

Review

## Immunoregulatory effects of homocysteine on cardiovascular diseases

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**Abstract:** Hyperhomocysteinemia (HHcy) has been recognized as an independent risk factor for atherosclerosis for more than 30 years, but the mechanisms by which HHcy leads to atherosclerosis are not well fully understood. In this review, we will summarize the immunoregulatory effects of homocysteine on cardiovascular diseases from humoral immunity, monocyte/macrophage and T lymphocyte activity. Homocysteine can induce chemokine and cytokine secretion in monocytes and T lymphocytes and also directly stimulate B lymphocyte proliferation and IgG secretion. In addition, the cellular mechanisms that may explain the pro-inflammatory effect of HHcy are included. Homocysteine may directly or indirectly lead to oxidative stress or endoplasmic reticulum (ER) stress. Elevated levels of homocysteine also decrease the bioavailability of nitric oxide and modulate the levels of other metabolites including *S*-adenosyl methionine and *S*-adenosyl homocysteine which may result in cardiovascular diseases.

**Key words:** homocysteine; inflammation; cardiovascular diseases

## 同型半胱氨酸在心血管疾病中的免疫调节作用

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**摘要:** 高同型半胱氨酸血症是动脉粥样硬化的独立危险因素, 但是其致病机制尚未完全阐明。本文将从体液免疫、单核巨噬细胞以及T细胞活性等几方面归纳总结同型半胱氨酸在心血管疾病中的免疫调节作用。同型半胱氨酸可以诱导单核细胞和T细胞分泌趋化因子和细胞因子, 还可以直接刺激B细胞增殖及IgG分泌。此外, 本文还总结了高同型半胱氨酸致炎作用的细胞内机制。同型半胱氨酸可以直接或间接导致氧化应激或者内质网应激, 还可以降低一氧化氮的生物活性, 影响包括*S*-腺苷蛋氨酸和*S*-腺苷同型半胱氨酸的水平, 从而导致心血管疾病的发生。

**关键词:** 同型半胱氨酸; 炎症; 心血管疾病

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### 1 Introduction

Atherosclerosis is the leading cause of cardiovascular morbidity and mortality in the world. It is not simply an inevitable degenerative consequence of aging but is, rather, a chronic inflammatory disease. The inflammation theory of atherosclerosis was raised by Virchow in 1856 for the first time. And it is now widely recognized as a disease characterized by inflammatory reaction in the inner-wall of large- and medium-sized arteries, especially in areas of arterial

branching and non-laminar blood flow. There are many kinds of immune cells can be detected in atherosclerotic lesions, such as monocytes/macrophages, T lymphocytes, mast cells and few B lymphocytes. Leukocyte recruitment and expression of pro-inflammatory cytokines may accelerate inflammatory reaction further<sup>[1]</sup>.

Hyperhomocysteinemia (HHcy) has been recognized as an independent risk factor for atherosclerosis for more than 30 years, but the mechanisms by which HHcy leads to atherosclerosis are not well fully elucidated<sup>[2]</sup>. In this

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review, we will summarize the immunoregulatory effects of homocysteine (Hcy) on cardiovascular diseases. In addition, the cellular mechanisms that may explain the pro-inflammatory effects of HHcy are included.

## 2 Overview of Hcy and vasculature

Hcy was first described by Butz and du Vigneaud in 1932<sup>[3]</sup>. An association between elevated Hcy levels and human diseases was first suggested in 1962 by Carson and Neil<sup>[4]</sup>. They had found high concentration of Hcy in the urine of some children with mental retardation. The elevated Hcy levels in these patients were caused by severe enzyme defects blocking the Hcy metabolism. The inborn error of Hcy metabolism which caused homocystinuria was later found to be associated with premature occlusive cardiovascular diseases, even in childhood, and about 25% of the patients died before the age of 30 in cardiovascular events. Since McCully, in 1969, linked elevated plasma Hcy concentration with vascular diseases, many investigations have been conducted to find the mechanisms by which atherothrombotic complications were induced in HHcy<sup>[5]</sup>. HHcy is now defined as a pathological condition characterized by an increase in plasma concentration of total Hcy that is higher than 15  $\mu\text{mol/L}$ . Pathophysiologic harmful effects of HHcy are very complex, including (1) causing endothelial injury or dysfunction; (2) inducing the proliferation of vascular smooth muscle cells; (3) promoting platelet accumulation and platelet-rich thrombus formation; (4) impairing bioavailability of nitric oxide (NO) and so on.

