REVIEWS

Cellular and Molecular Mechanisms of Atherogenesis. CD40-CD40L Immunoregulatory Signal

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 128, No. 10, pp. 364-379, October, 1999
Original article submitted April 5, 1999

Contact-dependent interactions of CD40 receptor and its ligand CD40L are regarded as a stimulator of atheroma-associated cells. A new T lymphocyte-dependent pathway of activation of immune inflammation in the vascular wall is discussed, which is presumably maintained by self-regulation of antiinflammatory cytokine production.

Key Words: *CD40-CD40L immunoregulatory signal; leukocyte adhesive molecules; cytokines; immune inflammation*

In the early 1970s we for the first time summarized the data disclosing the role of immunological factors in the development of experimental atherosclerosis in rabbits [1]. "The Phenomenon of Production of Autoimmune Complexes in Human and Animal Blood in Atherosclerosis" was registered as a discovery [3].

Unfortunately, this research received little attention. Only after publications in foreign journals [32, 33] demonstrating the role of apoprotein B-containing lipoproteins (low-density lipoproteins, LDL) and immune complexes in the development of atherosclerosis the results obtained at the Institute of Experimental Medicine began to be cited in Russian and foreign publications.

In the early 1990s we published a series of reviews in which atherogenesis was regarded from a viewpoint of immune inflammation in the vascular wall [6,7,22,23,51]. The term immune inflammation was introduced by A. I. Strukov, Member of Academy of Medical Sciences [10] and developed by V. V. Serov, Member of Academy of Medical Sciences [9]. We widely used this term when discussing the problem of atherogenesis [5,7].

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The central issue in the discussion on the possible role of immunity in atherogenesis is the nature of antigens and the type of cell interactions. Several key issues are discussed now, which explain the onset of immune inflammation in atherogenesis: 1) formation of peroxide-modified LDL (mLDL) acquiring antigenic properties [4,42,44]; 2) immune response to vascular antigens [7,23]; 3) immune response to viral and bacterial antigens [21]; and 4) complement complex (C5b-9) and pentraxines [8,23].

Lipoproteins modified by peroxidation (oxygenation), abundant in the macrophage-enriched atherosclerotic zone, activate chemokine production, which stimulates T and B cell migration into plaques, antibody production, and *in situ* differentiation of cytotoxic cells [2,7,51]. Atherosclerotic plaques contain a great number of $CD4^+$ T cells and macrophages, which suggests that cell-mediated immunity plays an important role in atherogenesis. The fact that $CD4^+$ T cells produce proinflammatory interferon-γ (IFN-γ) in response to antigenic stimulation suggests that atherosclerotic plaque is a result of immune inflammation [16,23,38, 51,56]. This concept is now intensively discussed [8].

Thus, T cells can initiate immune inflammation in atherogenesis, and their transmitters, both soluble and contact-dependent, play a crucial role in atherosclerosis [6,7,23]. The majority of investigations elucidat-

0007-4888/99/0010-0971$22.00

ing the role of immunological factors in the atherosclerosis development have been focussed on the role of soluble inflammatory transmitters rather than surface-associated and immunoregulatory molecules. Recent data help to explain some of these assumptions by deciphering the role of contact-dependent interactions between CD40 receptor and its ligand CD40L, regarded as a stimulant for atheromatous cells [40].

Immunoregulatory signal CD40-CD40L. Numerous *in vivo* and *in vitro* studies demonstrated that cell surface protein CD40 and its ligand CD40L are the integral part of humoral immune response to thymus-dependent antigens [17] and regulation of antigen-presenting cells.

CD40 is a type 1 cell surface 50 kD protein expressed mainly on B cells. It was also found on various thymic cells and monocytes [14,15]. Recently expression of CD40 was detected on endothelial, epithelial, stellate, and smooth muscle cells (SMC) [11,27, 55]. The ligand for CD40 (CD40L) was cloned and identified as a $CD4^+$ T cell activating antigen. CD40L (gp39, CD154) is a type 2 membrane protein 30-33 kD [17,25,41].

