrophages (Figure 1C) into plaque in HHcy apoE<sup>−/−</sup> mice. However, the collagen content in lesions was not affected by Treg transfer (Figure 1D).

3.2 Adoptive transfer of Tregs reversed the HHcy-accelerated proliferation of T cells

T cells can be polyclonal activated, regardless of specificity. Anti-CD3 antibody and concanavalin A (ConA) are commonly used as polyclonal T-cell activators. Our previous work suggested that HHcy may be involved in the pathogenesis of atherosclerosis by enhancing ConA-induced T-cell proliferation. In the present study, we used anti-CD3 antibody to activate T cells and found that Hcy increased the proliferation of T lymphocytes in vitro as well (data not shown). Since Tregs negatively regulate the function of Th1 and Th2 effector cells, we performed an in vivo adoptive cell transfer study to determine whether Treg transfer could attenuate HHcy-increased T-cell proliferation. T-cell proliferation rate was significantly increased in apoE<sup>−/−</sup> mice with HHcy at 2 (Figure 2A), 4 (Figure 2B), and 6 weeks (Figure 2C). However, T-cell proliferation was significantly lower in HHcy apoE<sup>−/−</sup> mice receiving normal Tregs from C57BL/6J mice than in those receiving saline, at 2 (Figure 2A), 4 (Figure 2B), and 6 weeks (Figure 2C).

3.3 HHcy decreased the proportion of Tregs in splenic T cells in vivo

Since adoptive transfer of Tregs could reduce atherosclerotic plaque size and reverse the HHcy-promoted proliferation of T cells, we hypothesized that Hcy treatment might alter Treg frequency and accelerate atherosclerosis. In agreement

Figure 1  Adoptive transfer of Tregs reduced atherosclerotic plaque size and infiltration of macrophages and T cells into plaque. apoE<sup>−/−</sup> mice, 6 weeks old, were fed normal mouse chow with or without water supplemented with Hcy (1.8 g/L Hcy) for 2, 4, and 6 weeks. Serial sections of dissected aortic roots were stained with Oil red O (magnification ×100), anti-Mac-3 antibody, anti-CD4 antibody, and Sirius red (magnification ×200). (A) Quantification of the mean aortic root lesion areas in each group. (B) Representative photomicrographs of T-cell infiltration in lesions. Arrows indicate CD4<sup>+</sup> T cells. (C) Representative photomicrographs of macrophage infiltration in lesions. (D) Representative photomicrographs of collagen in lesions. *P < 0.05 compared with control. #P < 0.05 compared with HHcy group.
with this hypothesis, the proportion of CD4^+CD25^{high} T cells in splenic T cells was reduced, by 41.4, 60, and 27.7%, at 2 (Figure 3A), 4 (Figure 3B), and 6 weeks (Figure 3C), respectively, in HHcy apoE2/2 mice.

3.4 Foxp3 mRNA and protein levels were down-regulated in HHcy apoE2/2 mice

Since Foxp3 is not only a faithful marker for CD4^+CD25^{+} Tregs but also is necessary and sufficient for their development and function,24,25 we tested the effect of HHcy on Foxp3 mRNA expression by real-time PCR and protein expression by western blot analysis in T cells from murine spleens both in vitro and in vivo. Foxp3 mRNA and protein expressions in T cells were reduced at 2 (Figure 4A and E), 4 (Figure 4B and F), and 6 weeks (Figure 4C and G) in apoE2/2 mice treated with HHcy. In addition, Foxp3 mRNA and protein expressions in T cells were significantly decreased after treatment with Hcy 30 μM for 48 h in vitro (Figure 4D and H). These results suggest that reduced Foxp3 expression is responsible for HHcy-induced Treg down-regulation in apoE2/2 mice, and the decreased Foxp3 protein expression was due, at least in part, to reduced mRNA expression.

3.5 HHcy impaired the suppressive function of Tregs in vitro and in vivo

To investigate the HHcy effect on the suppressive function of Tregs, we mixed sorted CD4^+CD25^{+} Tregs and CD4^+CD25^{−} effector T cells (Teffs) in different ratios in the presence or absence of HHcy (30 μM) for 72 h and proliferation or cytokine expression was measured by co-culture assay, which has been well validated to assess Treg function. The in vivo co-culture assay revealed Teff proliferation was greater in apoE2/2 mice treated with HHcy than in controls at 2 (Figure 5A), 4 (Figure 5B), and 6 weeks (Figure 5C), which suggests that the function of Tregs was impaired in HHcy apoE2/2 mice. The in vitro co-culture assay revealed Teff proliferation enhanced by 30 μM Hcy treatment for 72 h, which indicates that the suppressive activity of CD4^+CD25^{+} Treg was decreased as well (Figure 5D). In addition, CD4^+CD25^{+} Teffs showed normal proliferation and CD4^+CD25^{+} Tregs...
Foxp3 mRNA and protein level were down-regulated in HHcy apoE−/− mice. Quantitative Foxp3 mRNA expression (relative to that of GAPDH) of T lymphocytes purified from murine spleens using specific magnetic beads in apoE−/− mice with and without (control) HHcy at 2 (A), 4 (B), and 6 weeks (C). (D) Effect of Hcy (with or without 30 μM Hcy treatment for 48 h) on Foxp3 mRNA expression in primary T lymphocytes from C57BL/6J mice by real-time PCR (n = 3). Western blot analysis of Foxp3 protein expression was determined in splenic T lymphocytes from apoE−/− mice with and without (control) HHcy at 2 (E), 4 (F), and 6 weeks (G). (H) Western blot analysis of Foxp3 protein expression was determined in splenic T lymphocytes from C57BL/6J incubated with Hcy (30 μM) in the presence of anti-CD3 antibody for 48 h. Denitometric analysis of Foxp3 protein expression (relative to eIF5) is shown in the right panel. *P < 0.05 compared with control.
showed lower proliferation than did controls after treatment with 30 μM Hcy for 72 h (Figure 5D), which indicates that Hcy directly inhibits Treg but not Teff function in vitro. In vivo, HHcy treatment attenuated Treg activity and promoted Teff proliferation (Figure 5A–C).