## 3 Endothelial cells interact with blood cells

Endothelial dysfunction is commonly detected in the early stage of atherosclerosis. Hcy has direct toxic effect on endothelial cells. The von Willebrand factor (vWF) and thrombomodulin (TM) can be detected in the plasma of HHcy patients<sup>[6]</sup>. At the meanwhile, pyridoxine plus folic acid treatment appears to ameliorate endothelial dysfunction through normalizing Hcy metabolism in these patients. Many studies using animal models and human subjects have demonstrated that HHcy impaired flow-mediated endothelium-dependent vasodilatation by inhibiting endothelium-derived NO<sup>[7]</sup>. Moreover, Hcy could induce cell cycle G<sub>1</sub> phase arrest in endothelial cells via inhibiting the PI3K/Akt pathway<sup>[8]</sup>.

In normal status, there are few white blood cells adhering to endothelial cells. On Hcy-induced injury, endothelial

cells are activated and are capable of producing various adhesion molecules and chemokines such as, vascular cell adhesion molecule (VCAM-1)<sup>[9,10]</sup>, intercellular adhesion molecule-1 (ICAM-1)<sup>[11]</sup>, E-selectin<sup>[10]</sup>, P-selectin<sup>[12]</sup>,  $\beta$ 1-integrin<sup>[13]</sup>, interleukin-8 (IL-8)<sup>[10,14]</sup>, monocyte chemoattractant protein-1 (MCP-1)<sup>[14]</sup> that participate in inflammatory reactions in the arterial wall. Hcy also activates IkB- $\alpha$  resulting in nuclear translocation of NF- $\kappa$ B and enhanced NF- $\kappa$ B/DNA interaction in endothelial cells, thus, causing an imbalance in intracellular signaling.

Endothelial dysfunction ultimately loses a balance between the magnitude of injury and the capacity for repair. A variety of evidence suggested that circulating endothelial progenitor cells (EPCs) constituted one aspect of this repair process<sup>[15,16]</sup>. Hcy impaired EPC proliferative, migratory, adhesive and *in vitro* vasculogenesis capacity<sup>[17]</sup>. HHcy may induce the reduction in EPCs with decreased functional activity. HHcy not only directly impairs endothelial cells but also affects EPC number and function simultaneously, thus influence endothelial repair process and disturb the balance between the magnitude of injury and the capacity for repair, which lead to endothelial dysfunction.

## 4 Monocyte/macrophage activity

The recruitment of monocytes/macrophages into the artery wall is one of the earliest and key events in the pathogenesis of atherosclerosis. The cell recruitment is mainly regulated by adhesion molecules and chemokines. The most notable chemokines MCP-1, a prototype of CC chemokines, and IL-8, a prototype of CXC chemokines, were found to be highly expressed in human atherosclerotic lesions which stimulate the migration of monocytes, T lymphocytes and neutrophils into the intima of the arterial wall<sup>[18]</sup>. Gu *et al.*<sup>[19]</sup> reported that the absence of MCP-1 greatly decreased the lesion size in LDLR<sup>-/-</sup> mice. Similarly, the absence of MCP-1 receptor and CC chemokine receptor 2 (CCR2) caused a reduction in lesion size in apoE<sup>-/-</sup> mice<sup>[20]</sup>.

Previous studies have suggested that Hcy could induce expression and secretion of MCP-1 and IL-8 in human aortic endothelial cells and smooth muscle cells<sup>[14,21]</sup>. Our group pays more attention to the macrophages in human lesions. Because MCP-1 and IL-8 have been shown to be expressed mainly by macrophages in human lesions, and activated macrophages in plaque in response to pathologic agents, such as Hcy, may play an important role in the production of MCP-1 and IL-8. We and others both reported that Hcy could induce MCP-1 and IL-8 secretion in human monocytes and THP-1 cells<sup>[21,22]</sup>. Our study