CD40-CD40L is more intensely studied in the context of T and B cell interactions [20,41]. The signal induced by CD40L interaction with B cell CD40 regulates B cell proliferation and differentiation [13, 17]. Detection of CD40 on thymic cells suggested that CD40-CD40L interaction can be functionally important in selection of autoreactive T cells [26]. In particular, CD40-CD40L stimulates the production of interleukin-2 (IL-2) and IFN-γ by type 1 T helpers (Th1) and of IL-4, IL-5, and IL-10 by type 2 T helpers (Th2) [43]. CD40L is involved in the T cell-dependent immune response by expressing costimulants, such as B7.1- and B7.2-molecules on antigen-presenting cells essential for antigen recognition [53]. The CD40-CD40L interaction stimulates cytokine production in monocytes [11] and enhances the expression of adhesive molecules for leukocytes on the endothelium [27,39,55].

The absence of CD40L function leads to total loss of thymus-dependent humoral immune response and prevents the formation of B cell memory. Genetic observations confirmed that CD40-CD40L interaction plays an important, if not the key role in the thymus-dependent response. For example, in humans with immunodeficiency syndrome caused by hyper-IgM, mutations of gp39 gene lead to changes in gp39 protein, impairing its binding to CD40. As a result, T helpers cannot trigger B cell activation and production of immunoglobulins (Ig). That is why this disease is characterized by a decrease or even complete absence of IgA, IgG, and IgE in the blood and atrophy of the germinal centers in secondary lymphoid follicles [12,34]. In CD40 knock-out mice no germinal centers are formed in lymphoid organs and the formation and maturation of B cells is disordered [29].

Role of CD40-CD40L in atherogenesis. Some recent publications offer direct proofs that CD40-CD40L interactions stimulate inflammatory response in atherosclerosis [39,40]. One of these reports was published under an intriguing title "CD40 Signaling in Vascular Cells: A Key Role in Atherosclerosis?" [40]. The initial stage of this signaling pathway is presented in Fig. 1.

It is unclear what and where causes activation of $CD4^+$ T cells with expression of CD40L? The following mechanism seems to be plausible. LDL penetrating into the vascular wall through intact endothelial monolayer by means of nonspecific receptor-mediated endocytosis undergo peroxide modification in the intima with the formation of mLDL. Some mLDL with lymph enter regional lymph nodes and activate T cells. Presumably $CD4^+$ T cells stimulated in lymph nodes (expressing CD40L and inducing production of proinflammatory cytokines by vascular wall cells) migrate into the zone of mLDL accumulation in the vascular wall. It is also possible that CD4 cells are activated in the vascular wall and express CD40L under the effect of antigen-MHCII complex.

On the other hand, mLDL generated in the intima stimulate the expression of IL-1β and CD40L and activate CD40 on endotheliocytes and (through costimulating molecules B7.1 and B7.2) induce expression of MHCII. In the endothelium, CD40 can stimulate the expression of CD40L with autocrine regulation [55]. At the earliest stage of atherogenesis the interaction between CD40L-positive T cells and $CD40^+$ on endotheliocytes can increase the expression of CD40L on $CD4^+$ cells and endotheliocytes [36] with subsequent activation of adhesive molecules.

Atherosclerotic lesions in arteries are characterized by increased number of leukocytic adhesive molecules on the endothelium and SMC. Immune inflammation in atherogenesis develops through expression and involvement of adhesive molecules in cell-cell interactions. A signal triggering this reaction is the activation of the CD40-CD40L system, which induces the expression of E-selectin (CD62R), VCAM-1 (CD106), ICAM-1 (CD54), M-CSF, IL-6, but not B7.1 (CD80), B7.2 (CD86), or MHCII on the endothelium [35, 36] and migration into the vascular wall through binding of agranular leukocytes to adhesive molecules. This reaction is maintained due to leukocyte recirculation and production of inflammatory cytokines. In particular, activated T cells and monocytes produce cytokines stimulating CD40 expression by the endothelium and activating the production of adhesive molecules, thus maintaining endotheliocytes in a functionally active state. This activation pathway led F. March *et al.* [39,40] to a hypothesis that CD40-