3.6 HHcy increased pro-inflammatory and decreased anti-inflammatory cytokine production from activated splenic T cells in early atherosclerosis

To further study the mechanisms of the HHcy-induced inflammatory responses, we investigated whether Hcy could affect the secretion of cytokines from activated T cells. ELISA assay revealed significantly increased IL-2, IFN-γ, and IL-17 levels (Figure 6A–C) but decreased IL-10 and TGF-β levels at 2 (Figure 6E and I), 4 (Figure 6F and J), and 6 weeks (Figure 6G and K) in activated splenic T cells from apoE−/− mice with HHcy. In response to anti-CD3-antibody stimulation, purified splenic T cells from mice treated with normal Tregs showed lower levels of IL-2, IFN-γ, and IL-17 (Figure 6A–C) and higher levels of IL-10 (Figure 6E–G) and TGF-β (Figure 6I–K) than did T cells from mice without Treg cotransfer. In vitro, Hcy treatment resulted in enhanced IL-2, IFN-γ, and IL-17 secretion by T cells (Figure 6D), but IL-10 production by activated T cells was not affected (Figure 6H). Within a concentration range of 30–300 μM Hcy, TGF-β production by activated T cells was slightly reduced (Figure 6I).

4. Discussion

As a potent pro-inflammatory factor, HHcy accelerates the development of atherosclerosis by promoting inflammation both in vitro and in vivo.20,22 T-cell- and macrophage-produced cytokines may play an important role in this process. However, different subsets of T cells exert their effect on the initiation of HHcy-accelerated atherosclerosis is unknown. In the current study, we demonstrated that HHcy leads to reduced proportion and suppressive function of Tregs, with increased atherosclerotic lesion area and accumulation of macrophages and T cells in plaques of apoE−/− mice. Importantly, the adoptive transfer of Tregs from age-matched normal C57BL/6J mice to HHcy apoE−/− mice significantly abrogated the atherosclerotic lesions and attenuated the HHcy-induced infiltration of macrophages and T cells in plaques. Our data demonstrate for the first time that HHcy-induced Treg reduction in proportion and function may be responsible, at least in part, for HHcy-accelerated atherosclerosis in apoE−/− mice.

Atherosclerosis is a chronic inflammatory disease.1 As a potent pro-inflammatory factor, HHcy can accelerate atherosclerotic development in the early stages of hypercholesterolemia.22 In the process, HHcy causes significant promotion of ConA-induced proliferation of T lymphocytes21 and lipopolysaccharide-induced proliferation of B lymphocytes.26 An increasing body of evidence suggests that Tregs, which control immunological tolerance, suppress both Th1 and Th2 immune responses and prevent the development of various immunoinflammatory diseases and may play an important role in controlling atherosclerosis development. Treg depletion by the use of an anti-CD25 antibody also enhances atherosclerosis in apoE−/− mice.9 de Boer et al.10 recently reported low levels of Tregs in all developmental stages of human plaques, which highlight the relevance of this T-cell subset in human atherosclerosis.
HHcy increased pro-inflammatory and decreased anti-inflammatory cytokine production from activated splenic T cells in early atherosclerosis. In lymphocyte proliferation assays, IL-2, IFN-γ, IL-17, IL-10, and TGF-β production in the supernatant was measured by ELISA, respectively. Purified spleen-derived T cells were isolated from apoE−/− mice with and without (control) HHcy at 2 (A, E, and I), 4 (B, F, and J), and 6 weeks (C, G, and K). Purified T cells from C57BL/6J mice (D, H, and L) were cultured with or without Hcy (30–300 μM) for 48 h in anti-CD3 antibody-coated microplates (n = 3 for each). All cultures were performed in triplicate. *P < 0.05 compared with control. †P < 0.05 compared with HHcy group.
Both the number and functional properties of Tregs are important in mediating immune and inflammatory responses. Experimental models revealed an impaired Treg-regulated immune mechanism in atherosclerosis. We hypothesize that HHcy may alter the number and function of Tregs, thus leading to accelerated atherosclerosis. In support of this hypothesis is our finding that transfer of Tregs from normal age-matched C57BL/6J mice significantly reduced atherosclerotic lesion area in HHcy apoE−/− mice and reversed the HHcy-enhanced proliferation of T cells. Notably, cotransfer of Tregs resulted in a marked reduction in infiltration of T cells and macrophages into plaques, which suggests reduced plaque inflammation. This finding, validated by measuring plaque size in the aortic roots and analysis of lesion macrophage and T-cell infiltration, suggests that the normal function of Tregs may be essential for maintaining the homeostasis of different subsets of T cells in the vessel wall.