demonstrates that Hcy significantly enhances MCP-1 and IL-8 in healthy human monocytes that can increase leukocyte chemotaxis. The intracellular oxidative products and subsequent activation of multiple signaling mediators, including MAPKs and NF- $\kappa$ B, are involved in the effects of Hcy in human monocytes. Our another study suggests that the MCP-1 level is apparently elevated both in the plasma and the monocyte in response to low-dose of endotoxin in the patients with angiographically confirmed coronary artery disease with mild HHcy<sup>[23]</sup>. Holven *et al.*<sup>[24]</sup> and we<sup>[23]</sup> reported that folic acid treatment reduced release of chemokines from peripheral blood mononuclear cells in HHcy patients by reducing plasma Hcy levels, but did not have direct effect on human monocytes at such low-dose of folic acid. These data suggest that Hcy might exert its atherogenic effect by enhancing inflammatory response of immunocytes in the blood vessels. Lipopolysaccharide (LPS) stimulation of TNF- $\alpha$  synthesis by peritoneal macrophages was inhibited by *S*-adenosyl-*L*-homocysteine hydrolase inhibitor, and treatment with *S*-adenosyl-*L*-homocysteine hydrolase inhibitor resulted in a modest decrease in major histocompatibility complex class II (MHC-II) determinant expression by IFN- $\gamma$ -activated macrophages, while the expression of other cell surface markers was not altered<sup>[25]</sup>. The processing of antigen and its presentation by MHC-II-positive macrophages to a T-cell hybridoma was also not affected<sup>[25,26]</sup>.

In methionine-rich diet-induced moderate HHcy animal model, the adhesion of monocytes to endothelial cells is also significantly increased, and folic acid treatment can also inhibit the adhesion<sup>[10,11]</sup>.  $\beta$ 2-integrins including LFA-1, p150/95 and Mac-1 play an important role in adhesion of monocytes to endothelial cells. Sotiriou *et al.*<sup>[27]</sup> reported that through its apo(a) moiety lipoprotein(a) specifically interacts with the  $\beta$ 2-integrin Mac-1, thereby promoting the adhesion of monocytes and their transendothelial migration in a Mac-1-dependent manner and the interaction between Mac-1 and lipoprotein(a) was strengthened in the presence of Hcy. These findings can illustrate why both lipoprotein(a) and Hcy were present in women, and the associated risk for cardiovascular diseases was greater than what would be expected if these two risks were simply acting independently<sup>[28]</sup>.

## 5 T lymphocyte activity

Substantial evidence suggests that cellular immune system is involved in atherogenesis. Atherosclerosis fulfills many criteria of a chronic inflammatory process. It is recognized

that T lymphocytes accumulate in the lesions during the earlier stages of atherosclerosis, perhaps even preceding monocytes. In the advanced atherosclerotic plaque, T lymphocytes represent up to 20% of the cells, 10% of which are in an activated state<sup>[29]</sup>. Once resident in the arterial intima, the T cell may encounter antigens such as oxidized low-density lipoprotein (ox-LDL) and heat-shock proteins (HSPs) of endogenous or microbial origin. Upon activation by engagement of the receptor and antigen, the T cell can produce cytokines that can influence the behavior of other cells present in the atheroma. Notably, CD154 binding to CD40 ligand, particularly on macrophages, may induce the expression of tissue factor, matrix metalloproteinases (MMPs) and pro-inflammatory cytokines. The production of these mediators provides an amplification loop resulting from crosstalk between the prototypical cell of acquired immunity (the T lymphocytes) and that of innate immunity (the mononuclear phagocytes). Within the atheroma, as in other tissues, the helper T cells can polarize into those secreting generally pro-inflammatory cytokines (known as TH1 cells) and/or those secreting predominantly anti-inflammatory cytokines (denoted TH2 cells). In general, TH1 cells predominate in the atheroma. But experimental data in mice suggest that with extreme levels of hypercholesterolaemia the balance may shift towards TH2 predominance. Recent evidence indicates that in abdominal aortic aneurysms, TH2 cytokines predominate in contrast with the situation in occlusive atherosclerotic disease.

Several lines of evidence have suggested that Hcy may exert a stimulatory effect on T cell functions. First, our group reported that Hcy potentiated Con A-induced proliferation and inhibited cellular apoptosis in mouse spleen T lymphocytes. ApoE-knockout mice with HHcy had an enhanced susceptibility of T cell mitogen-induced T lymphocyte proliferation and secreted IFN- $\gamma$  and IL-2 compared to the control group. Hcy increased the production of reactive oxygen species (ROS) from T lymphocytes. The potentiating effect of Hcy on Con A-induced T lymphocyte proliferation was significantly reduced by antioxidants<sup>[30]</sup>. The study of our group in HHcy patients showed that the plasma level of RANTES (regulated upon activation normal T cell expressed and secreted), mainly secreted from T lymphocytes was higher than that in non-HHcy control group<sup>[24,31]</sup>. Second, subjects with folic acid or vitamin B12 deficiency, in which Hcy levels become quite elevated, demonstrate an increase in frequency of circulating lymphocytes, suggesting that nuclear fragmentation and micronuclei elevated plasma levels of Hcy. It has been