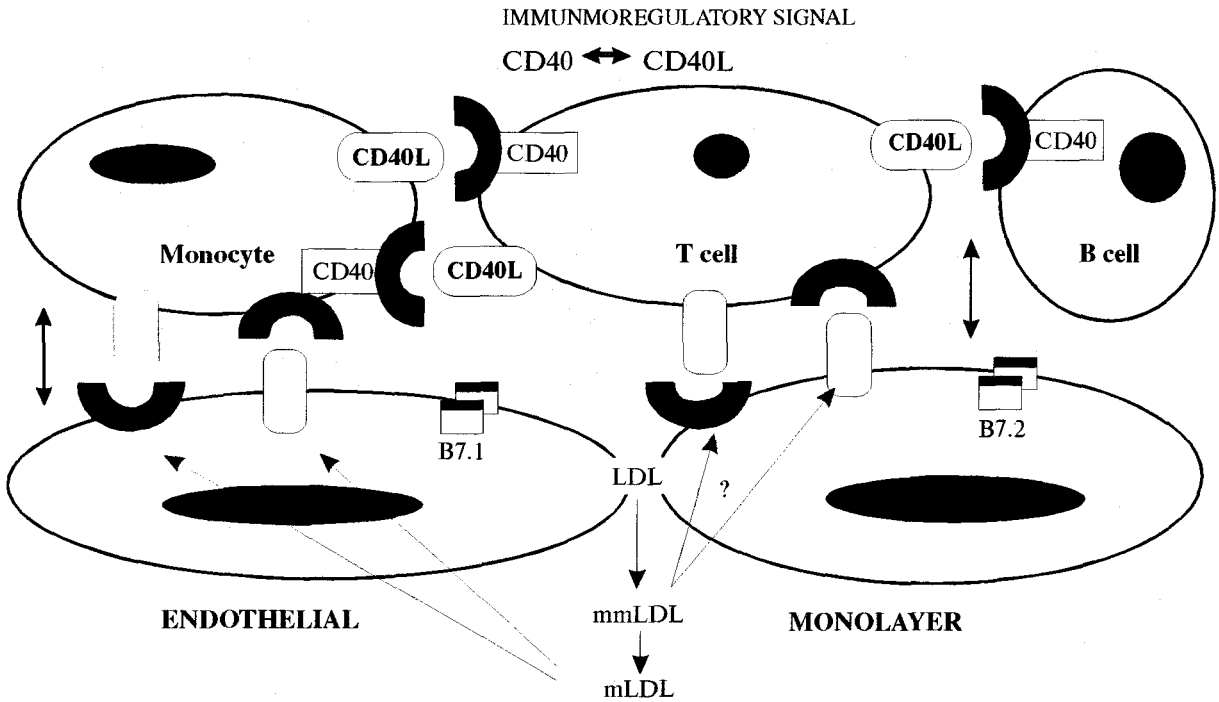

Fig. 1. Involvement of CD40-CD40L immunoregulatory signal in atherogenesis initiation in response to formation of peroxide-modified LDL (mLDL). Stimulates expression of CD62 (ELAM-1, E-selectin) on endothelium — endothelial leukocyte-adhesive molecule 1 and CD106 (VCAM-1) — vascular cell adhesive molecule 1; induces activation of endothelium *in vivo*; CD40 increases macrophagal production of tumor necrosis factor-α and interleukin-6, stimulating the expression of CD40 on endotheliocytes. mmLDL: minimally modified LDL.

CD40L immunoregulatory signal plays an important role in the initiation and development of atherosclerotic lesions in arteries. We support this viewpoint.

Normally endotheliocyte culture expresses little CD40 antigen detectable with monoclonal antibodies. The level of endothelial expression of CD40 increases 3-fold after 24-h incubation in a medium containing optimal concentrations of tumor necrosis factor-α (TNF-α), IL-1, IFN-β, or IFN-γ. Interferon induces a greater expression of CD40 than TNF-α or IL-1. Recombinant CD40L increases the expression of leukocytic adhesive molecules on the endothelium. On the other hand, CD40 can act as a signal receptor in the development of T cell-induced inflammatory reaction [27]. Similarly to B cells and monocytes, endothelial cells interact with T cells in the course of immune response.

Some cytokines of agranular leukocytes (IFN-γ, TNF-α, and IL-1β) can induce endothelial expression of adhesive molecules without CD40. The production of IL-1β by these cells, as a sign of immunoregulatory activation of endotheliocytes, was observed during the very first stages in the formation of lipid spots in human aorta in the zone of mLDL accumulation and/or formation (Fig. 2, *a, b*). Adhesion and migration of mononuclear cells into the intima through endothelial channels in the absence of endothelial damage was observed in the zone of IL-1β production (Fig. 3).

Initial stages of formation of lipid spots is associated with migration of blood cell (monocyte/macrophage and lymphocyte) and SMC from the deep layers of the intima or media to the zone of mLDL deposition or *in situ* formation. Accumulation of T cells in atherosclerotic lesions [6,16,23,38] even surpasses the presence of monocytes/macrophages [24].