In agreement with our findings that HHcy treatment significantly reduced the proportion of Tregs in splenic T cells of apoE−/− mice, others have reported on a low number of Tregs in human atherosclerotic lesions. However, how the number and function of Tregs are regulated during atherosclerosis is unclear. Because Foxp3 expression is strongly associated with a Treg phenotype and is pivotal for the acquisition of regulatory properties, we reasoned that HHcy-induced Treg reduction in number and function might be mediated by Foxp3. HHcy indeed down-regulated the mRNA and protein expression of Foxp3 in murine splenic T cells, which suggests that reduced Foxp3 expression is responsible for HHcy-induced Treg reduction in apoE−/− mice.

apoE−/− mice usually have hypercholesterolaemia. We wondered whether the effect of HHcy on Tregs was influenced by a hypercholesterolaemic environment. We found the concentration of total plasma cholesterol significantly elevated in apoE−/− mice, regardless of Hcy supplementation (see Supplementary material online, Table S1). However, total cholesterol levels were not changed among all groups. So the effect of HHcy was not associated with severe hypercholesterolaemia. Furthermore, we investigated the HHcy effect on Tregs in C57BL/6J mice with normal plasma total cholesterol level and found the Treg number reduced and suppressive activity impaired in such mice (data not shown). These data indicate that Hcy alone inhibited the number and function of Tregs and the HHcy-induced Treg suppressive function in apoE−/− mice may not be associated with hypercholesterolaemia.

Several experimental studies have clearly shown a critical pathogenic role for a Th1-cell-mediated response in atherosclerosis by their secreting pro-inflammatory cytokines such as IL-2 and IFN-γ. IL-10 and TGF-β can mediate the Treg suppressive function in pathogenic T cells in vivo, although other cell–cell contact mechanisms of suppression have been identified in vitro. The adopted transfer of Tregs with specific deletion of TGF-β fails to inhibit T-cell-induced colitis in vivo. Disruption of TGF-β signalling in T cells accelerates atherosclerosis. In addition, stimulation of peripheral CD4+CD25+ T lymphocytes with TGF-β can up-regulate Foxp3 expression and result in a suppressive function in vitro, which indicates that TGF-β is required for Treg activation/induction. In our study, incubation of Tregs with Teffs at different ratios revealed that HHcy significantly reduced the suppressive properties of Tregs, as evidenced by enhanced proliferation of effector CD4+CD25− cells both in vitro and in vivo. In addition, the production of the anti-inflammatory cytokines TGF-β and IL-10 in the culture by activated T cells was decreased but that of the pro-inflammatory cytokines IL-2, IFN-γ, and IL-17 was increased in HHcy apoE−/− mice. Therefore, our data indicate that decreased Treg function may be responsible for the initiation of atherosclerosis in the early stage of the disease. Our data provide an explanation for HHcy attenuating Treg activity and accelerating atherosclerosis by inhibiting TGF-β and IL-10 secretion and promoting IL-2, IFN-γ, and IL-17 production from activated T cells in vivo.

Superoxide anions are present in the aortic wall of HHcy mice. Our group has reported that Hcy increases the production of ROS from T lymphocytes. The potential effect of Hcy on ConA-induced T-cell proliferation is significantly reduced by antioxidants. Furthermore, increased ROS level generated by the thiol (-SH) of Hcy auto-oxidation can damage and modify proteins, for example, by inducing the production of oxidized-LDL, which results in activation of specific T cells and contributes to the development of atherosclerosis. Recent studies have shown that the effect of oxidized-LDL on atherosclerosis is associated with reduced frequency and compromised suppressive function of Tregs. Induced oral tolerance to oxidized-LDL resulted in the suppression of early atherosclerosis, which may be explained by a significant increase in Treg level. Thus, oxidative stress may be one of the mechanisms of HHcy-induced reduction in Treg level and accelerated atherosclerosis. Our laboratory is working on further understanding the mechanism of HHcy-induced Treg reduction in atherosclerosis.

In summary, a specific subset of CD4+ T cells, Tregs, play an important role in the initiation of HHcy-accelerated atherosclerosis. The proportion and suppressive function of Tregs are reduced in HHcy apoE−/− mice, which suggests that HHcy modulates atherosclerosis through its effect on Treg responses. Transfer of normal Tregs in an atherosclerotic model results in insignificant attenuation of atherosclerosis. These studies suggest novel potential therapeutic avenues for atherosclerosis in terms of modulation of Treg response. As is known, regulatory pathways are likely to be complex. Further investigations are needed to clarify the exact mechanism by which Tregs ameliorate HHcy-accelerated atherosclerosis.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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References