observed in several diseases where there is altered immune function including HIV, common-variable immunodeficiency, systemic lupus erythematosus, inflammatory bowel disease and rheumatoid arthritis. Finally, Hcy has been shown to be quite cytotoxic to cells in culture and may be one of the underlying causes of its postulated role in atherogenesis and neuronal degeneration<sup>[32]</sup>. Dawson *et al.*<sup>[32]</sup> also reported that Hcy could activate T lymphocytes and induce cytokine secretion, especially type 1 cytokines, including IFN- $\gamma$ , IL-2, TNF- $\alpha$  and IL-10 but not type 2 cytokines IL-4 or IL-5 (Fig.1). The precise mechanism involved in the generation of these cytokines is still under investigation but they believe the Hcy effect is being mediated, in part, by specific stress-associated signals post Hcy treatment.

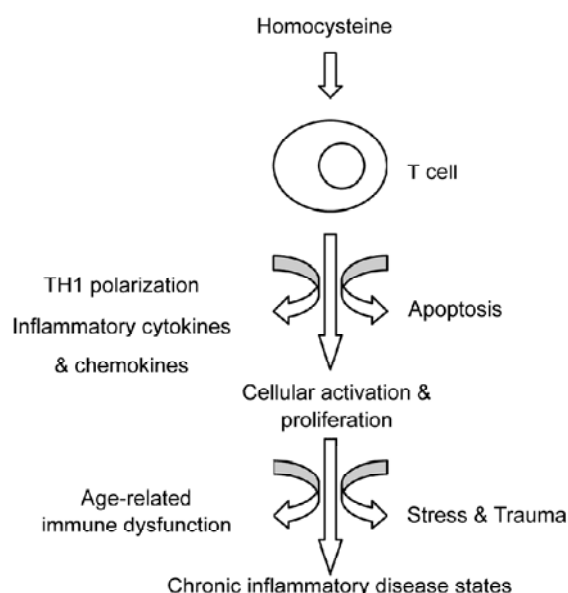


Fig.1. Summary of effects of Hcy on T cell function. Hcy is a T cell activator facilitating T cell proliferation, expansion and inducing cytokine secretion at certain concentration and significant apoptosis at other concentration<sup>[32]</sup>.

## 6 Humoral immunity

Humoral immune system is also involved in the atherogenesis. Previous studies reported the presence of B lymphocytes in every stage of atherosclerotic lesions<sup>[33]</sup>. Adventitial inflammation in atherosclerosis has long been recognized, and the presence of B lymphocytes was described in 1981<sup>[34]</sup>. Immunoglobulin-secreting B cells, immunoglobulins and C5b-9 terminal complexes resulting from the activation of the complement system have all been detected in human atherosclerotic lesions, and IgG and IgA levels are significantly

higher in the fibrous plaque intima<sup>[35]</sup>. In lesions of hypercholesterolemic rabbits and mice, B cells are relatively abundant, and clones of immunoglobulin-producing cells can be found<sup>[36]</sup>. In humans and animals, IgG accumulation is prominent in lesions<sup>[37]</sup>. B cells also participate in atherosclerosis in ApoE-knockout mice; thus, these cells may accumulate through VCAM-1 expression from surrounding cells and may produce antibodies and pro-inflammatory cytokines<sup>[38]</sup>.

According to our previous results, Hcy not only activates resting B lymphocyte proliferation but also acts as a modulator to potentiate LPS-induced B lymphocyte proliferation. ApoE-knockout mice with HHcy showed significantly increased B cell proliferation in response to LPS. The ROS generated by thiol (–SH) auto-oxidation of Hcy are essential, and PKC, p38 MAPK and NF- $\kappa$ B are involved in Hcy-induced B lymphocyte proliferation. Hcy also significantly increased the production of IgG antibodies from resting B lymphocytes<sup>[39]</sup>. Furthermore, Hcy-induced formation of ROS, activation of NF- $\kappa$ B, and secretion of IgG could be inhibited by the liver-X receptor (LXR) agonist TO 901317. HHcy may increase B lymphocytes susceptibility to inflammatory progression of atherosclerotic lesions. Recent evidence shows that autoantibodies may specifically recognize the N-Hcy-Lys epitope on Hcy-containing proteins in humans<sup>[40]</sup>. Therefore, Hcy may act as an antigen that stimulates B lymphocytes to produce specific antibodies.