Endothelial cells, macrophages, and SMC play an important role in atherogenesis. The mechanisms by which these cells participate in atherogenesis have been described in our monograph [7]. Recent observations extended our knowledge on the role of these cells in atherogenesis. Analysis of human endotheliocyte, macrophage, and SMC cultures showed variations in the expression of CD40L and CD40 in these cells [40], which increased after stimulation (incubation) of these cells with cytokines, such as IL-1β, TNF-α, and IFN-γ.

Immunohistochemical analysis of atherosclerotic lesions in the aorta in young and elderly people showed that T lymphocytes predominate over macrophages in the zone of initial lesions (transitory zone between normal intima and fatty strips) irrespective of the age [52]. T lymphocytes constitute up to 20% cell population in atherosclerotic plaques and about 90% in the atheromatous nucleus zone (together with macrophages). T lymphocyte population are primarily presented by $CD4^+$ cells, the majority of which

express MHC HLA-QR antigen, IL-2 receptor, and integrines [48].

Migration of T cells into atherosclerotic lesions, their *in situ* proliferation, and secretion of cytokines are regarded as an immune response to mLDL [47]. Activation of the CD40 signal pathway can play an important role in the formation of atherosclerotic lesions by regulation of the antigen-activated T lymphocytes. Detection of CD40 and CD40L expression on mononuclear cells in the surface and deep layers of atherosclerotic plaques confirms this assumption (Fig. 2, *c, d*). $CD40^+$ T lymphocytes activate vascular wall cells and stimulate the expression of molecules presumably involved in atherogenesis (adhesive molecules, cytokines, tissue factors, and some proteinases). This implies a new T lymphocyte-dependent pathway of induction of immune inflammation via expression of CD40 and CD40L in the vascular wall autoregulated (Fig. 4).

Activated T lymphocytes in atherosclerotic lesions express CD40L [40], participate in regulation of cytokine production by T cells and via IFN-γ secretion induce the expression of CD40 on SMC and fibroblasts, stimulating the production of adhesive molecules CD54 and CD106 on these cells [55] and inducing activation and proliferation of these cells, followed by collagen synthesis, i. e. promote the formation of connective tissue cap and stroma of the plaques.

Many studies demonstrated that the high level of CD40 expression on macrophages during interaction with CD40L can activate a series of effector functions characterizing the development of chronic inflammation in arteries, similar to autoimmune vascular reac-

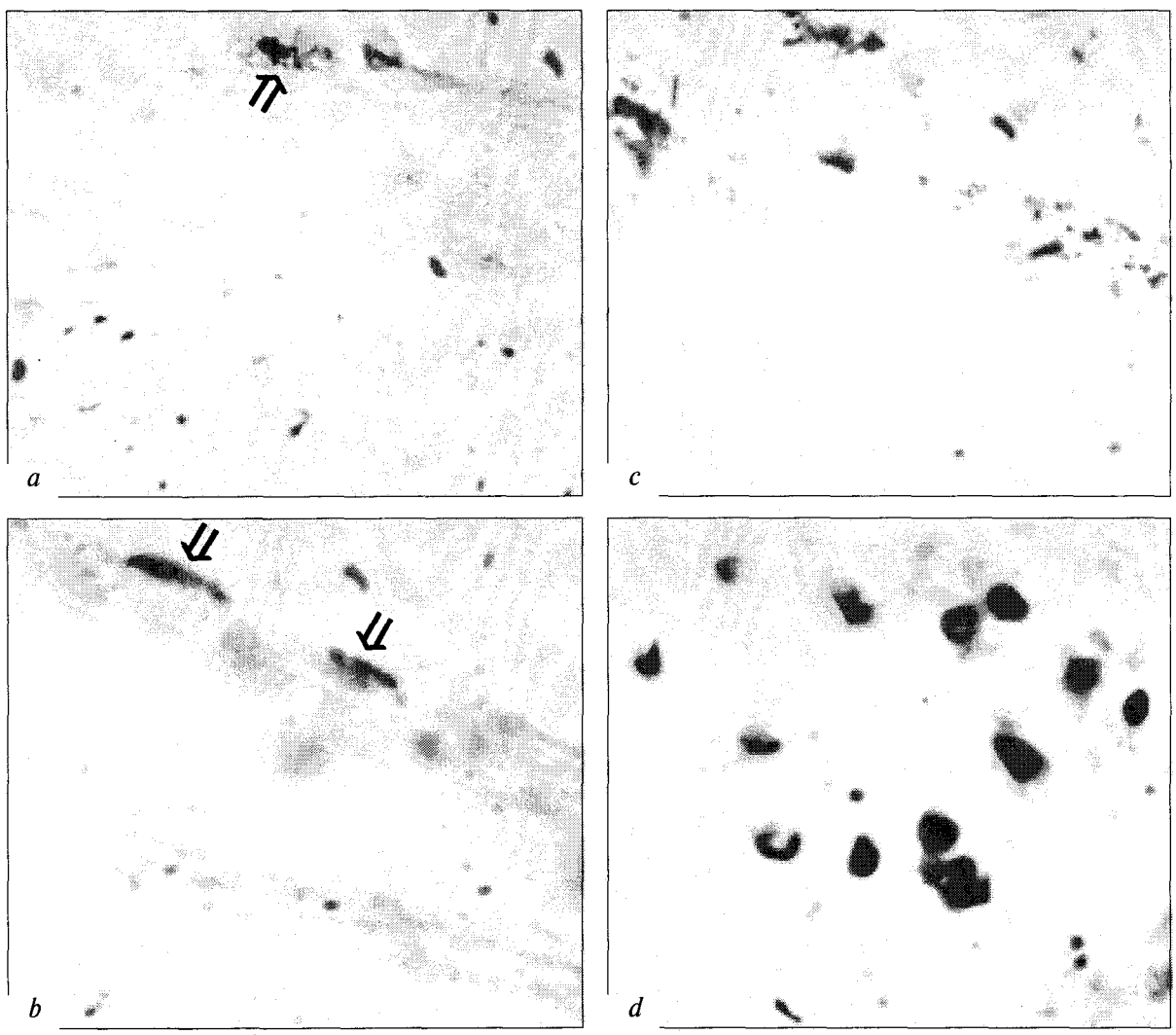

Fig. 2. Expression of cytokines and CD40-CD40L immunoregulatory signal in human coronary (*a, b*) and carotid (*c, d*) arteries in the zone of deposition of peroxide-modified LDL (mLDL) in atherogenesis. Immunohistochemistry with monoclonal antibodies, ×1200. *a*) focal accumulation of mLDL in subendothelial layer (arrow); *b*) focal production of interleukin-1β in endotheliocytes (arrows); *c*) CD40 positive staining in subendothelial layer; *d*) CD40L positive staining in lipid spot mononuclear cells.

tions [19]. The population of antigen-presenting macrophages increased due to autocrine regulation of CD40 expression and enhanced expression of CD54, CD86, and MNCII [31]. CD40L provides a costimulatory signal for macrophages and activates macrophage production of cytokines TNF-α, IL-1β, IL-6, IL-8, and IL-12 [28,30]. Moreover, CD40-CD40L can stimulate production of NO [49] and free radicals ($O_2^{\bullet-}$, $OH^{\bullet}$) by macrophages inducing or enhancing peroxide modification of LDL in atherosclerotic plaques.

In situ immunohistochemical analysis showed expression of both CD40 and CD40L in atherosclerotic plaques in man [39]. On cross-sections of the carotid artery immunoreactive CD40 and CD40L were found mainly in the marginal zones of plaques and between the involved and intact zones of the artery. Specific location of CD40L and CD40 was observed on endothelial cell, macrophages, and SMC. $CD4^+$ lymphocytes in the plaque also expressed CD40L [39,54]. Sections of intact arteries exhibit no CD40L and demonstrated only minor expression of CD40 on the endothelium.

Since plaques contain little B cells, macrophages are the main cells expressing CD40 *in situ*. On the other hand, the presence of B cells in atherosclerotic lesions is an important factor indicating the possibility of local antibody production [2,36,46]. Stimulation of B cells via CD40L can cause focal antibody production and isotypical differentiation [50].

The exposure of CD40L on vascular wall stimulates the biological activity of cells. CD40L is a signal for the production of the major inflammatory cytokines in macrophages and resident cells of the vascular wall. Interactions of CD40 with its ligand on the endothelium and SMC induces the production of IL-1β, IL-6, and IL-8 [39,45]. The same signal reactions on macrophages induce the production of IL-1β, IL-6, IL-8, TNF-α, and NO [39,45,49].