The role of B lymphocytes in HHcy remains unclear. Hansson and colleagues<sup>[37]</sup> have shown the accumulation of IgG and complement factor C3 in the human arterial endothelium and atherosclerotic lesions. In contrast, protective immunity seems to be associated with the development of IgG antibodies to ox-LDL, although this finding remains controversial<sup>[41]</sup>.

## 7 Potential cellular mechanisms

### 7.1 Oxidative stress

The presence of superoxide anions was detected in the aortic wall of HHcy mice<sup>[42]</sup>. There are two pathways in Hcy-induced oxidative stress. The reactivity of the sulfhydryl group of Hcy has been implicated in molecular mechanisms underlying the increased risk of atherosclerosis and thrombosis, ROS generated by thiol (–SH) auto-oxidation of Hcy can damage cell ability; Hcy can also inhibit cellular anti-oxidant effect. The auto-oxidation of thiol produces many kinds of ROS, including superoxide anion, hydrogen peroxide and hydroxy. These ROS damage protein

structure, affect enzyme activity and induce cell dysfunction which in turn accelerate the toxic effect of ROS. Moreover, ROS are a second messenger which can regulate different signal pathways. In endothelial cells, Hcy-induced ROS activate ERK1/2, Src, Syk and NF- $\kappa$ B and then affect downstream molecules<sup>[43]</sup>. Our previous study reveals that the NADPH oxidase-dependent ROS activate a signaling pathway involving NF- $\kappa$ B mediating Hcy-induced MCP-1 and IL-8 secretion from human monocytes *in vitro*<sup>[5]</sup>. And our recent report provides direct evidence to demonstrate the role of Ref-1 which is a ubiquitously expressed bifunctional protein involved in repairing DNA damage and facilitating the DNA binding ability of many redox-sensitive transcription factors, including AP-1 and NF- $\kappa$ B in HHcy-accelerated atherosclerosis by promoting MCP-1 secretion<sup>[44]</sup>. Our results validate that in monocytes/macrophages, Hcy promotes ROS production via NADPH oxidase, which induces the translocation and upregulation of Ref-1. Ref-1 in turn enhances the DNA binding ability of NF- $\kappa$ B, thereby increasing the expression of its corresponding target genes, such as MCP-1. These data suggest that HHcy, together with hypercholesterolemia, can accelerate atherosclerotic lesion formation by promoting MCP-1 secretion via Ref-1.

## 7.2 NO

The loss of NO bioactivity also contributes to endothelial dysfunction in mild HHcy. There is increasingly compelling evidence that thiols react in the presence of NO and endothelium-derived relaxing factor (EDRF) to form *S*-nitrosothiols, compounds with potent vasodilatory and antiplatelet effects<sup>[45]</sup>. Hcy directly modulates vascular function by reducing NO bioavailability through the generation of superoxide<sup>[46,47]</sup>, because NO also has anti-inflammatory effect. Hcy-induced oxidative stress will lead to further damage of endothelial cell function<sup>[48]</sup>.

## 7.3 Hypomethylation

Hcy levels are regulated by folate bioavailability and also by the methyl donor *S*-adenosyl methionine (SAM) and its metabolite *S*-adenosyl homocysteine (SAH). Transmethylation reactions mediated by *S*-adenosyl-*L*-methionine are required for the chemotaxis of mononuclear leukocytes. Some researchers reported that SAH-induced inhibition of transmethylation could inhibit chemotaxis of macrophages *in vitro*<sup>[49-52]</sup>. Heterozygous cystathionine  $\beta$ -synthase-deficient (CBS<sup>+/-</sup>) and wild type (CBS<sup>+/+</sup>) mice were fed a folate-replete, methionine-enriched diet. Plasma levels of total Hcy were elevated in CBS<sup>+/-</sup> mice compared with that in CBS<sup>+/+</sup> mice, relaxation of aortic rings to acetylcholine was selec-

tively impaired by 35%. Plasma levels of SAH were elevated 2-fold in liver and brain of CBS<sup>+/-</sup> mice<sup>[53]</sup>.