The presence of CD40 and its ligand on macrophage clusters in atheroma promotes the release of proteases destroying plaque endothelium and stroma (collagen and elastin) and initiate thrombosis by expressing the tissue factor [39].

On the whole, *in vivo* and *in vitro* experiments showed that endothelial cells, macrophages, and SMC of the vascular wall can be regarded as a new source of CD40L and proved the possibility of T cell-independent pathway of activation of CD40 expression and extension of the functional role of this pathway in the regulation of nonimmune cells, as an illustration of potential auto- and paracrine activation in atherogenesis.

Hence, new data on CD40-CD40L involvement in atherogenesis are presented:

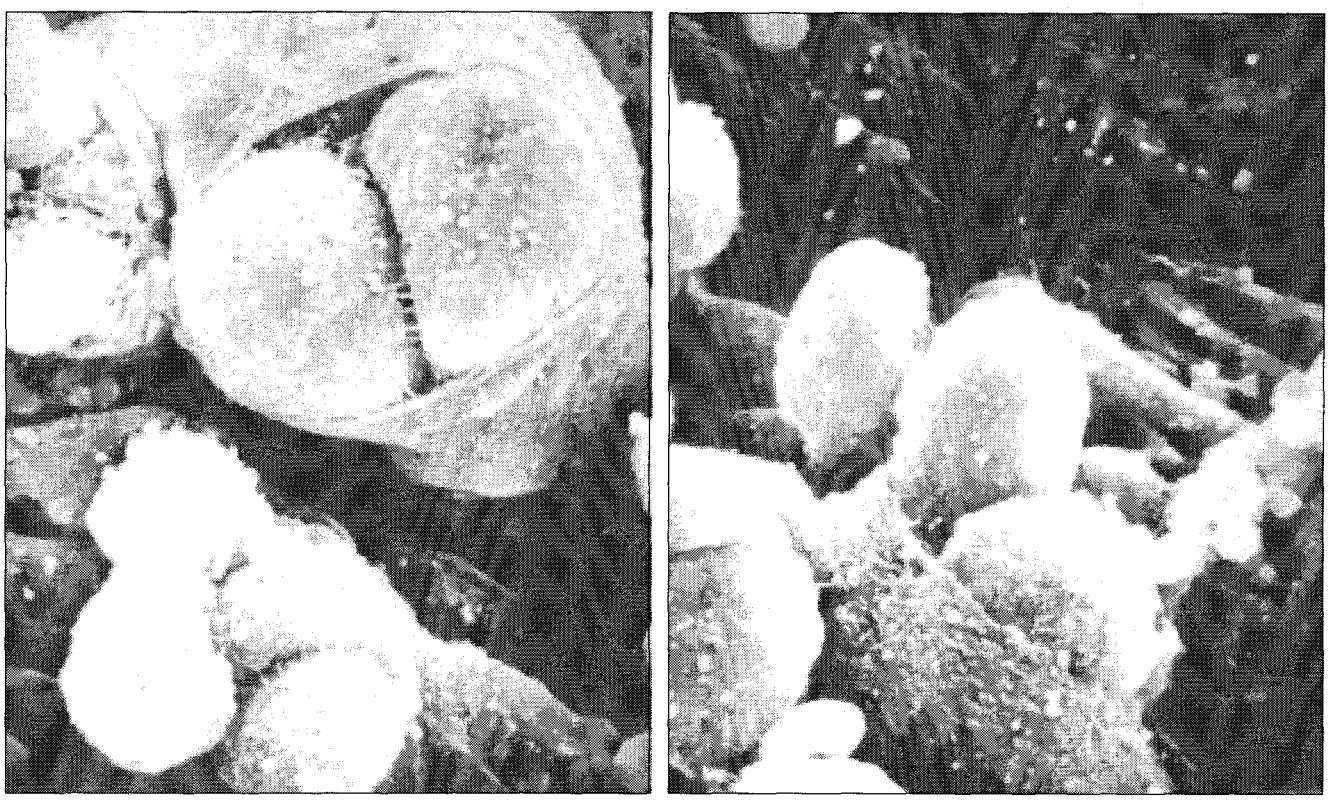

Fig. 3. Initial stage of coronary artery atherogenesis: penetration of monocytes through endothelial monolayer. Scanning electron microscopy, ×4600.