## 7.4 Endoplasmic reticulum (ER) stress

The ER is one of the important organelles, and it serves several important functions, including post-translational modification, folding and assembly of newly synthesized secretory proteins, and a cellular calcium store. Various conditions can disturb ER functions, including inhibition of protein glycosylation, reduction of formation of disulfide bonds, calcium depletion from the ER lumen, impairment of protein transport from the ER to the Golgi and expression of misfolded proteins. Such ER dysfunction causes proteotoxicity in the ER, collectively termed "ER stress"<sup>[54]</sup>.

In cultured endothelial cells, smooth muscle cells and mesangial cells, Hcy can upregulate GRP78, GRP94 and protein disulfide isomerase (PDI) expression via activating PKR-like ER kinase (PERK) and IRE21 expression, suggesting that misfolded proteins would accumulate in the ER because of redox potential changes caused by Hcy<sup>[55-57]</sup>. Moreover, Hcy-induced ROS production and reducing NO bioavailability can lead to ER stress<sup>[58]</sup>. Hcy-induced ER stress may also promote cyclic-AMP-responsive-element-binding protein H (CREBH) cleaved upon ER stress and result in activating expression of acute phase response (APR) genes, and then promote inflammatory response<sup>[59]</sup>.

## 8 Anti-inflammatory therapy in HHcy-induced atherosclerosis

Atherosclerosis is a kind of chronic inflammatory disease. Hcy could induce atherosclerosis via regulating immunoregulatory process. Therefore, anti-inflammatory therapy might offer some effects on Hcy-induced atherosclerosis. *In vivo* studies showed that antibodies recognizing MCP-1, VCAM-1, or E-selectin can abolish the enhanced monocyte binding to the aortic endothelium of HHcy rats<sup>[10]</sup>. Folic acid is a well-known reagent that can decrease Hcy level. Low-dose of folic acid administration which reduces the plasma Hcy levels increases NO-mediated endothelium-dependent vasomotor responses, reduces vascular superoxide production, and improves enzymatic coupling of endothelial NO synthase through availability of the cofactor tetrahydrobiopterins as well as improves vascular function through abolishing inhibition of endothelial NO synthase and vascular oxidative stress comparable to daily intake and dietary fortification<sup>[60]</sup>.

Recently, it has been shown that a nuclear factor peroxisome

proliferator-activated receptor (PPAR) can reduce inflammation by inducing I $\kappa$ B binding to NF- $\kappa$ B. PPAR- $\gamma$  activator prevents Hcy-induced MCP-1, IL-8 and IL-6 production in monocytes<sup>[22,61]</sup>. Hcy competes with activator of PPAR- $\alpha$  and PPAR- $\gamma$  for binding PPAR- $\alpha$  and PPAR- $\gamma$ , respectively, indicating a role of PPAR in amelioration of Hcy-mediated endothelial cell dysfunction<sup>[62]</sup>. MMP-9 was specifically induced in CBS<sup>-/+</sup> mice and inhibited by PPAR- $\alpha$  activator treatment<sup>[63]</sup>. Mondal *et al.*<sup>[64]</sup> reported that development of intimal hyperplasia was 4-fold higher in HHcy rats; this augmentation was significantly reduced in rosiglitazone-treated animals (rosiglitazone is a kind of widely used PPAR- $\gamma$  activator). The PPAR- $\gamma$  activator rosiglitazone can attenuate Hcy-stimulated increase in the rate of development of intimal hyperplasia indirectly by increasing the rate of catabolism of Hcy by CBS and directly by inhibiting vascular smooth muscle cell proliferation. In addition, erythromycin can decrease Hcy-induced extracellular MMP-2 production in cultured rat vascular smooth muscle cells<sup>[65]</sup>. These results also indicate that anti-inflammatory therapy has some effects in Hcy-induced cardiovascular diseases.

## 9 Summary

Inflammation and immunity are two important effects in Hcy-induced atherosclerosis, especially in the early stage. Hcy could induce inflammatory response in different cell types through many kinds of mechanisms. Sharma *et al.*<sup>[66]</sup> traced many references and studied Hcy-related genes. The comprehensive network collated has led to the identification of genes that are modulated by Hcy indicating that Hcy exerts its effect not only through modulating the substrate levels for various catalytic processes but also through regulation of expression of genes involved in complex diseases. But the exact intracellular signal transduction mechanism is still not fully understood. Therefore, to study HHcy and its related diseases via its immunoregulatory effects could provide an insight into new findings.

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