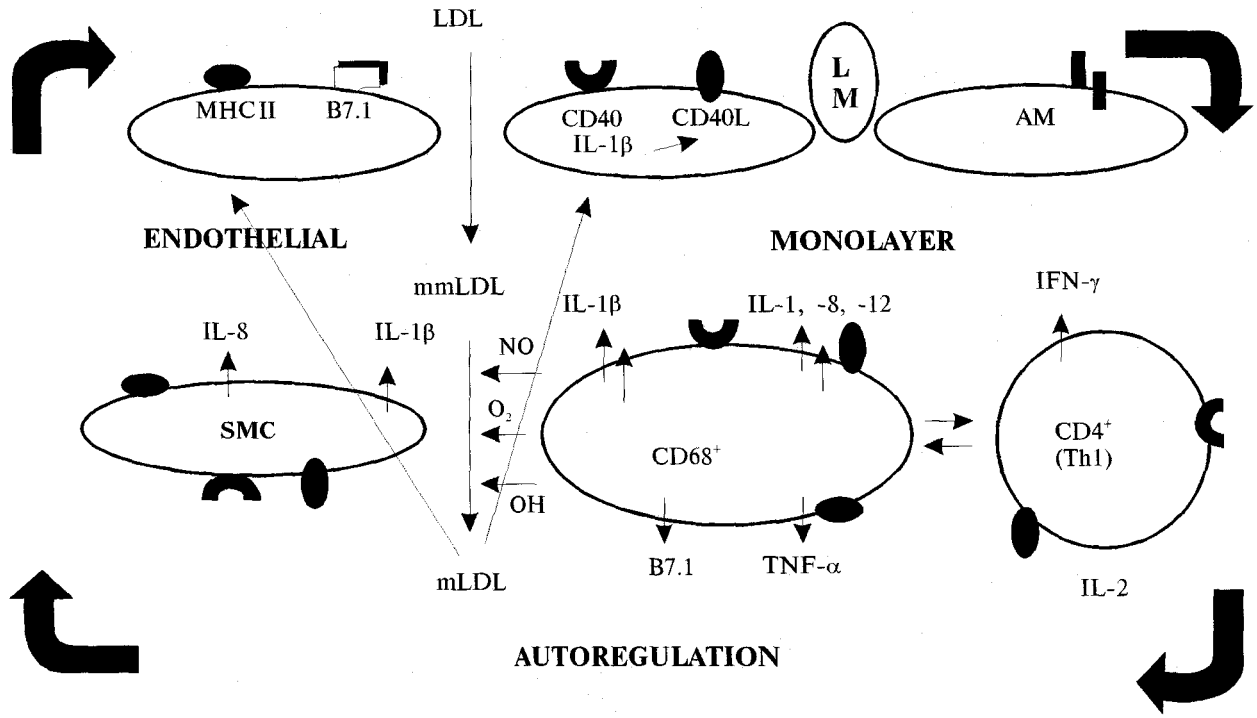

Fig. 4. Autoregulation of immune inflammation in atherosclerosis of the vascular wall and the role of inflammatory cytokines. L) lymphocytes; M) monocytes; AM) adhesive molecules; mLDL) peroxide-modified LDL; mmLDL) minimally modified LDL. Interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) stimulate expression of CD40L in endotheliocytes, monocytes/macrophages, and smooth muscle cells (SMC); CD40-CD40L signal stimulates expression of adhesive molecules on endotheliocytes, monocytes/macrophages, SMC; activates expression of inflammatory cytokines in vascular wall cells and ensures antigen presentation; induces activation and *de novo* expression of CD40L on endotheliocytes, monocytes/macrophages, SMC; promotes proliferation and *in situ* phenotypical cell differentiation.

— human endothelial cells and SMC express CD40L, a molecule previously detected only on activated agranular leukocytes;
— possibility of CD40 expression in SMC of the vascular wall;
— coexpression of CD40L and CD40 by intimal cells in atherosclerotic lesions *in situ*.

The data on the functional role of CD40 in human SMC extended our knowledge about vascular wall biology, particularly at the early stage of atherogenesis. CD40L enhances the expression of cytokines involved in atherogenesis in atherosclerotic plaques [37].

Therefore, CD40-CD40L signal pathway can play different roles in atherogenesis. The first is the regulation of antigen-specific cell response to subsequent activation. The other is the expression of CD40L on the vascular wall involved in atherosclerotic lesions; this demonstrates a new T cell-dependent pathway of inflammatory activation, a component of atherogenesis which can be no longer doubted.

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